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Research Article

Determination of the Effect of GDF9, BMP15, BMPR1B Gene Polymorphism, and Environmental Factors for Fecundity by Logistic Regression Analysis in Kangal Akkaraman Sheep [1]

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Abstract

Polymorphisms identified on the BMPR1B, BMP15, and GDF9 genes tend to increase multiple birth and ovulation rates in sheep. In the planned study, the productivity records of the flocks (approximately 41.000) collected from 2016 to 2022, belonging to the Public Breeding of Kangal Akkaraman Sheep in Sivas province TAGEM/58KAK2012-08 sub-project of the Public Animal Breeding National Project, were used. Accordingly, the ear numbers of sheep that gave birth to at least two twins (n=96) and at least two singletons (n=96) were determined from 15 different farms relevant records. According to similar feeding characteristics, environmental variables were grouped as location, year-round feeding type, and seasonal feeding type. DNA groups were genotyped by the PCR-RFLP method for BMPR1B (FecB), BMP15 ($FecX^B$, $FecX^G$, $FecX^I$, $FecX^H$) and GDF9 (FecG) alleles. Accordingly, among the 6 SNPs examined, only the GDF9 gene-FecB SNP was determined polymorphic. Genotypic effect (FecB allele) and environmental effect variables (location, year-round feeding type, seasonal feeding type) were also examined with a logistic model. It was determined that the relevant alleles and environmental variables did not have a statistically significant effect on the twinning phenotype. According to the results obtained, it was thought that the genes associated with multiple births in Kangal Akkaraman breed may have different variants specific to the breed. In addition, it is suggested that this character, which is affected by multiple genes, should be included in the planned breeding studies by considering the interaction of environmental variables and determining the variation of the related genes. In this respect, it is concluded that our study will guide the sequencing studies and multivariate analyses to be planned.

Keywords: BMP15, BMPR1B, Fecundity, GDF9, Gene polymorphism, Logistic regression analysis, Sheep

Introduction

The genes involved in controlling fecundity in sheep have been defined as bone morphogenetic protein receptor 1B (BMPR1B) [1], bone morphogenic protein 15 (BMP15) [2,3], and Growth differentiation factor 9 (GDF9) [3]. Polymorphisms identified on reported genes tend to increase multiple birth and ovulation rates in sheep [4]. All these genes belong to the TGF-β superfamily (transforming growth factor beta) [5], which plays an important role in the process of embryo development, ovulation rate, and offspring number. It has been assumed that marker-assisted selection using both genes were guaranteed to increase the number of lambs per litter in ewes and will have significant economic value for sheep breeders. Numerous fertility-related polymorphisms have been identified in various sheep breeds up to this time. However, few polymorphisms have been consistently detected across different breeds. The Booroola gene polymorphism (FecB) was identified as the first major gene for fertility in sheep in 1980. Later studies have shown that ovulation rate and the number of lambs born

per litter can be genetically regulated by a number of different genes, collectively referred to as fecundity (Fec) genes [6,7]. Actually, in a study conducted by Xu et al.[8], many different alleles of the genes examined in different breeds were reported.

The sheep BMPR1B gene has 11 exons and 10 introns on Chromosome 6. Many single nucleotide polymorphisms (SNPs) have been identified for the sheep BMPR1B gene [9], among these, the c.746A>G, c. 864 T>C and c. 1,113 C>A have been associated with sheep reproduction [10,11]. A large study on the nonsynonymous SNP of c.746A>G, which was also examined in our study and characterized as the FecB allele, showed that damage to the BMP system during follicle development increased mean ovulation in Australian Booroola Merino [12], Small-tailed Han sheep [13] and Hu sheep [14]. It has also been reported that this allele has an additive effect on offspring and ovulation rate, but has negative effects on fetal growth and development and body mass during pregnancy [15]. The BMP15 gene (GDF9B, also known as the FecX gene) is on the X chromosome [2] and encodes bone morphogenetic protein 15, which plays an important role in follicular development in sheep [3]. It consists of an intron and 2 exons. BMP15 gene significantly affects fertility [16]. The same allele that causes reproduction in heterozygous Romneys is called the Inverdale allele ($FecX^{I}$). Polymorphism of this gene, identified in various breeds, are called by different names such as $FecX^{I}$, $FecX^{H}$, $FecX^{G}$, $FecX^{B}$, $FecX^{L}$, $FecX^{R}$, $FecX^{Gr}$ and $FecX^{O}$ [17-19]. Sheep with two inactive copies of the BMP15 gene (homozygous animals) have been reported to be infertile [2,3] and display a similar ovarian phenotype. It has been reported that sheep with a single inactive BMP15 gene (heterozygous animals) exhibit increased fertility and an increased ovulation rate and an increased incidence of twin or triplet births [18,20,21]. The GDF9 gene, a member of the Transforming Growth Factor Beta (TGF-β) superfamily, consists of 1 intron and 2 exons located on chromosome 5 in sheep. It is a necessary gene for regular standard follicular development in sheep. It has been reported that there are 8 different polymorphisms (G1-G8) on the gene [22]. Concerning this gene, ovulation rates in sheep are higher in animals with a heterozygous genotype (1.88-1.78) than in animals with a homozygous (1.22-1.16) genotype [22-24].

