

## Investigation of SNPs in BMP15 and GDF9 genes in “Çepni” and “Of” sheep in the Black Sea region of Turkey

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**Abstract:** Sheep fertility genes have been studied in Turkey for many years. But so far it cannot be said that a satisfactory result has been achieved in terms of the number of detected polymorphisms on fertility genes. Exhibiting the genetic structure of local gene resources adapted to a particular region is very important for sustainability, conservation and breeding studies, and many other issues such as profitability. Therefore, the primary aim of this study was to investigate the single nucleotide polymorphisms (SNPs) on the BMP15 and GDF9 genes in two new Turkish sheep breeds, Çepni and Of. DNA sequencing was performed to identify SNPs in both genes of Çepni and Of sheep. One novel SNP (T755C) in the BMP15 gene and five known SNPs (c471C > T (G2), c477 G > A (G3), c721 G > A (G4), c978 A > G (G5), and c994 G > A (G6)) in the GDF9 gene, and a total of six SNPs were identified. Çepni and Of breeds showed a highly polymorphic structure for the genes examined. This finding shows that these two sheep breeds have great potential for future association studies. As a conclusion, the findings of this study were the first for the Çepni and Of breeds, and it should be examined for novel and other SNPs for more convincing results, especially litter size association studies, in new and other native sheep breeds in Turkey.

**Key words:** BMP15, GDF9, Çepni sheep, Of sheep, SNP, T755C

### 1. Introduction

Archaeological findings and genetic studies demonstrate that Turkey is one of the most critical domestication centres—Iraq, Turkey, Syria, and Jordan—for *Ovis aries* [1,2]. According to data from the Turkish Statistical Institute and the Food and Agriculture Organization—Domestic Animal Diversity, Turkey’s sheep population is nearly 42 million, and the breed number is 40 [3,4]. The number of sheep breeds in Turkey has expanded in recent years through registration studies supported by the General Directorate of Agricultural Research and Policies of the Republic of Turkey (TAGEM).

In 2020, TAGEM registered “Of” and “Çepni” breeds as new local sheep breeds in Turkey. These two breeds have been bred in Trabzon in Turkey’s Black Sea region, for centuries [5]. The Çepni breed is predicted to have a pure-bred population with a herd size of approximately 3500–4000 heads. The Of breed is also predicted to have a pure-bred population size of around 1800–2000 heads. These two breeds account for 1.40% and 2.80% of the total sheep in the region, respectively.

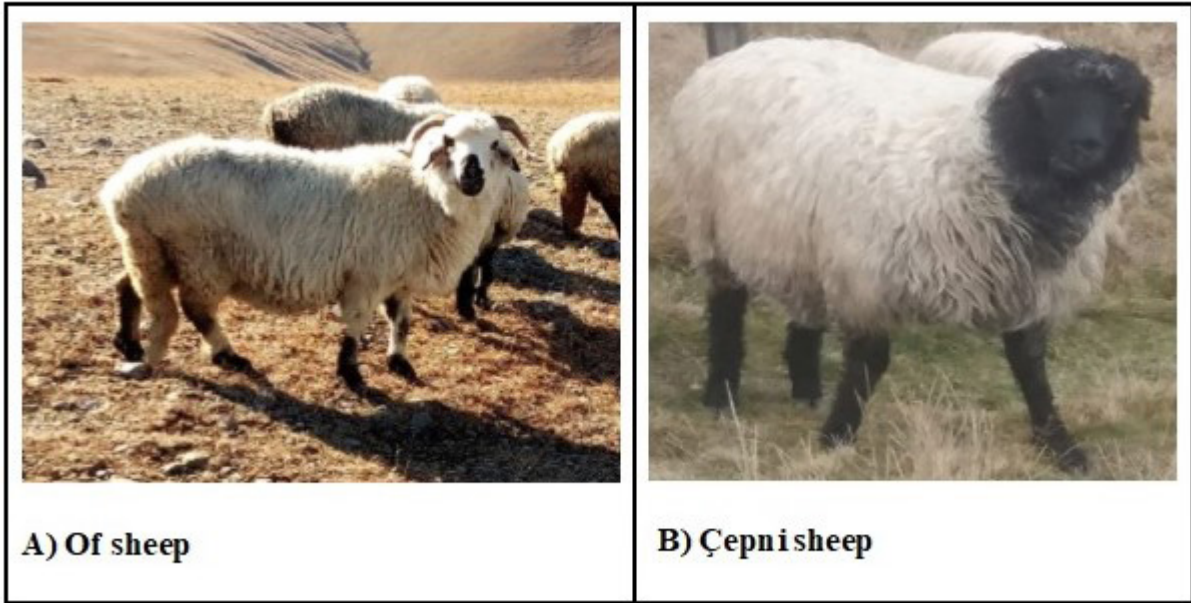
To summarize the morphological characteristics of these two breeds (Figure 1), Çepni has a white body with a whip and a slight grey or black color from the beginning

