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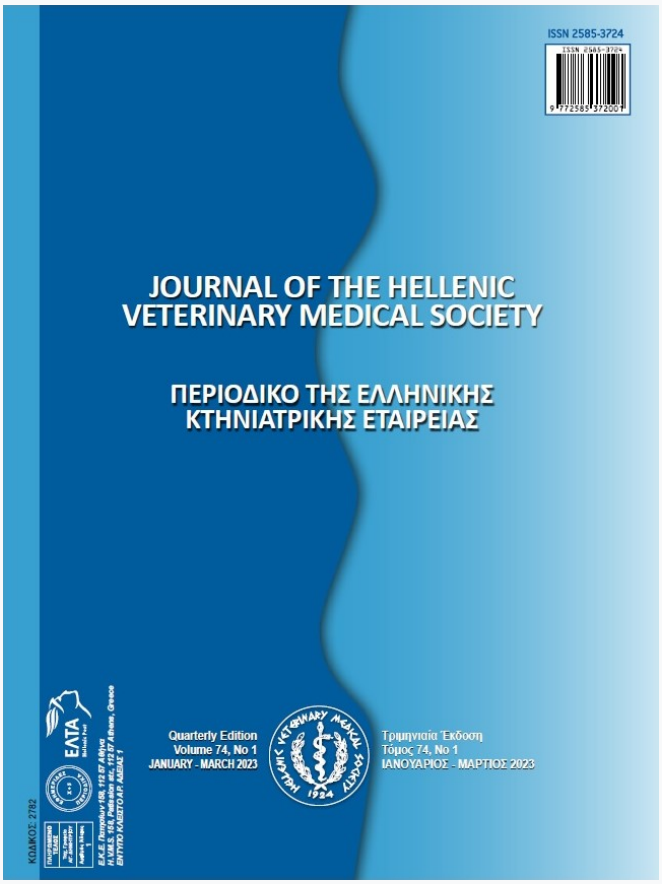

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Research article
Ερευνητικό άρθρο

Determinatıon of the *MSTN/Hae*III gene polymorphism in indigenous Morkaraman sheep

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ABSTRACT: Polymorphic information on native gene resources determined by molecular methods can play a leading role in breeding future new breeds. The myostatin gene, an important gene in sheep breeding, has a polymorphism in the third exon. The present research aimed to investigate the polymorphic structures of the myostatin (*MSTN*) gene locus in indigenous Morkaraman sheep and reveal the distribution of genotype and allele frequencies. The *MSTN*/*Hae*III gene polymorphisms were detected in the DNA that was isolated from blood samples collected from 262 Morkaraman sheep utilized in the research by employing the PCR-RFLP method. The Hardy-Weinberg genetic balance test showed that the distribution of genotype frequencies was not balanced ($P<0.01$) in the studied population. The MM, Mm, and mm genotype frequencies of the *MSTN* gene in the population were determined as 11.1%, 62.6%, and 26.3%, respectively, whereas the frequencies of the M and m allele were 0.42 and 0.58, respectively. The observed heterozygosity (Ho) calculated for *MSTN* in the whole population was significantly higher than the expected heterozygosity (He) values. The PIC values of the studied population showed a moderate polymorphism with an average of 0.37. While the F_{IS} value was found as -0.312, the F_{IT} value was determined as -0.303. The genotype and allele frequencies found with regard to the *MSTN* gene polymorphism were sufficient in order to reveal the breed's genotype diversity.

Keywords: *MSTN*, polymorphism, PCR-RFLP, Morkaraman, sheep

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INTRODUCTION

The existence of sheep and their yield potential with a very significant position in the livestock sector are of great importance in meeting the nutritional needs of the increasing world population. Fat-tailed breeds constitute approximately 87% (25.4 million heads) of Turkey's sheep population. The sheep population in the eastern and northeastern regions of Turkey is mainly comprised of the indigenous Morkaraman breed. It is considered that the mentioned sheep have evolved through natural selection under severe environmental conditions. Morkaraman sheep is a strong and large breed resistant to environmental conditions as a domestic gene source, with high adaptability. Breeders use the Morkaraman breed for meat, milk, and wool production (Turkyilmaz and Esenbuga, 2019).