In addition to the normal genotype of the Akkaraman breed, which constitutes approximately 40% of the sheep raised in Türkiye, the Kangal Akkaraman variety, which is reared in Sivas and Malatya provinces as a local type, was registered as a separate breed with the Communiqué published in the Official Gazette dated 14.08.2012 and numbered 28384 [25]. PCR-RFLP analysis for FecB, $FecX^{G}$, $FecX^{H}$ alleles in the Kangal breed (n: 42), was performed by Karslı et al.[26] but alleles associated with multiple births could not be detected. The average twin birth rate of Kangal breed sheep has been reported as 22% [27].

In the present study, it was aimed to detect the alleles reported to be associated with twinning in the BMPR-1B, BMP15 and GDF9 genes of Kangal Akkaraman breed sheep that have given birth to at least twice twins and at least twice singletons by PCR-RFLP method and to determine the effect of the relevant SNPs on the number of lambs per birth by logistic regression analysis.

Material and Methods

Ethical Statement

This study was approved by the Erciyes University Animal Experiments Local Ethics Committee (ERU-HADYEK, Approval no: 21/243-01.12.2021).

Experimental Design and Examination of Animal Data Records

According to the results of the power analysis the number of animals was determined as 192. Taking into account the litter size record of the herds (approximately 41.000) collected from 2016 to 2022, belonging to the Public Breeding of Kangal Akkaraman Sheep in Sivas province of the Public Animal Breeding National Project, the "TAGEM/58KAK2012-08" sub-project, Data records in the relevant project were examined in Excel format. Ewes giving birth to twins at least twice and singletons at least twice were determined by sorting and filtering in Excel according to years, birth type, and mating characteristics. In order to minimize the effect of inbreeding in the selection of animals showing twin and singleton phenotypes, sampling was carried out from 15 different farms in Şarkışla and Gemerek districts. Since the districts where the farms where the samples were collected may show similar environmental (pasture) conditions, they were also classified into two groups as Location 1 (n:99) and Location 2 (n:93) for logistic regression analysis. Information on the feeding type of the farms year-round and according to the months during the year was also obtained from the public hand breeding project. According to this, it was determined that the feeding types of the farms were different in December-February, different in March, and on pasture in April-November. Therefore, farms with similar feeding conditions during the year and throughout the year were categorized into 5 groups. Related information is reported in *Table 1*.

Collection of Blood Samples and DNA Isolation

Blood samples for genetic studies were collected from the *V. jugularis* of the animals into EDTA vacuum tubes. Blood samples were delivered to the laboratory via cold chain and stored at -20°C until DNA isolation. DNA isolation was performed according to the phenol-chloroform-isoamyl

Table 1. Year-round feeding type group and seasonal feeding type groups

Location	Year-Round Feeding Type Group	First Seasonal Feeding Type Group (December-February)	Second Seasonal Feeding Type Group (March)
1 (n:99)	Group 1 (n:20)	Barley-wheat-clover (Group 1, n:20)	Barley-wheat-clover (Group 1, n:20)
	Group 2 (n:9)	Barley-wheat-triticale (Group 2, n:9)	Barley-wheat-triticale (Group 2, n:9)
	Group 3 (n:21)	Barley-wheat-grass (Group 3, n:21)	Barley-wheat-grass (Group 3, n:21)
	Group 4 (n:49)	Barley-wheat-grass (Group 3, n:49)	Concentrate feed-wheat-grass (Group 4, n:49)
2 (n:93)	Group 5 (n:93)	Barley-wheat-clover (Group 1, n:93)	Concentrated feed-wheat-clover (Group 5, n:93)

Table 2. Genes, regions, primers, band sizes list of PCR-RFLP regions

Gene	Allel	Primers (Forward- Reverse)	Restriction Enzyme	PCR product size, type	Ref.
BMPR1B	FecB	F: CCAGAGGACAATAGCAAAGCAAA R: CAAGATGTTTTCATGCCTCATCAACACGGTC	*Ava*II	Mutant: 160 bp Non-carrier: 190 bp	[6]
BMP15	$FecX^B$	F: GCCTTCCTGTGTCCCTTATAAGTATGTTCCCCTTA R: TTCTTGGGAAACCTGAGCTAGC	*BstDE*I	Wild: 122, 31 bp Mutant:153 bp	[19]
	$FecX^G$	F: CACTGTCTTCTTGTTACTGTATTTCAATGAGAC R: GATGCAATACTGCCTGCTTG	*Hinf*I	Wild type: 112, 29 bp Mutant: 141 bp	
	$FecX^I$	F: GAAGTAACCAGTGTTCCCTCCACCCTTTTCT R: CATGATTGGGAGAATTGAGACC	*Xba*I	Mutant: 124, 30 bp Non-carrier:154 bp	
	$FecX^H$	F: TATTTCAATGACACTCAGAG R: GAGCAATGATCCAGTGATCCCA	*Ah*1I	Mutant: 218, 22 bp Non-carrier: 240 bp	
GDF9	FecG	F: GAAGACTGGTATGGGGAAATG R: CCAATCTGCTCCTACACACCT	*Hha*I	AA: 410, 52 bp GG: 254, 156,52 bp AG: 410, 254, 156, 52 bp	[3]