of the shoulders or on the tail, while others are completely black or grey. The head is black, and it is occasionally combined with the neck. In Of sheep, the body and head are typically white, whereas the area surrounding the eyes is often black. This breed may have black and white or brown and white freckles on its face. The Çepni breed is more agile than other breeds in the region, whereas the Of breed is bigger, has a higher milk yield, and is better resistant to extreme winter conditions [5].

The Çepni and Of breeds, which have adapted well to the Black Sea region in which they are bred, are a valuable source of income for breeders despite their smaller population size [6]. In this sense, it is essential to create techniques to protect and expand the number of valuable gene resources. By including an important gene in the breeding studies, an increase in yield and twin rates per animal is conceivable in a short time [7, 8]. Therefore, finding any mutation that can be used in breeding programs is a significant advantage.

The bone morphogenetic protein 15 (BMP15) and the growth differentiation factor 9 (GDF9) genes, members of the transforming growth factor- $\beta$  superfamily (TGF -  $\beta$ ), enhance the lambing rate per ewe due to their impact on follicle and granulosa cell development, cell proliferation

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**Figure 1.** A general view of the Of and Çepni sheep breeds.

and other factors. Numerous studies have revealed the involvement of those genes in reproduction in various animals, including sheep [9], goats [10], pigs [11].

According to breeder observations and information, the twinning rates for the Çepni range from 20% to 25% and 35% to 40% for the Of breed. There was no published molecular study for either breed, with the exception of one study examining the G1 mutation [12] which reported a significant level of heterozygosity, indicating that the Of breed may have further mutations in GDF9 or other fecundity genes [12]. This discovery inspired the current study. Therefore, the present investigation aimed to detect the single nucleotide polymorphisms (SNPs) in exon 2 of the BMP15 and GDF9 genes as both gene regions have many SNPs compared to other gene regions.

## 2. Material and methods

### 2.1. Collecting blood samples and DNA isolation

A total of 50 ewes (Çepni, n = 25, and Of, n = 25, Figure 1), raised in the Trabzon province of Turkey, were used in the study. All individuals were at the ages of 3–4 and their feeding primarily was based on the extensive conditions. The sampling process was performed under veterinary supervision, and blood was taken into 15 mL tubes containing ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. The DNA isolation from the whole blood was carried out following a related protocol (GeneJet Whole Blood Genomic DNA Purification kit, Thermo Fisher Scientific, USA).

