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Polymorphism of the Calpastatin (CAST) and Growth Differentiation Factor 9 (GDF9) genes in Akkaraman Sheep Breed

Akkaraman Koyun Irkında Kalpastatin (CAST) ve Büyüme Farklılaşma Faktörü 9 (GDF9) Genlerin Polimorfizmi

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Akkaraman sheep, *CAST/Msp*I, *GDF9/Hhal*, PCR-RFLP

Anahtar Kelimeler:
Akkaraman koyunu, CAST/*Msp*I, GDF9/*Hhal*, PCR-RFLP

ABSTRACT

Objective: In this study, the gene polymorphisms of Calpastatin (CAST) and Growth Differentiation factor 9 (GDF9) were determined in Akkaraman sheep breed.

Material and Methods: Genomic DNA was obtained from blood samples of 50 Akkaraman sheep. All samples for *CAST* and *GDF9* were genotyped by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method using the enzymes *Msp*I and *Hha*I.

Results: Three (MM, MN, and NN) and two (GG and GA) genotypes, respectively, were detected at the *CAST* and *GDF9* genes. The frequencies of the genotypes MM, MN and NN were determined to be 44 %, 54 % and 2 %, respectively. The frequencies of the GG and GA genotypes were found to be 84 % and 16 %, respectively. The AA genotype was not found.

Conclusion: The study showed for the first time that the Akkaraman breed carries the *GDF9* G1 mutation and has sufficient variation in the *CAST*. Previous studies provided that both genes can be used as biomarkers for increasing lambing yield and growth rates. Therefore, in order for the studied genes to be used as biomarkers in the Akkaraman breed, association studies should first be performed.

ÖZ

Objective: Bu çalışmada, Kalpastatin ve Büyüme Farklılaşma Faktörü 9 gen polimorfizmleri Akkaraman koyun ırkında belirlenmiştir.

Materyal ve Metot: Genomik DNA 50 baş Akkaraman koyun ırkına ait kan numunelerinden elde edilmiştir. Kalpastatin ve Büyüme Farklılaşma Faktörü 9 genleri için tüm örnekler, sırasıyla *Msp*I ve *Hha*I enzimleri kullanılarak Polimeraz Zincir Reaksiyon- Restriksiyon Parça Uzunluk Polimorfizm yöntemi ile genotiplendirilmiştir.

Bulgular: Kalpastatin ve Büyüme Farklılaşma Faktörü 9 geninde sırasıyla üç (MM, MN ve NN) ve iki genotip (GG ve GA) grupu tanımlanmıştır. Kalpastatin locusunda, MM, MN ve NN genotiplerin frekansları sırasıyla % 44, % 54 ve % 2 olarak belirlenmiştir. Büyüme Farklılaşma Faktörü 9 lokusunda ise GG ve GA genotiplerin frekansı ise % 84 ve % 16 olarak bulunmuştur. AA genotipine ise rastlanılmamıştır

Sonuç: Çalışma ilk defa Akkaraman koyun ırkının GDF9-G1 mutasyonunu taşıdığını ve CAST geni bakımından yeterli derecede genetik varyasona sahip olduğunu göstermiştir. Önceki araştırmalar her iki genin kuzu verimi ve büyüme oranlarının arttırılabilmesi amacıyla biyomarkör olarak kullanılabileceğini göstermiştir. Sonuç olarak, incelenen genlerin Akkaraman ırkında biyomarkör olarak kullanılabilmesi için öncelikle ilişkilendirme çalışmalarının yapılması önerilmektedir.

INTRODUCTION

Global consumption of sheep meat is increasing in parallel with the rise of human population. In 2029, the average per capita consumption of sheep meat is expected to reach 4.2 kg (OECD, 2021). To meet the demand for sheep meat, the main focus is to increase the number and yield of lambs (Hossain et al. 2020). Therefore, studies to increase the number of lambs or the twin rate per sheep and to improve live weight are likely to become more important in the future. To achieve the desired level of production, genetic improvement studies need to be conducted worldwide, as well as conventional breeding methods. In this sense, it can be said that polymorphism studies, which provide information on the genetic variation of a particular trait, are important as a starting point.

The reproductive and developmental performance of an animal is the result of its genotypic structure and environmental effects (Gbangboche et al. 2006). Although growth and litter size in sheep are influenced by many genes with minor effects as well as environmental conditions, there are important genes that have a significant effect on these traits. The Calpastatin (*CAST*) and Growth differentiation factor 9 (*GDF9*) are some of the most investigated genes for the traits of growth rate and litter size in sheep.