alcohol method. Quality and quantity determination of DNAs were determined with the help of nanodrops (Shimadzu, Japan).

PCR-RFLP

The 20 µL total reaction volume of the solution used for the PCR consisted of 1 X buffer solution, $MgCl_2$ (2.0 mmol/L), 0.5 U Taq DNA polymerase, dNTP (0.25 mmol/L) and 3 µL DNA (50 ng/µL). The PCR reactions consist of 1 cycle of pre-denaturation at 95°C for 5 min, followed by 35 cycles of 30 sec at 95°C 30 sec at annealing temperature (annealing temperature specific to each primer), and 30 sec at 72°C for the elongation step. Finally, the process is terminated by waiting at 72°C for 10 min.

The PCR bands length, restriction enzymes and resulting restricted fragment length of the obtained PCR products are presented in *Table 2*. Restriction endonuclease enzyme restriction protocols of the obtained PCR products were carried out according to the manufacturer's protocol. The resulting restriction and PCR products were run through 2% agarose gel electrophoresis and visualized under UV light.

Statistical Analysis

The created logistic regression model;

$$\log\left(\frac{P(Y=1\mid X}{1-P(Y=1\mid X)}\right)=\beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_4X_4+\beta_5X_5+\beta_6X_6$$

where; Y: Birth type (1: having at least two times single births, 0: having had at least two times multiple births), X_1: BMPR1B, X_2: BMP15, X_3: genotypes of GDF9 gene and X_4: Location effect, X_5: Year-round feeding type effect, X_6: Seasonal feeding type effect.

Coefficients of univariate logistic models were calculated using the maximum probability estimation method. Data analysis was performed with the ISLR package in software R 4.1.2 *(https://www.r-project.org/)*. The enter method was used in the applied logistic regression model. The coefficients in the model were obtained using the maximum probability method. Goodness of fit was tested using the chi-square statistic. Univariate logistic regression analysis was used to identify candidate variables and multivariate logistic regression analysis was used to determine the final model. Statistical significance of variables in the multiple

model was tested using the Wald test. The value of the goodness of fit test of the final model was evaluated using the Hosmer Lemeshow test value. Whether the population was in Hardy-Weinberg equilibrium was evaluated using the chi-square statistic. The significance level was taken as P<0.25 in univariate logistic regression models and P<0.05 in multivariate logistic regression models. Chi-square analyses were also performed with phenotype and polymorphic genotype only.

Results

As a result of the PCR analysis, bands reported in the literature belonging to 6 SNPs were obtained in our study. According to the obtained PCR-RFLP Gel-electrophoresis imaging results, the FecG SNP of the GDF9 gene *(Fig. 1)* was found polymorphic, and the restriction images of the other 5 SNPs were found monomorphic. According to these findings, it was determined that $FecX^{B}$ *(Fig. 2)*, $FecX^{G}$ *(Fig. 3)*, $FecX^{I}$ *(Fig. 4)*, $FecX^{H}$ *(Fig. 5)* alleles belonging to the BMP15 gene, and the FecB *(Fig. 6)* allele belonging to the BMPR1B gene examined in all genotypes in Kangal Akkaraman breed sheep was wild type.

Since 5 of the 6 SNPs examined were monomorphic, only the FecB SNP of the GDF9 gene and the location, year-round feeding type effect, seasonal feeding type effect were examined in the logistic regression analysis. In the resulting logistic regression model, the dependent variable was birth type, while the independent variables were evaluated as farm and FecB SNP of the GDF9 gene. In the applied logistic regression model, no significant effect of farm and FecB SNP of GDF9 gene was found on birth type *(Table 3)* (P>0.05).