The first issue emphasized in breeding studies based on yield in livestock is to provide ideal environmental conditions that will reveal the yield potential of the animal's genotype. On the other hand, although the efforts to improve the genotype in yield increase are more effective, profitable, and continuous, it is more difficult, specific, and time-consuming to implement them. It is possible to create a superior phenotype in animals by detecting good and productive genes that impact the character, increasing the frequencies of the wanted genotypes of particular genes of interest and using the interaction between genes. Since the characters of the yield acquired from farm animals are both controlled by many genes and are considerably influenced by environmental factors, the genotypic value is frequently not reflected by the phenotypic value in the said characters, and phenotype-based selection also decreases productivity (Özdemir et al., 2021).

Technological advancements in the area of molecular genetic techniques and analysis in recent years have made it possible to examine genes effective on quantitative characters, especially in farm animals, at the molecular level (Mahmoud et al., 2020; El-Komy et al., 2021; El-Magd et al., 2021; Sonmez and Özdemir, 2021). Molecular biology techniques offer opportunities to determine genetic variation at specific loci and research the relationships between variation in QTL (quantitative trait locus) and yield traits. The aim is to estimate the animal's genetic value with higher accuracy and increase the genetic gain through selection. To this end, it seems necessary to use MAS (marker assisted-selection). As a matter of fact, studies have stated that genetic variations on genes that impact phenotype-related physiological events can affect quantitative variations in the relevant phenotype (El-Magd et al., 2017; Raina et al., 2020; Özdemir et al., 2021). The purpose of MAS is to improve selection response. The successful application of this type of QTL in selection programs requires identifying the specific polymorphisms that are responsible for the effect observed (El-Magd et al., 2016; Özdemir et al., 2021).

GDF (growth and differentiation factor) has a regulatory effect on growth and differentiation events. GDF-8, one of the subtypes of GDF, is also expressed as the myostatin (*MSTN*) gene. Especially synthesized in skeletal muscles, myostatin inhibits muscle development. It was revealed for the first time that the myostatin gene affects skeletal muscle growth in mice (McPherron et al., 1997). Although mutation in the myostatin gene also causes double muscle syndrome, it is shown as one of the genes affecting the meat performance of animals (Allais et al., 2010; El-Magd et al., 2019). The sheep (Ovis aries) *MSTN* gene exists in chromosome 2 as three exons and two introns (Rodgers and Garikipati, 2008). A single nucleotide polymorphism has been detected in the *MSTN* exon3 and is characterized by the *Hae*III restriction enzyme cutting site. Extensive studies have been recently conducted on this polymorphic region of the myostatin gene, which is important in sheep. In their study on Sanjabi sheep in Iran, Soufy et al. (2009) observed all 3 genotypes in the myostatin polymorphism in exon 3 and found MM, Mm, and mm genotype frequencies as 2.00%, 1.33%, and 96.70%, respectively. Jamshidi et al. (2014) found the frequencies of Mm and mm genotypes as 0.53 and 0.47, respectively, in the Mehreban sheep breed, while Akbari et al. (2015) determined the frequencies of Mm and mm genotypes as 0.15 and 0.85, respectively, in Kordi sheep. Dimitrova et al. (2016) detected MM, Mm, and mm genotype frequencies in Bulgarian Merino sheep as 2.00%, 1.33%, and 96.70%, respectively, whereas Lazar et al. (2016) found mm and Mm genotype frequencies as 16.67% and 83.33% in Black-Headed Teleorman sheep, and Bayraktar (2020) reported the mm and MM genotype frequencies of 0.85 and 0.15 in the Awassi ram breed in Iraq. In contrast, Azari et al. (2012) in Dalagh sheep, Dehnavi et al. (2012), in Zel sheep, Bozhilova-Sakova et al. (2016) in Bulgarian Karakachan sheep, and Dimitrova et al. (2017) in Bulgarian Karnobat Merino sheep reported that the myostatin gene was in the mm genotype and monomorphic.