The *CAST* gene, mapped on sheep chromosome five, is a specific inhibitor of the enzyme calpain, which regulates muscle tenderness after animals have slimmed down. Calpain plays a role in the breakdown of muscle structure in mammals and the gene *CAST* shows its effect as disrupting the activity of calpain (Bozhilova-Sakova et al. 2020). The *CAST* gene is one of the most studied genes for meat quality to improve because of its effects on meat tenderness (Jawasreh et al. 2019). Due to its role, studies of genetic variations within the *CAST* gene are of interest to researchers in farm animals.

The *GDF9* gene in sheep, a member of the transforming growth factor (TGF-B) family and is located on chromosome 5, has been shown to have an effect on primordial follicle development and granulose cell proliferation (Abdoli et al. 2016). To date, eight different mutations (G1 to G8), five of which alter the amino acid sequence, have been reported by Hanrahan et al. (2004). Many studies have demonstrated that ewes carrying a heterozygous mutant allele of the GDF9 gene have a higher litter size than homozygous ones. Hossain et al. (2020) reported ewes with AA genotype had the highest litter size than in ewes with GG genotype (2.00 vs. 1.59) in indigenous sheep of Bangladesh. Due to the functional properties of the CAST and GDF9 genes, they are of interest to the scientific community.

Although several major mutations associated with litter size have been investigated in native Turkish sheep breeds, no study investigated the G1 mutation in exon 1 of the *GDF9* gene as well as there are few studies on the CAST gene in the Akkaraman breed. Therefore, the current study aimed to investigate the presence of the *GDF9*-G1 mutation and genetic polymorphism for the CAST gene in the Akkaraman sheep breed.

MATERIAL and METHOD

Blood sample's collection and DNA isolation

Blood samples were collected using vacuumed tubes with K2-EDTA from 50 Akkaraman breed sheep from seven distinct flocks belonging to two subprojects (TAGEM/66 AKK2011-01 and AKK2012-02) in the Yozgat (Ethical approval number: 2021/3, The Ethics Committee of Ahi Evran University, Kırşehir, Türkiye) region supported by the General Directorate of Agricultural Research and Policy (TAGEM). DNA was obtained from the whole blood using a DNA extraction kit according to the manufacturer's instructions.

PCR analysis

A 622 bp fragment of the *CAST* gene and 462 bp of the *GDF9* gene exon 1 were amplified by polymerase chain reaction (PCR) using the primers in table 1.

Table 1. Primer sequences used in the study

Çizelge 1. *Çalışmada kullanılan primer dizileri*

Gene	Primers	References
CAST	Forward; 5'TGGGGCCCAATGACGCCATCGATG3' Reverse; 5'GTGGAGCAGCACTTCTGATCACC3'	Palmer et al. (1998)
GDF9	Forward; 5'GAAGACTGGTATGGGGAAATG3', Reverse; 5'CCAATCTGCTCCTACACACCT3'	Kasiriyan et al. (2020)

PCR reactions were performed in a final volume of 25 µl, including 13 µl Taq DNA polymerase master mix red (2x), 1 µl of each primer (10 pmol/ µl), and distilled water to final volume. PCR conditions were performed as described: predenaturation at 95°C for 5 minutes, followed by denaturation at 95°C for 1 minute, annealing at 63 °C for the CAST and 60 °C for the GDF9 genes, 72 °C for 2 minutes extension, and final extension at 72 °C for 7 minutes.

Genotyping of the CAST and GDF9 genes.

All PCR products of the genes studied were screened with restriction enzymes to identify the possible genotypes. Genotyping for the CAST or GDF9-G1 gene was performed in a final volume of 30 µl consisting of 10 µl PCR product, 2 µl green buffer, 1 µl enzyme (*Msp*I / *Hha*I) and distilled water to the final volume. The reaction mixture was incubated at 37 °C for 20 minutes and then inactivated at 65 °C for 10 minutes. After digestion, samples were run in 3% agarose gel electrophoresis and genotypes were screened with EtBr (500 µL/mL in H_2O) under UV transilluminator.

Statistical analysis

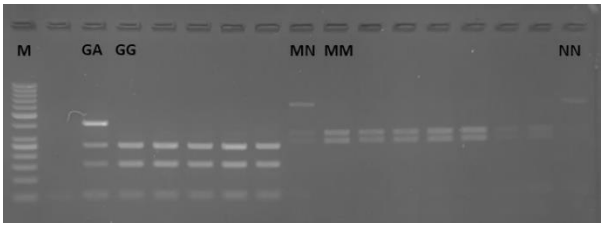
PopGene32 software was used to determine allele and genotype frequencies, as well as observed and expected heterozygosity levels of the genes studied (Yeh et al. 1997). The chi-square test was used to analyze whether the studied population were in Hardy-Weinberg equilibrium.

RESULTS

The present study investigated the *CAST* gene polymorphism and, for the first time, the *GDF9*-G1 polymorphism in the Akkaraman sheep breed. PCR-RFLP technique was used to determine possible alleles and genotypes of the studied genes.