### 2.2. Amplification and sequencing of the BMP15 and GDF9 genes

To amplify the exon 2 part of BMP15 (Gene ID: 100141303) and GDF9 (Gene ID: 100217402) genes, the following primer pairs by Abdoli et al. [13] 5'-CGCTTGCTCTTGTTCCTC - 3' and 5' - TAGCTGCACCTTTGCCGTC-3', 5' -TGGCATTACTGTTGGATTGTTT - 3' and 5' - GGTTTTACTTGACAGGAGTCTG - 3', were used in the study, respectively. A fragment of 906 bp for BMP15 and 1019 bp for the GDF9 gene was amplified by polymerase chain reaction (PCR). PCR reactions were performed in a final volume of 30 µL containing 13 µL of Taq DNA polymerase Master Mix (2X) (Ampliqon, USA), 1 µL of each primer, 2 µL DNA, and 13 µL of distilled water. Post-PCR products were all controlled in 2% agarose gel electrophoresis and then subjected to DNA sequence analysis, which performed using the BigDye Terminator v3.1 Cycle kit in the ABI3730XL Sanger DNA Sequencer (Applied Biosystem Foster City, CA).

### 2.3. Data analysis

The obtained sequences were aligned with the Mega X (Molecular Evolutionary Genetics Analysis) software [14], and then the polymorphic loci were detected by comparing the samples with each other and with reference sequences (accession numbers for GDF9 and BMP 15 genes; HE8666499.1 and NM\_001114767.2, respectively) obtained from NCBI (National Center for Biotechnology Information) GenBank. Allele and genotype frequencies

for each locus were estimated for the BMP15 and GDF9 genes using the PopGene32 program [15]. A Chi-square analysis was performed to test the Hardy-Weinberg equilibrium (HWE) of the populations. Sequences showing high similarity to the samples in the study were collected by the NCBI, and a phlogenetic tree was constructed using a neighbor-joining tree with MEGA software [14].

### 3. Results

#### 3.1. DNA sequence analysis

The present study demonstrated Çepni and Of sheep carried the T755C SNP in the BMP15 gene. The result of the DNA chromatogram of the T755C SNP is shown in Figure 2. As for the GDF9 gene, five SNPs (G2 to G6) were discovered in Çepni and Of sheep. Figure 3 shows the DNA chromatogram results for the identified SNPs. In the study, all SNPs were first findings for the studied breeds.

#### 3.2. Allele and genotype frequencies and Hardy-Weinberg equilibrium (HWE)

The BMP15 gene was found to have two genotypes (TT and CT) in the study. The frequencies of the corresponding genotypes for Of sheep were 0.72 and 0.28, respectively, and 0.79 and 0.21 for Çepni sheep. For the whole population, frequencies of TT and CT genotypes were 0.77 and 0.23. As seen in Table 1, the TT genotype had the highest frequency, while the CT genotype had low and similar frequency in either breed. According to Chisquare analysis, both breeds were in HWE for the BMP15 gene.

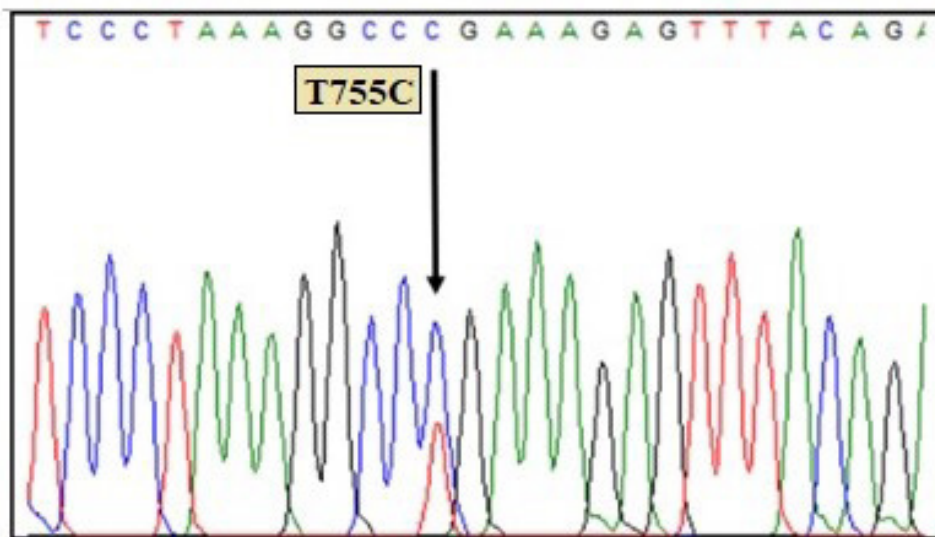
The five known SNPs for exon 2 on the GDF9 gene [16] were also detected in Çepni and Of sheep and their