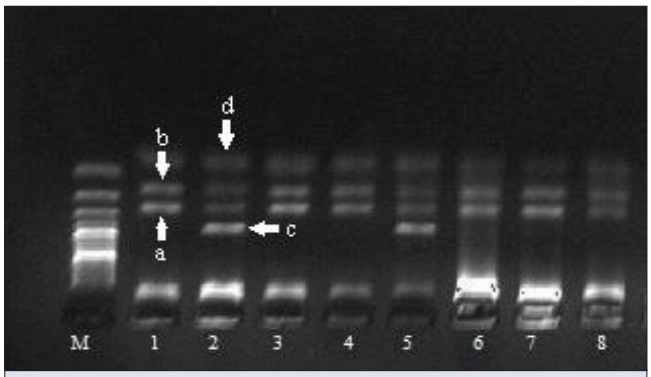

Fig 1. Gel-electrophoresis image of restricted PCR products for GDF9 gene FecG SNP; M: Marker with 100 base pairs; a:254 bp, b: 156 bp, c: 410 bp, d: 52 bp; GG: a, 3, 4, 6, 7, 8 (254, 156, 52 bp) AG: 2, 5 (410, 254, 156, 52 bp)

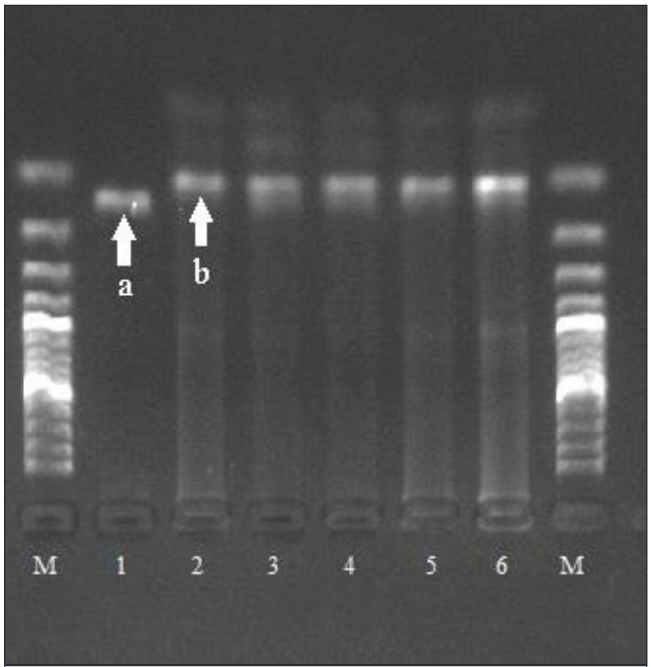

Fig 3. Gel-electrophoresis image of PCR and restricted PCR products for BMP15 gene FecXG SNP; M: Marker with 100 base pairs; a:141 bp, b: 112 bp; PCR product: 1 (141 bp); Wild type RFLP products: 2, 3, 4, 5, 6, 7 (112 bp)

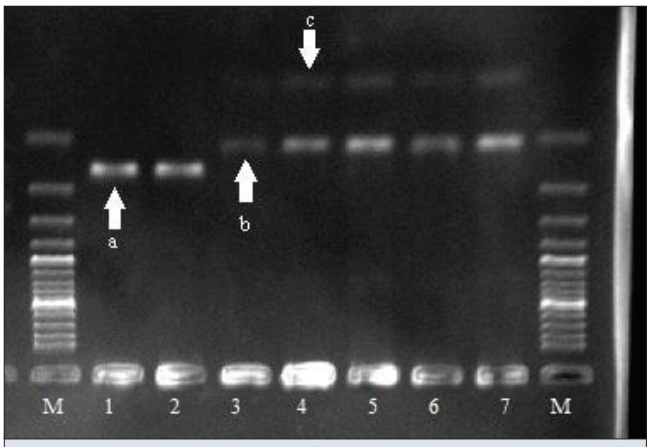

Fig 2. Gel-electrophoresis image of PCR and restricted PCR products for BMP15 gene FecXB SNP; M: Marker with 100 base pairs; a:153 bp, b: 122 bp, c: 31 bp; PCR products: 1, 2 (153 bp); Wild type RFLP products: 3, 4, 5, 6, 7 (122, 31bp)

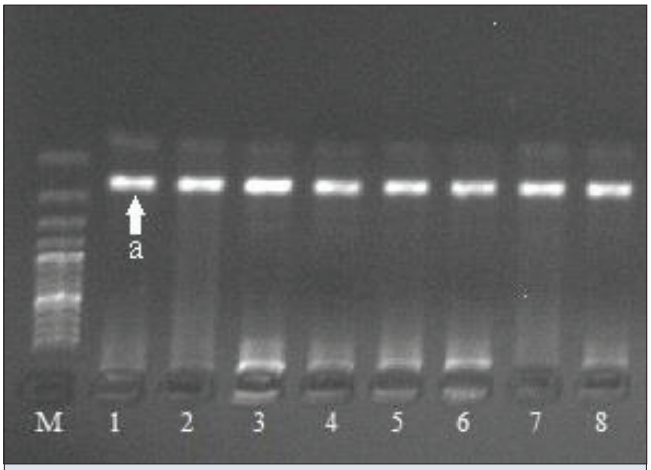

Fig 4. Gel-electrophoresis image of restricted PCR products for BMP15 gene FecXI SNP; M: Marker with 100 base pairs; a:154 bp; PCR product: 1; Non-carrier RFLP products: 2, 3, 4, 5, 6, 7, 8