A 622 bp fragment of the *CAST* gene was successfully amplified by PCR and all samples were subjected to the restriction enzyme *Msp*I to find possible genotypes of the Akkaraman breed. The results showed two alleles (M and N) and three genotypes (MM, MN and, NN) in Figure 1.

The frequency of heterozygous genotypes with three fragments (622, 336 and 286 bp) was 0.54, while the frequency of homozygous MM (336 and 286 bp) was 0.44. The NN genotype was observed in only one individual with a frequency of 0.02 in Table 2. The frequency of M and N alleles were 0.68 and 0.32, respectively.



M: 50 bp ladder

Figure 1. The gel image of the identified genotypes for the genes of *CAST* and *GDF9*.

Şekil 1. *CAST ve GDF9 genleri için tanımlanan genotiplere ait jel görüntüsü.*

Table 2 shows the frequencies of allele and genotype and heterozygosity values in Akkaraman sheep.

Observed (Ho) and expected (He) heterozygosity values for the *CAST*/*Msp*I were 0.5400 and 0.4160. The Chi-square results showed that the studied population was not in Hardy-Weinberg equilibrium (HWE) for the *CAST*/*Msp*I in Table 2.

A 462 bp fragment of *GDF9* exon-1 was amplified by PCR and then digested with the restriction enzyme *Hha*I. The results showed four fragments with sizes of 52, 156, 254, and 410 bp. The ewes that did not carry the mutation had three banding patterns, 52, 156, and 254 bp, while the heterozygous ones (GA) that carried the mutation had four banding patterns in Figure 1.

Two genotypes for the *GDF9* gene were found, GG and GA with frequencies of 0.84 and 0.16, respectively, in the Akkaraman sheep breed in Türkiye. The genotype AA was not observed in the study. The frequencies of the A and G alleles were 0.92 and 0.08, respectively, and the population was in HWE for the *GDF9*-G1 polymorphism (Table 2).

Table 2. The allele and genotype frequencies and the heterozygosity values

Çizelge 2. *Allel ve genotip frekansları ile heterozigozite değerleri*

Gene	N	Allele frequency		Genotype frequency			Heterozygosity		Chi-squared (df=1)
		M	N	MM	MN	NN	Ho	He	X^2=4.5774
CAST	50	0.68	0.32	0.44	0.54	0.02	0.5400	0.4160	P=0.032
		G	A	GG	GA	AA	0.1600	0.1187	X^2=0.3278
GDF9	50	0.92	0.08	0.84	0.16	0.00			P=0.5669

DISCUSSION and CONCLUSION

Fertility and body weight are characteristics composed of many genes and affected by environmental factors. It is well known the improvement of these quantitative traits is limited by conventional methods due to their inheritance pattern, expressed in later life, low heritability and time-consuming nature (Calus et al. 2013). Genetic progress of litter size and growth rate by the conventional breeding methods is varied from 1 to 2% as for many other quantitative traits (Bradford, 1985). It is important to reveal genetic variability and detect major mutations in economically important traits before making a decision on genetic improvement of a breed. Therefore, the objective of present study was to reveal the genetic polymorphism of two important traits, litter size and body weight, in Akkaraman sheep breed.

The frequency of heterozygous individuals was 0.54, indicating reasonable genetic variability within the *CAST* gene, in Akkaraman sheep. This could be due to the fact that the animals originated from national genetic breeding flocks with rams rotating for at least two to three years.

The *CAST* gene has been extensively studied in Turkish native sheep breeds, whereas studies investigating its effects on meat quality and yield have been relatively rare (Kırıkçı et al. 2021; Bayram et al. 2019; Yılmaz et al. 2014a; Balcıoğlu et al. 2014).

In the present study, the MN genotype's frequency of *CAST*/*Msp*I was highest in the studied breed. This frequency value was also higher than in previously reported for several Turkish sheep breeds; Akkaraman, Kıvırcık, Karayaka, İmroz and Hemsin breeds (Kırıkçı et al. 2021; Bayram et al. 2019; Avanus, 2015), while it was similar in Karakul and Kıvırcık sheep (Avanus, 2015). The frequency of ewes with homozygous MM genotype was lower than in some Turkish sheep breeds (Bayram et al. 2019; Avanus, 2015; Yılmaz 2014b), in Indian Nellore Brown and Palla breeds (Ramadevi et al. 2020) and in Russian sheep breed (Kulikova et al. 2018). According to obtained results from this study, it can be said that the frequency of heterozygous ewes for the *CAST* /*Msp*I gene is higher than the frequency of heterozygous ewes in most of the breeds mentioned above and in the study of Avanus (2012).

Genetic variability is important for maintaining and improving various quantitative traits and must be present in both breeding programs (Hill, 2000) and association studies. Thus, it could be concluded that the current study provides an important opportunity for association studies in Akkaraman as it provides evidence of reasonable genetic variation for *CAST* /*Msp*I.