frequencies are estimated as shown in Table 2. Since the number of related loci is high, the important ones were mentioned here instead of describing the whole frequency values for all SNPs. Briefly, what is noteworthy here is that heterozygous mutant individuals have been identified in both breeds for the G2-G6 SNPs. Particularly, the frequency of heterozygotes genotypes for the G3 was considerably high for both breeds: Çepni (0.70) and Of (0.76) compared to other loci.

The results of the Chisquare test indicated that both breeds were in HWE for the studied loci. For the G3 locus, only Of sheep was not in HWE.

#### 3.3. Phylogenetic tree of the BMP15 and GDF9 genes

Six breeds of sheep Balochi (accession number: JN655672.1 for BMP15), Chinese (accession number: KR063137.1 for GDF9), Lori Bakhtiari (accession number: KT013294 for BMP15), Norwegian White (accession number: HE8666499), Pelibuey (accession numbers: KT853038 for BMP15, NM\_001142888 for GDF9), Santa Ines (accession number: FJ42911 for GDF9), and *Bos taurus* as an outgroup (accession numbers: DQ463368.1 for BMP15 and GQ922451.1 for GDF9) were used for the phylogenetic study of BMP15 and GDF9 genes in Çepni and Of breeds. The result of the topology of the neighbor joining tree for GDF9 and BMP15 is shown in Figures 4 and 5, respectively. The phylogenetic tree showed that Bolochi sheep and Norwegian white sheep differed from the others in the GDF9 and BMP15 genes, respectively. Çepni was closer to Chinese sheep for the GDF9 gene and to Lori Bakhtiari sheep for the BMP15 gene.