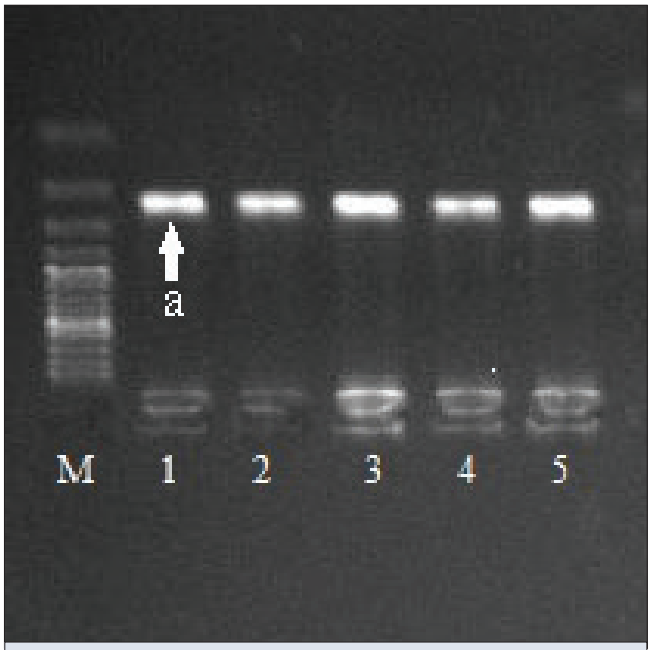

Fig 5. Gel-electrophoresis image of restricted PCR products for BMP15 gene FecXH SNP; M: Marker with 100 base pairs; a:240 bp; PCR product: 1; Non-carrier RFLP products: 2, 3, 4, 5

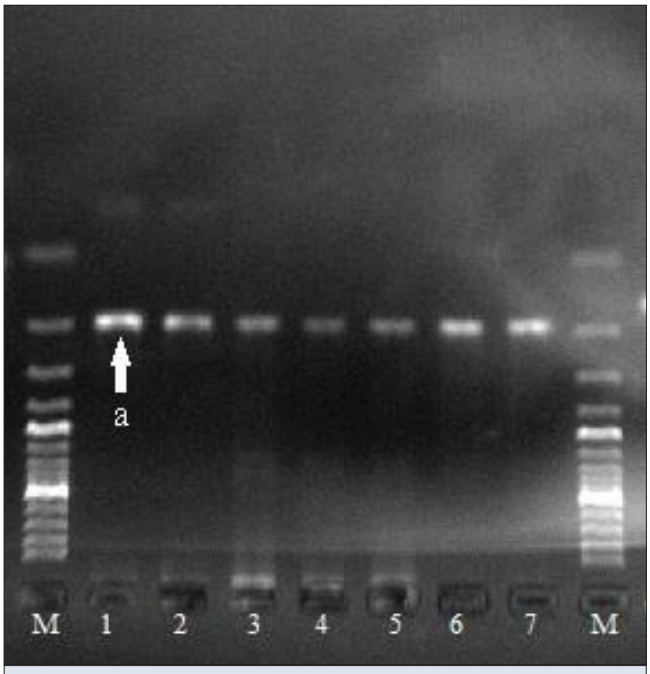

Fig 6. Gel-electrophoresis image of PCR and restricted PCR products for BMPR1B gene FecB SNP; M: Marker with 100 base pairs; a:190 bp; PCR product: 1; Non-carrier RFLP products: 2, 3, 4, 5, 6, 7

Table 3. *Regression model for birth type and genotype, year-round feeding type, seasonal feeding groups*

Independent Variables	β Coefficient	St Error	Wald Statistic	P Value	Exp(β)	95% CI Exp(β)	
						Upper Limit	Lower Limit
Genotype GG	-0.138	0.346	0.159	0.690	0.871	0.442	1.716
Location 1	-0.432	0.502	0.743	0.389	0.649	0.243	1.735.
FSFTG			1.081	0.582			
FSFTG 1	-0.538	0.540	0.992	0.319	0.584	0.203	1.682
FSFTG 2	-0.367	0.732	0.252	0.616	0.693	0.165	2.905
SSFTG			0.005	0.943			
SSFTG 3	-0.038	0.523	0.005	0.943	0.963	0.345	2.685
Constant	0.592	0.587	1.015	0.314	1.807		

FSFTG: *First seasonal feeding type group,* ***SSFTG:*** *Second seasonal feeding type group*

Table 4. *Correct classification table for birth type*

Observed		Predicted		
		Birth Type		Correct Classification Rate
		Singleton	Twin	
Birth Type	Singleton	72	24	75.0
	Twin	65	31	32.3
Total Correct Classification Rate				53.6

The correct classification rate was found to be 55.2% *(Table 4)*. As a result of the Hosmer and Lemeshow test, the chi-square statistic was found 30.681. Frequencies and Chi-square analysis results for all genes are reported in *Table 5.* For the FecB allele of GDF9 gene, the population was found to be in Hardy-Weinberg equilibrium *(Table 6)* (P=0.073). In addition, sample numbers of locations and phenotype frequencies according to GDF9 genotypes for locations are reported in *Table 7*.