The population was not in HWE for the *CAST*/*Msp*I polymorphism ($P < 0.05$). One of the possible reasons for this result could be one or more factors affecting the Hardy-Weinberg equilibrium, such as migration, mutation, etc. Moreover, since the study was performed on animals from national breeding herds, the probable cause could be controlled mating. Similar results were also observed in Karayaka, Morkaraman and İvesi sheep breeds (Balcıoğlu et al. 2014).

The observed heterozygosity value was similar to some Turkish sheep breeds; Sakız, Karakul, Kivircik and Bulgarian Merino sheep (Bozhilova-Sakova et al. 2020; Avanus, 2015; Yilmaz et al. 2014b). The observed heterozygosity value for Akkaraman was also higher than the values reported for some Turkish sheep breeds: Karayaka, Hemşin, Imroz, Red Karaman (Avanus, 2015) and Colombian Creole hair sheep (Montes et al. 2019). Jawasreh et al. (2017) reported that Awassi lambs with genotype MN had higher average daily gain (0.167 vs. 0.128 kg/d) and body weight (32.31 vs. 31.78 kg) than lambs with genotype MM.

Several studies of multiple births have been conducted in indigenous Turkish sheep breeds (Gursel 2011; Karslı et al. 2011; Karsli et al. 2012). However, the Akkaraman breed has hardly been studied for major mutations compared to other indigenous breeds. The lack of study of this breed might be due to the fact that it is a nonprolific breed.

Akkaraman sheep is one of the most commonly reared breeds in Türkiye and accounts for 40-45% of the total sheep population. It has a fat-tailed structure and high adaptability to different climates. The twin birth rate of Akkaraman has been reported in the range of 13.5% to 37% (Ceyhan et al. 2019; Aktaş et al. 2016; Tekerli et al. 2002). Multiple birth is one of the most desired traits, especially in breeds with low fertility. The studied breed in this study, Akkaraman, is one of the breeds with low fertility. It is known that some farmers are interested in twin births, although they study on breeds with low fertility. Therefore, to determine the polymorphism of the GDF9 gene, the current study first aimed to work with the animals of farmers who want to increase the rate of twin births.

In the *GDF9* exon 1 gene of Akkaraman sheep, GA and GG genotypes were detected with frequencies of 0.16 and 0.84, respectively. The frequency of heterozygous genotypes, indicating low frequency, was similar to

other studies (0.12-0.16) (Kirikci et al. 2021; Gorlov et al. 2018; Eghbalsaied et al. 2017) and was monomorphic in the study by Aboelhassan et al. (2021). Obtained frequency result for GA genotype was lower than the one reported in Garole sheep (Polley et al. 2010). Some studies have shown that the ewes with heterozygous genotype produce more lambs despite having low frequency of GA genotype (Gorlov et al. 2018; Moradband et al. 2011). On the other hand, some studies did not find an association between ewes with heterozygous genotypes for this gene and multiple births for various reasons (Eghbalsaied et al. 2017). In contrast to this result, a study showed that ewes with homozygous genotype had a higher lambing rate. It can be inferred that the genotypic structure and its effects on lambing rates varies according to the sheep breeds studied. Therefore, it is necessary to demonstrate associations between genotype and phenotype at the breed level before making decision on genomic selection. In a study which carried out by Aboelhassan (2021), it was suggested that selection for *GDF9* exon-1 gene might increase the lambing rates per ewe.

In the present study, the animals with homozygous AA genotype for *GDF9*-G1 were not detected, which is in agreement with the results of other studies conducted on different breeds of sheep from Russia (Salsk and Volgograd), India (Garole), Iran (Lori-Bakhtyari), Türkiye (Karayaka) and Egypt (Barki, Osseimi, Rahmani, Saudanez and Awase) (Aboelhassan et al. 2021; Kirikci et al. 2021; Gorlov et al. 2018; Eghbalsaied et al. 2017; Koloskov et al. 2015).

Observed (Ho) and Expected (He) heterozygosity values for *GDF9*/ *Hha*I were calculated as 0.1600 and 0.1187, respectively. The population was in HWE for the *GDF9* gen (P >0.05). Expected heterozygosity value, an indicator of genetic variation, was low for the Akkaraman sheep breed.

Genetic variations on genes could alter the structure and functions of genes and the affinity of binding to their receptors. For this reason, studies investigating polymorphism, especially in the genes of traits that are economically important in sheep, are important for future breeding strategies. The number of samples used in the study constitutes the limitations of the research. However, it is important to note that the current study showed that the Akkaraman breed has the G1 mutation in exon 1 of the *GDF9* gene and a reasonable genetic variation of the *CAST* gene compared to other native sheep breeds. Consequently, it is recommended to perform association studies with a larger number of samples for both genes in Akkaraman sheep breed.

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