Sheep breeding has an important share in animal production. Due to its proximity to the Fertile Crescent, the first domestication region, it is important to conduct genetic studies on the Morkaraman native sheep breed as a gene resource and introduce them to the literature. Therefore, the primary purpose of the present research was to examine the polymorphic structures of the myostatin (*MSTN*) gene locus *Hae*III belonging to the Morkaraman sheep breed, the dominant indigenous breed of the eastern regions of Turkey, and reveal the distribution of genotype and allele frequencies.

MATERIALS AND METHODS

The Republic of Turkey Ministry of Ataturk University, Agriculture Faculty Local Ethics Committee (AEC approval number: 3/2021) approved the experimental protocol of this study.

Sampling and DNA isolation

The collection of blood samples was performed in 10 ml vacuum tubes containing K3EDTA from the vena jugularis of 262 heads of unrelated Morkaraman sheep, as 135 reared in the Food and Livestock Application and Research Center (GHUAM), Sheep Breeding Branch at Ataturk University, 66 reared on private sheep farms in Erzurum province and 61 in Bingöl province. Genomic DNA samples were isolated following the manufacturer's procedure using the Qiagen Genomic DNA Purification Kit (Qiagen Inc., Chatsworth, CA, USA). The quality of every DNA sample was determined by a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). DNA was diluted to 50 ng/µl and kept at 4 °C. Genomic DNA isolation procedures were repeated for the samples whose DNA quality and purity measurement results did not reach the desired level.

Polymerase chain reaction (PCR)

The primer pairs for the sheep *MSTN* gene were as follows: forward primer; 5´- CCG GAG AGA CTT TGG GCT TGA-3´ and reverse primer; 5´- TCA TGA GCA CCC ACA GCG GTC-3´ (Smith et al., 1997). The PCR reaction was carried out in 4 µl of 10x buffer, 1 µl (10 pmol) of each primer, 0.5 µl of $DNTP_s$, 1 µl of $MgCI_2$, 2.4 µl of Taq DNA polymerase (BioLabs, M0273L), 2 µl (50-100 ng/µl) of DNA and finally added ultrapure water until a total volume of 20 µl. The PCR program was run with an initial denaturation step at 94 °C for 5 min, followed by 34 cycles of denaturation at 94 °C for 45 s, annealing at 59 °C for 45 s, and an extraction step at 72 °C for 45 s, followed by a final extension step at 72 °C for 7 min. The PCR products of the *MSTN* gene were run on 1%-agarose gel electrophoresis and checked under UV light.

Genotyping of the *MSTN* gene

The PCR products of the amplified *MSTN* gene were digested by the *Hae*III restriction endonuclease enzyme (BioLabs, R0108S). The incubation of the PCR products was carried out for a period of 10-12 h at a temperature of 37 °C in a final volume of 20 µl, including 8-10 µl of the PCR product, 5 µl of the buffer R, 2.5 µl of the buffer Tango, and 6 U *Hae*III restriction enzyme. The digested PCR products were run to 2-2.5% agarose gel electrophoresis stained with Ethidium bromide (500 µ/ml in H2O) up to 2.5 h, and the digested PCR products were genotyped under UV light.

Data analysis

The *MSTN* locus allele gene and genotype frequencies of the examined Morkaraman sheep breed, HW genetic balance test of genotype frequencies, observed (Ho) and expected (He) heterozygosity levels of the population, polymorphism information content (PIC), effective allele number (Ne), within-population (FIS) and between-population (FST) genetic difference values, and statistical analyses were computed in the GenAlEx 6.5 (Peakall and Smouse, 2012) package program and by utilizing the GDIcall Online Calculator (http://www.msrcall.com/Gdicall.aspx) (Wu et al., 2014).

RESULTS AND DISCUSSION

PCR was conducted on every of the DNA samples acquired from Morkaraman sheep blood samples, and DNA bands were achieved. The agarose gel image of the PCR products under UV light is displayed in Figure 1.

Polymorphic regions of the *MSTN* gene were determined by digestion with the *Hae*III restriction endonuclease enzyme-producing genotypes of MM:337/83 bp, mm:131/123/83 bp, and mm:254/131/123/83 bp. Figure 2 shows an exemplary agarose gel image of the PCR-RFLP result under UV light.