**Figure 2.** DNA chromatogram result of the identified T755C SNP in the exon 2 region of the BMP15 gene.

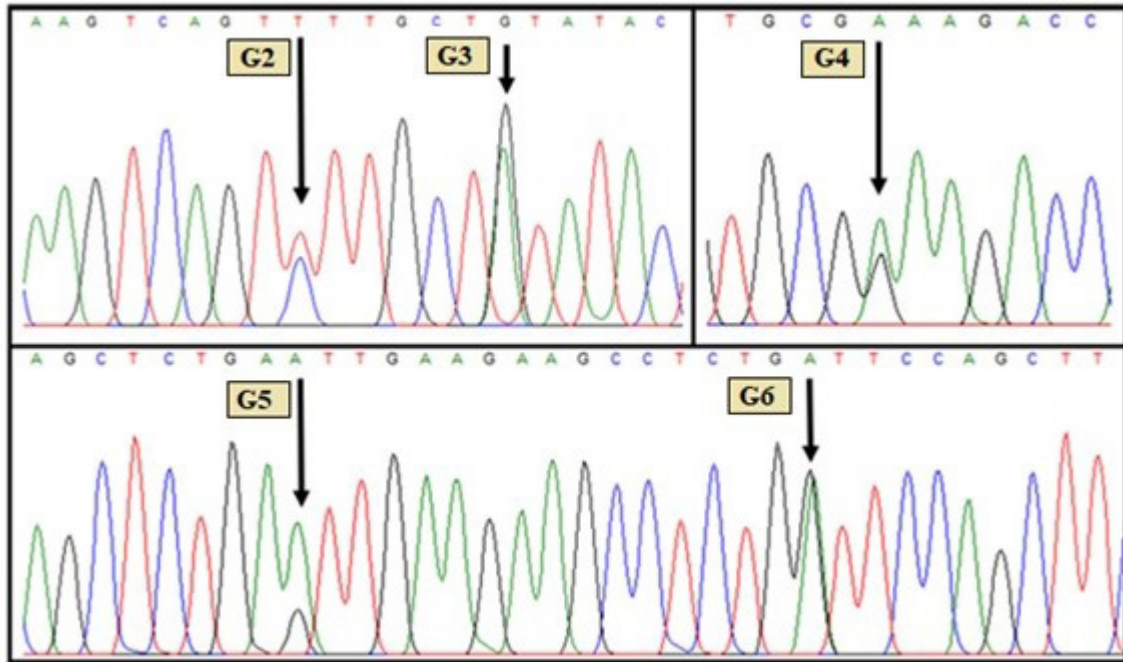


Figure 3. DNA chromatogram results of the identified SNPs (G2–G6) in the exon 2 region of the GDF9 gene.

Table 1. Allele and genotype frequencies of the identified T755C SNP in the exon 2 region of the BMP15 gene.

Breed	Genotypic frequency		Allele frequency		Ho	He	X <sup>2</sup>
	CT	TT	C	T			
Of	0.28	0.72	0.13	0.87	0.2609	0.2319	0.4231
Çepni	0.21	0.79	0.10	0.90	0.2083	0.1906	0.2548

Ho: Observed heterozygosity, He: Expected heterozygosity, x<sup>2</sup>: Chi-square

Table 2. Allele and genotype frequencies of the identified SNPs in the exon 2 region of the GDF9 gene.

SNPs	Breed	Genotype frequency			Allele frequency		Ho	He	X <sup>2</sup>
G2	Çepni	CC (0.80)	CT (0.20)	TT (0.00)	C (0.90)	T (0.10)	0.200	0.1837	0.2424
	Of	CC (0.57)	CT (0.43)	TT (0.00)	C (0.78)	T (0.22)	0.440	0.3502	1.7814
G3	Çepni	AA (0.20)	GA (0.70)	GG (0.10)	A (0.55)	G (0.45)	0.700	0.5077	3.024
	Of	AA (0.08)	GA (0.76)	GG (0.16)	A (0.46)	G (0.54)	0.760	0.5069	6.493
G4	Çepni	AA (0.00)	GA (0.15)	GG (0.85)	A (0.08)	G (0.92)	0.150	0.1423	0.08
	Of	AA (0.00)	GA (0.12)	GG (0.88)	A (0.06)	G (0.94)	0.120	0.1151	0.066
G5	Çepni	AA (0.70)	GA (0.20)	GG (0.10)	A (0.80)	G (0.20)	0.200	0.3282	3.4202
	Of	AA (0.74)	GA (0.26)	GG (0.00)	A (0.87)	G (0.13)	0.2609	0.2319	0.4231
G6	Çepni	AA (0.10)	GA (0.20)	GG (0.70)	A (0.18)	G (0.82)	0.1818	0.3044	4.005
	Of	AA (0.00)	GA (0.30)	GG (0.70)	A (0.15)	G (0.85)	0.3043	0.2638	0.6235

SNPs: Single nucleotide polymorphisms, G2: c471C > T, G3: c477 G > A, G4: c721 G > A, G5: c978 A > G, G6: c994 G > A, Ho: Observed heterozygosity, He: Expected heterozygosity, x<sup>2</sup>: Chi-square