Table 5. *X^2 analysis of phenotype and genotype for each gene allele*

Genes	Genotype	Phenotype		Chi-square P Value
		Twin Observed Count (Expected Count)	Single Observed Count (Expected Count)	
BMPR1B (FecB)	WW	96	96	Monomorphic
BMP15 $FecX^B$	WW	96	96	Monomorphic
BMP15 $FecX^G$	WW	96	96	Monomorphic
BMP15 $FecX^I$	WW	96	96	Monomorphic
BMP15 $FecX^H$	WW	96	96	Monomorphic
GDF9 FecG	AG	23 (22.0)	21 (22.0)	0.118 P=0.731
	GG	73 (74.0)	75 (74.0)	

Table 6. *Hardy-Weinberg equilibrium of the GDF9 gene population*

Gene	Genotypes	Expected	Observed	A Allele	G Allele	Chi-square P Value
GDF9	AA	2.5208	0	0.1146	0.8854	3.2155 P=0.073
	AG	38.9583	44 (0.23)			
	GG	150.5208	148 (0.77)			

Table 7. *Sample numbers of location groups and phenotype frequencies according to GDF9 genotypes*

Location	Phenotype			Genotype		
				AG	GG	Total
Location 1	Phenotype	Twin	Count	12	38	50
			% within genotype	54.5%	49.4%	50.5%
			Count % within phenotype	24.0%	76.0%	100.0%
		Singleton	Count	10	39	49
			% within genotype	45.5%	50.6%	49.5%
			Count % within phenotype	20.4%	79.6%	100.0%
	Total		Count	22	77	101
			% within genotype	100.0%	100.0%	100.0%
			Count % within phenotype	22.2%	77.8%	100.0%
Location 2	Phenotype	Twin	Count	11	35	46
			% within genotype	50.0%	49.3%	49.5%
			Count % within phenotype	23.9%	76.1%	100.0%
		Singleton	Count	11	36	47
			% within genotype	50.0%	50.7%	50.5%
			Count % within phenotype	23.4%	76.6%	100.0%
	Total		Count	22	71	93
			% within genotype	100.0%	100.0%	100.0%
			Count % within phenotype	23.7%	76.3%	100.0%

Discussion

Many studies on DNA regarding sheep fertility and litter size have been reported. The first studies conducted with molecular methods on this character reported that the polymorphism known as the Booroola allele was on exon 8 of the BMPR1B gene. Previous studies reported that the polymorphism of this gene was identified in 48 breeds living in 19 countries [28]. In addition, later studies have also showed the polymorphism of this gene in different breeds [29-31]. On the other hand, in some of the studies, it has been reported that this polymorphism was not

detected in some breeds [32-37]. In a study of Xu et al.[8] on different breeds with high and low offspring fertility, different candidate gene clusters related to offspring size were identified. For example, in the reviewed study, it was reported that BMPR1B, FBN1 and MMP2 gene clusters in the Wadi breed may play a role in variation according to breeds. Pourali et al.[36] reported no FecB polymorphism in Markhoz goats, but there may be new alleles in exon 8 of the relevant gene. Karslı et al.[26] reported that the FecB allele could not be detected in Kangal Akkaraman (n:42) and South Karaman (n:29) sheep. Similarly, in our study, no polymorphism related to the allele examined in the Kangal Akkaraman breed was detected. Therefore, its relationship with offspring productivity could not be determined. It was determined that all samples examined had wild type allele. Therefore, the related gene was not subjected to logistic regression analysis. It was concluded that the related allele was not found in Kangal Akkaraman breed sheep and could not be associated with twinning.