Gene and Genotype Frequencies and Genetic Equilibrium Test Results:

The study identified three genotypes MM, Mm, and mm by the *Hae*III enzyme in the *MSTN* gene polymorphic region. Table 1 presents the detected genotypes and allele gene frequencies, and Table 2

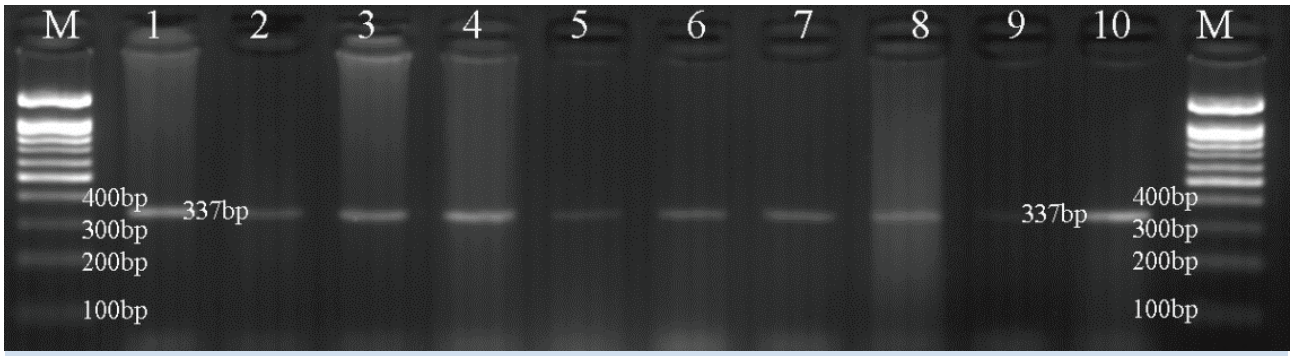

Figure 1. Image of PCR products in 1% agarose gel under UV light (M: marker, line 1-10 PCR products: 337 bp)

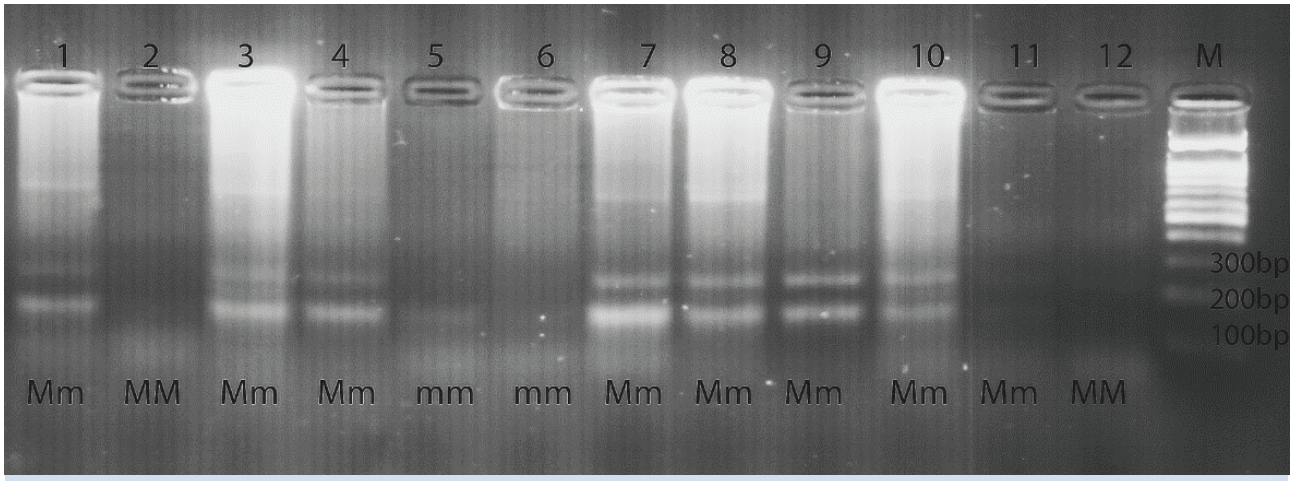

Figure 2. PCR-RFLP image of the MSTN gene in 2% agarose gel: MM; 254/83 bp, Mm; 254/131/123/83/83 bp, mm; 131/123/83 bp