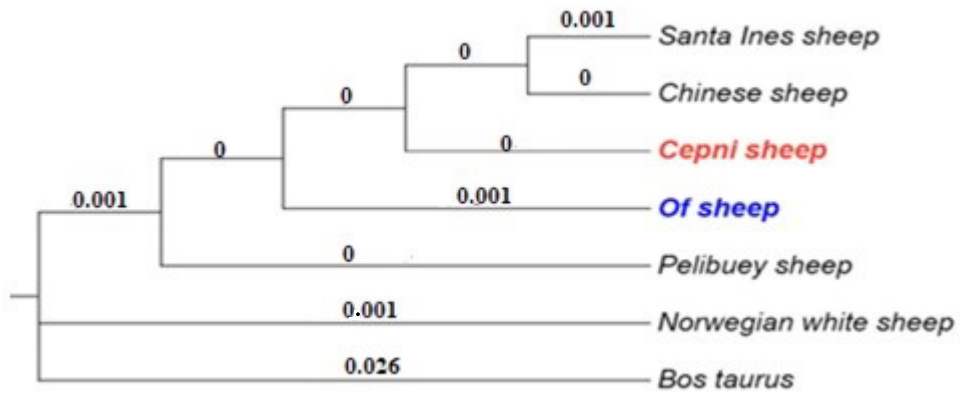


Figure 4. Neighbor - joining tree for GDF9 gene exon 2 region between breeds.

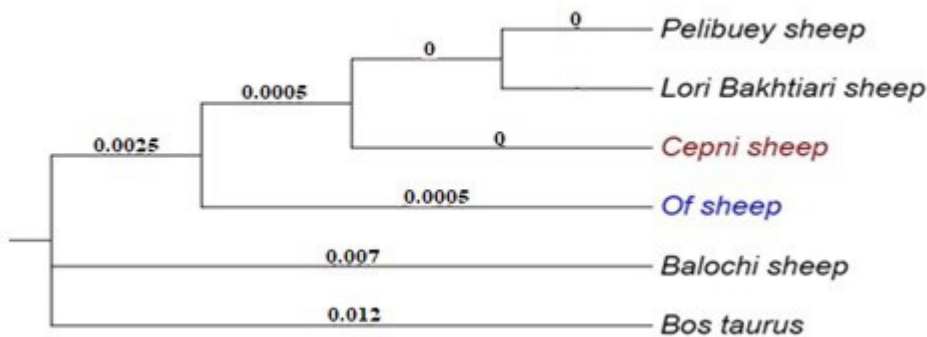


Figure 5. Neighbor-joining tree for BMP15 gene exon 2 region between breeds.

#### 4. Discussion

A high number of SNPs in exon 2 of the BMP15 gene were reported by researchers in sheep, and some of them cause fertility or infertility due to abnormalities in the early stages of folliculogenesis in homozygous ewes. Apart from known SNPs in the recent years, a few novel SNPs have also been identified in sheep BMP15 gene [17,18]. One of them is called a 755 T > C which was initially found in Iranian sheep by genome-wide association analysis (GWAS) and then in a study by Amini et al. [17], which reported that the mutation has an increasing influence on litter size in ewes with the CT genotype. Afterwards, this mutation was reported in Cele Black sheep from China [19]. In the current study, a 906 bp fragment of the BMP15 gene was successfully amplified, and DNA sequencing findings revealed a 755T > C novel mutation in the examined breeds for the first time, which resulted

from proline to leucine formation at the position of exon 2 of the BMP15 gene.

According to earlier studies, BMP15 heterozygous animals have increased ovulation rates and lead to greater litter size than wild-type animals [16–19]. The current study revealed two genotypes CT and TT, but no evidence of a homozygous genotype (CC) in the examined samples, in contrast to the study of Amini et al. [17], who reported a low frequency for CC genotype. One possible explanation for this finding is that the population's frequency of the C allele was low (0.12) as well as the low sample size in the study. Similar findings were also observed for Cele black sheep in the study of Niu et al. [19] which demonstrated an increased litter size at heterozygous animals ( $2.20 \pm 0.129$  vs.  $1.87 \pm 0.066$ ). This result implies that the mutation has a dominant influence on the sheep litter size. The frequency of heterozygous sheeps having the 755T > C mutation was

given as 0.17 to 0.34 for Lori Bakhtiari and Afshari breeds [17], which is similar to the current study's finding that the frequency of heterozygous genotypes ranged from 0.21 to 0.28 for Çepni and Of breeds, respectively. Another investigation found that the mutation was present in 5% of the genotyped 20 Iranian sheep. Consequently, the present study's findings are comparable to or close to the earlier results.