The other two genes investigated with alleles in our study were BMP15, located on the X chromosome, and GDF9 gene, located on the 5th chromosome. In the present study, the $FecX^G$, $FecX^B$, $FecX^I$, $FecX^H$ alleles located in the Exon 2 region of the BMP15 gene and the $FecG^H$ allele reported on the exon 1 region of the GDF9 gene were examined. In the literature studies reviewed polymorphisms in the BMP15 and GDF9 genes were detected in Belclare and Cambridge sheep breeds by Hanrahan et al.[3]. Polymorphisms in the BMP15 ($FecX^I$, $FecX^H$, $FecX^G$, $FecX^B$) and GDF9 ($FecG^H$) genes in Sakız, Kıvırcık, Awassi and İmroz breed sheep were examined by Gürsel et al.[32], and it was reported that the SNPs examined, except for the $FecX^G$ SNP in the BMP15 gene and GDF9 ($FecG^H$), were determined monomorphic. In the study by Mullen et al.[38] examining BMP15 ($FecX^G$, $FecX^B$) and GDF9 ($FecG^H$) in Belclare and Cambridge sheep, it was reported that $FecX^B$ was determined only in hyper productive sheep breeds and $FecG^H$ was determined in the Lleyn breed. Saleh et al.[37] reported that BMP15 $FecX^G$ and GDF9 FeG^H SNPs were detected in Rahmani and Rahmani x Barki cross. Rezaei et al.[39] in which previously reported SNPs in the BMP15 and GDF9 genes in Persian-black sheep were examined, the wild type allele frequency for GDF9 was reported as (+) (75%) and the mutant allele frequency was reported as (-) (25%). The observed frequency for GG, G+, ++ genotypes was reported as 0.05, 0.40, 0.55, respectively. In a study conducted by Kırıkçı [40] in which SNPs in BMP15 and GDF9 genes were investigated in Of sheep living in the Black Sea region, the DNA of the relevant genes was sequenced. A novel SNP (T755C) in the BMP15 gene and five known SNPs in the GDF9 gene (c471C>T (G2), c477 G >A (G3), c721 G>A (G4), c978 A >G (G5) for a total of six SNPs were defined. It was reported that Çepni and Of breeds showed a highly polymorphic structure in the examined genes. Aymaz et al.[41] detected new SNPs in addition to existing SNPs in the BMP15 (in Kıvırcık, Karacabey Merino, Sakız, Gökçeada, Çine Çaparı, İvesi and Karakaçan breeds) and GDF9 gene regions (in Kıvırcık, Karacabey Merino and Sakız breeds). Tong et al.[30] reported a study examining new variants in the promoter region of the GDF9 gene in Mongolian sheep. In addition, in a study conducted on Of sheep breed with a twinning rate of up to 35-40%, it was reported that the genotypes of the FecG1 (GDF9) allele were found to be heterozygous by the PCR-RFLP method [42]. Polley et al.[29] reported that the G1 locus of the GDF9 gene was polymorphic, and two genotypes were detected: mutant (A) and wild type (G) with allele frequencies of 0.18 and 0.82, respectively. Gorlov et al.[22] reported that when the GDF9 G1 and G4 gene regions were examined, the GDF9 gene was found to have a high frequency of G allele and GG genotype in the G1 region, and A allele and AA genotype in the G4 region. Wang et al.[31] sequenced the entire coding region of the GDF9 gene in Luzhong sheep, and reported that g.41768501A>G, g.41768485 G>A polymorphisms in GDF9 and FecB were significantly associated with litter size in Luzhong sheep. In the study conducted by Kırıkçı [43] on the Akkaraman breed with BMP15 ($FecX^G$ and $FecX^I$) and GDF9 (G1 and G4) genes; the relationship between genotypes and the number of offspring were examined (n: 100). GDF9 G1 was reported to be the only polymorphic SNP among the examined genes. It was reported that the frequencies of GA and GG genotypes were 0.26, 0.74, and A and G allele frequencies were 0.13 and 0.87. According to the association analysis, there was no statistically significant difference between the investigated SNPs and offspring size in that study. Consistent with the finding of Kırıkçı [43] obtained in the Akkaraman breed, in our study, the G1 region on the exon 1 region of the GDF9 gene in the Kangal Akkaraman breed was examined and found to be polymorphic. In our study, it was determined that the A allele frequency (mutant) was 0.1146, the G allele frequency was 0.8854, the AG genotype frequency was 0.23 and the GG genotype frequency was 0.77. According to the results of the logistic regression analysis, it was determined that the effect of the genotypes of the GDF9 gene examined in the model, together with the farm effect, on twinning was not statistically significant. According to the results of logistic regression analyses performed in the presence of environmental variables and genotypic variables, it was determined that genotype and other environmental conditions examined did not have a statistically significant effect on the dependent variable of fecundity. It was determined that in-season and year-round feed type changes in the sample examined in this character, which is especially affected by environmental

effects and the effect of multiple genes, were not effective on the twinning phenotype. According to obtained results, the proportion of animals showing twinning phenotype with AG genotype was determined as 22.2% and 23.7% in locations 1 and 2, respectively. The fact that the farms had no effect on the twinning phenotype suggested that sampling was carried out under similar care and feeding conditions and the variation observed in twinning may be due to different genetic variations. By taking samples from different farms, it was aimed to minimise the genetic relationship and to ensure impartiality in the evaluation of the results. It was also concluded that the obtained results when the FecB allele of the GDF9 gene was subjected to Chi-square analysis only with the twin phenotype was not statistically significant *(Table 5)*.