\ **Table 1.** Morkaraman Breed's MSTN/HaeIII Genotypes and Allele Gene Frequencies

Genotype	GHUAM		Erzurum		Bingöl		Total	
	n	%	n	%	n	%	n	%
MM	12	8.9	10	15.2	7	11.5	**29**	**11.1**
Mm	79	58.5	42	63.6	43	70.5	**164**	**62.6**
mm	44	32.6	14	21.2	11	18.0	**69**	**26.3**
Allele gene	M	m	**M**	**m**	**M**	**m**	**M**	**m**
frequencies (%)	38	62	47	53	47	53	**42**	**58**

GHUAM: Food and Livestock Application and Research Center

presents the Hardy-Weinberg genetic equilibrium test and X^2 test results.

Upon examining the population with regard to allele frequencies, it was found that the M allele was at the frequency of 0.42 and the m allele was at the frequency of 0.58 in the Morkaraman sheep breed (Table 1). While the m allele in the breed was observed at a high frequency, in general, animals with 29 MM genotypes, 164 Mm genotypes, and 69 mm genotypes were determined. Whereas allele frequency superiority and genotype difference did not change in the subpopulations, MM, Mm, and mm genotypes were 8.9%, 58.5%, and 32.6% on GHUAM sheep farm, 15.2%, 63.6%, and 21.2% on Erzurum private farms, and 11.5%, 70.5%, and 18.0% on Bingöl private farms, respectively (Table 1). Considering allele gene and genotype frequencies, it can be said that heterozygous individuals in terms of the *MSTN*/*Hae*III gene polymorphism are observed more in individual and overall populations and the biological diversity is quite high.

Concerning different sheep breeds with regard to the *MSTN* gene, the Dalagh sheep breed (Azari et al., 2012), Zel sheep breed (Dehnavi et al., 2012), Karak-

achan sheep breed (Bozhila-Sakova et al., 2016), and the Bulgarian Karnobat Merino sheep breed (Dimitrova et al., 2017) were found to be monomorphic. While the Mehreban sheep breed (Jamshidi et al., 2014), Kordi sheep breed (Akbari et al., 2015), Black-Headed Teleorman sheep breed (Lazar et al., 2016), Nilagiri sheep breed (Sahu et al., 2019), and Awassi sheep breed (Bayraktar, 2020) were found to be polymorphic with only two genotypes, the Sanjabi sheep breed (Soufy et al., 2009), Bulgarian Merino sheep breed (Dimitrova et al., 2016), and Batur sheep breed (Haren et al., 2020) were reported to be polymorphic with three genotypes. While studies indicating the MSTN gene polymorphism reported the mm genotype at a higher frequency than the MM genotype, the findings were consistent with our study.

In studies, the m gene is seen at higher frequencies compared to the M gene (Soofy et al., 2009; Jamshidi et al., 2014; Akbari et al., 2015; Dimitrova et al., 2016; Lazar et al., 2016; Bayraktar, 2020; Haren et al., 2020), which is consistent with allele gene frequencies in our study.

Table 2 summarizes the Hardy-Weinberg genetic equilibrium test results of the Morkaraman sheep breed MSTN gene HaeIII polymorphism.

The Hardy-Weinberg genetic equilibrium test showed that the distribution of genotype frequencies of 262 Morkaraman sheep was not in equilibrium (P<0.01) (Table 2). This result did not change in the subpopulations of Morkaraman sheep either. This may have been due to breeding being done in herds or a sampling error.

Heterozygosity and Fixation Index:

The populations' heterozygosity rate and fixation index values were calculated according to the observed and expected heterozygosities and shown in Table 3.

Upon examining Morkaraman sheep populations separately and in general, it was determined that the average observed heterozygosity values were quite higher than the expected heterozygosity values. While the heterozygosity value (Ho) observed for the *MSTN* polymorphic region throughout the population was calculated as 0.632, the expected heterozygosity value (He) was calculated as 0.480. The Ho value was higher than 0.50 for all the populations studied, and the lowest observed heterozygosity value on the basis of herds was found in GHUAM (0.585), followed by Erzurum (0.636) and Bingöl populations (0.705) (Table 3).