Many SNPs on the GDF9 gene, another fertility gene, have been identified by Hanrahan et al. [16] in Cambridge and Belclare sheep. All SNPs reported on the related region of DNA sequenced at exon 2 were also detected in Çepni and Of sheep, which means that the studied breeds had a polymorphic structure for GDF9 gene. As a result, five SNPs were found in Çepni and Of sheep breeds: c471C > T (G2), c477 G > A (G3), c721 G > A (G4), c978 A > G (G5) and c994 G > A (G6).

In the study, the genotypes with the highest frequency value for G4 mutations were the GG ones for both breeds studied. This finding was similar to the Iranian breeds of sheep named Bahmei and Lak Ghashai [20]. Dolatabady and Habibizad [20] found the frequencies of heterozygous genotypes for G2 and G3 mutations to be 0.15 for CT and 0.20 for AG in Bahmei sheep, respectively. These values were significantly lower than those found in the current study, which showed 0.43 for CT in Of sheep and 0.70 and 0.76 for AG in Çepni and Of breeds, respectively. The frequency of the GA genotype for the G3 locus in Kermani sheep, 0.482, was also lower than in this study [21].

As for the G4 mutation, the frequency of the GA genotype determined for Çepni and Of sheep was lower than the values reported for Mehraban sheep (0.29) [22], Kermani sheep (0.481) [21] and Egyptian sheep (0.78) [23]. The frequency of heterozygous GA genotype for the G6 mutation in Çepni and Of sheep was higher than in Egyptian sheep (0.02) [23] and lower than in Kermani sheep (0.450) [21]. These mutations have also been observed in various sheep breeds, including the Garole [24], Poll Dorset, Suffolk, German Mutton Merino, and Chinese Merino [25]. The result of neighbor joining trees for both genes demonstrated that Çepni sheep are close to the Central Asian sheep breeds. These can be attributed to two reasons. One is that they might share the same SNPs [26] and another is that it is believed to have originated in Central Asia, to the knowledge of farmers living in the region.

There is a great gap in the number of identified fecundity mutations in Turkish native sheep breeds except for a few studies [12, 27–29]. The present study revealed all

known mutations (G2 to G6) in the sequenced region of GDF9 gene exon 2 [16] and one novel mutation (T755C) [17] on the BMP15 gene in Of and Çepni breed, which means the studied breeds have genetic variation that allows for association studies between litter size and related genes in the future.

The number of mutations to be identified is expected to be influenced by some factors, such as sample size, breed, and sampling method [30]. However, despite the limited number of samples in this study, a significant number of mutations in fertility genes were identified for Turkey's new sheep breeds, Of and Çepni. The study revealed how important it is for the genetic structure of new gene resources to be investigated.

## 5. Conclusions

The most striking and important conclusion of this study is that for the first time a novel T755C mutation and almost all mutations in the exon 2 regions of the BMP15 and GDF9 genes are found in two new sheep breeds, Çepni and Of. As indicated by earlier studies, these mutations may be potential molecular markers for the studied breeds. Furthermore, the findings hold important potential for future association investigations on the studied breed and other breeds in Turkey's Black Sea region.

## Data availability

The data are available from the corresponding author upon request.

## Acknowledgement/disclaimers/conflict of interest

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## Ethical statement

This research's blood materials were collected with ethical approval given by the Ondokuz Mayıs University Local Ethical Committee (OMU-HADYEK, Samsun, Turkey) (approval number: 2017/25).

## Conflict of interest

The author has no conflict of interest.

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