In Cele Black breed sheep, a SNP was detected at position L251P on the Exon 2 region of the BMP15 gene by Niu et al.[44]. This SNP has been shown to be a significant mutation affecting fertility in Cele Black breed sheep. A 3 bp (CTT) deletion was detected in exon 1. In a study performed by Davis et al.[20], no $FecX^{I}$ allele of the BMP15 gene in any of the tested sheep was reported. Karsli et al.[45] examined the sheep breeds of Akkaraman (24 samples), Dağlıç (19 samples), İvesi (19 samples), Tuj (15 samples) and Karakaş (19 samples) bred in Turkey, and they reported that the polymorphisms in the BMP15 gene ($FecX^{G}$, $FecX^{I}$, $FecX^{H}$, $FecX^{B}$) were monomorphic. In a study conducted on Malin and Dorper sheep by Somarny et al.[34], $FecX^{I}$, $FecX^{H}$, $FecX^{B}$ and $FecX^{G}$ polymorphisms were not determined. In the PCR-RFLP study conducted on the Awassi breed (n=88), the $FecX^{I}$ (Inverdale) allele was investigated, but no polymorphism was detected [46]. In a study performed by Karslı et al.[26] to examine the $FecX^{G}$, $FecX^{H}$ alleles in Kangal (n: 42) and South Karaman (29) sheep. Also another study of Karslı and Balcıoğlu [33] to examine the $FecX^{G}$, $FecX^{H}$, $FecX^{I}$, $FecX^{B}$ alleles in Akkaraman (24 samples), Dağlıç (19 samples), İvesi (19 samples), Tuj (15 samples) and Karakaş (19 samples) sheep breeds. In two study, it was reported that the relevant alleles could not be detected. Also, in our study, four alleles of the BMP15 gene were found as monomorphic wild type. This suggests that the analysed allele has no effect on the twinning phenotype in Kangal Akkaraman breed. Ghoreishi et al.[47] examined the BMP15 and GDF9 genes in Markose goats. They reported that two new mutations were discovered in the relevant genes, which were related to the number of offspring. In a study conducted by Çelikeloğlu et al.[48] , BMPR1B (Exon 9, 10, 13a, 13b), BMP15 (Exon 1, Exon 2) and GDF9 (Exon 1 and exon 2) genes in Pırlak sheep were found that relevant regions of all three genes have monomorphic structures. In a study conducted by Çelikeloğlu et al.[49] on BMPR1B, BMP15 and GDF9 genes from Ramlıç and Dağlıç local breeds, it was reported that many SNPs were detected in the sequencing study of the relevant genes in sheep that gave 60 single births and 60 twin births. These authors detected 36, 4 and 11 SNPs in the GDF9, BMPR1B and BMP15 genes in Ramlıç breed and 40, 3 and 11 SNPs in Dağlıç breed. A total of 16 SNPs in the Ramlıç breed and 10 SNPs in the Dağlıç breed were significant for three genes. Ultimately, from the analysis, four SNPs (g.49496G>A, c.1658A>C, c.2037C>T, c.2053C>T) were shown to exist in the BMPR1B gene and one deletion mutation (c.28-30delCTT) in the BMP15 gene. These authors determined five SNPs (c.1487C>A, c.2492C>T, c.2523G>A, c.2880A>G and c.2763G>A) of the BMPR1B gene of the Dağlıç breed as well as the Ramlıç breed. They suggested that the observed polymorphisms have the potential to be used as genetic markers in the selection of productive animals for both breeds.

It is of great importance to characterize the birth type characteristics that will contribute economically to our country's domestic sheep assets. According to all these literature studies and the results obtained from the present study, it can be seen that the variations of the genes examined vary in each breed. Accordingly, among the 6 SNPs examined in our study, only the GDF9 gene-FecB SNP was determined polymorphic. Genotypic effect (FecB allele) and environmental effect variables (location, year-round feeding type, seasonal feeding type) were also examined with logistic model. It was determined that the relevant alleles and environmental variables did not have a statistically significant effect on the twinning phenotype. According to the obtained results, it was thought that the genes associated with multiple births in Kangal Akkaraman breed may have different variants specific to the breed. In addition, it is suggested that this character, which is affected by multiple genes, should be included in the planned breeding studies by considering the interaction of environmental variables and determining the variation of the related genes. In this respect, it is concluded that our study will guide the sequencing studies and multivariate analyses to be planned.

Declarations

Availability of Data and Materials: The dataset used in the study is available from the corresponding author (E.G. Aksel) on reasonable request.

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Ethical Statement: This study was approved by the Erciyes University

Animal Experiments Local Ethics Committee (ERU-HADYEK, Approval no: 21/243-01.12.2021).

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Author Contributions: EGA, EÇG, MA, ÖOD contributed to the conceptualization, design, funding and supervision of the study. EGA conducted all experiments and wrote the first draft of the manuscript. MA, EÇG, ÖOD collected, analyzed and interpreted the data. All authors contributed to the critical revision of the manuscript and have read and approved the final version.

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