A marker's polymorphism information content (PIC) refers to its ability to identify a polymorphism among a population's individuals, and its value increases with an increase in the capacity. It is among the marker quality indicators in genetic studies. As stated by Botstein et al. (1980), markers with PIC values higher than 0.5 are accepted as very informative, values between 0.25 and 0.50 as somewhat informative, and values below 0.25 as not very informative. The PIC values of the studied population showed a moderate polymorphism at levels of 0.25<PIC<0.50 with an average of 0.37 and the average of Ne values of 1.95 (Table 3).

Table 2. Hardy-Weinberg genetic equilibrium test results

Population	n	Observed			Expected			X^2 test	P
		MM	Mm	mm	MM	Mm	mm		
GHUAM	135	12	79	44	19.6	63.7	51.6	7.78	**
Erzurum	66	10	42	14	14.6	32.9	18.6	5.08	*
Bingöl	61	7	43	11	13.3	30.4	17.3	10.55	**
Total	**262**	**29**	**164**	**69**	**47.0**	**127.9**	**87.0**	**20.80**	**

*: P<0.05, **: P<0.01

Table 3. Populations' genetic indices in the MSTN gene locus

Population	Ho	He	PIC	Ne	F_{IS}	F_{ST}	F_{IT}
GHUAM	0.585	0.472	0.3606	1.894	-0.240*		
Erzurum	0.636	0.498	0.3739	1.993	-0.277*	0.007*	-0.303*
Bingöl	0.705	0.498	0.3741	1.991	-0.416*		
General	0.632	0.480	0.3691	1.954	-0.312		

Ho: observed heterozygosity; He: expected heterozygosity; Ne: effective allele number; PIC: polymorphism information content; F_{IS}: intra-subpopulation genetic difference value; F_{ST}: between-subpopulation genetic difference value; F_{IT}: intra-total population genetic difference value, *:P<0.05.

The intra-population fixation index (F_{IS}) value for the *MSTN* polymorphic gene region, which gives the ratio of the observed heterozygosity level in the population to the deviation in Hardy-Weinberg equilibrium, was determined to be -0.242 in the GHUAM population, -0.277 on the Erzurum private farm, -0.416 on the Bingöl private farm, and -0.312 in the whole population. These values show that the levels of heterozygosity are quite high within and throughout the population.

The F_{IT} value, which measures whether the total observed heterozygosity level deviates from the required amount according to the Hardy-Weinberg principle, was calculated as -0.303 across the subpopulations. From these values, it is understood that the heterozygosity ratio value in the *MSTN* gene region is high since it is close to zero and negative.

The F_{ST} value, a measure of genetic differentiation between populations, was found to be 0.007 in the population. According to these values, it was determined that genetic differentiation was significant ($P<0.05$) among the Morkaraman sheep populations examined in terms of the *MSTN* gene region.

CONCLUSION

Three genotypes (MM, Mm, and mm) of the *MSTN* gene *Hae*III polymorphism were detected in the genomic DNA samples of 262 Morkaraman sheep using the PCR-RFLP method. The MM, Mm, and mm genotype frequencies of the *MSTN* gene in the population were 11.1%, 62.6%, and 26.3%, respectively, while the frequencies of the M and m allele were 0.42 and 0.58, respectively. The observed heterozygosity (Ho) value calculated for the *MSTN* gene in the whole population was considerably higher than the expected heterozygosity (He) value. The PIC values of the studied population showed a moderate polymorphism with an average of 0.37, and Ne values were higher than 1.95. While the F_{IS} value was found as -0.312, the F_{IT} value was determined as -0.303. The genotype and allele frequencies revealed with regard to the *MSTN* gene polymorphism were found to be adequate to determine the breed's genotype diversity. It is necessary to demonstrate its usability in animal breeding by associating the *MSTN* gene polymorphism determined by such studies with some performance traits of animals.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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