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MOLECULAR DETERMINATION OF THE ASSOCIATIONS BETWEEN MYOSTATIN GENE AND SOME GROWTH TRAITS OF RABBITS

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Abstract: This study investigates the myostatin (MSTN) gene polymorphisms and their associations with some growth traits in New Zealand white rabbits. A substitution from cytosine (C) to thymine (T) was observed at base position 847 in the second intron region of the gene, and the genotypes TT and CT were attained only. Genotype frequencies were calculated as 47% for TT and 53% for CT, while allele frequencies were determined as 74% for T and 26% for C. The genotype was found to be not in Hardy-Weinberg Equilibrium (χ^2=7.27, P<0.01). The research revealed that the significant associations of MSTN gene polymorphism on some growth traits such as live weight, shoulder-to-tail length, front leg length, and chest circumference. Rabbits belongs to the TT genotype were found to have significantly higher live weight and front leg length compared to rabbits with the CT genotype, while rabbits with the CT genotype were found to be longer in shoulder-to-tail length and chest circumference compared to rabbits with the TT genotype. No statistically significant differences were found among the genotypes for other growth traits such as neck-to-shoulder length, hind leg length, and ear length. These findings suggest that MSTN gene polymorphism may influence certain growth traits in rabbits, and the observed genotypic differences, especially in traits such as live weight, shoulder-to-tail length, front leg length, and chest circumference, should be considered in genetic breeding programs. The findings of this study contributes to better understanding of the potential of MSTN gene polymorphisms in rabbit breeding programs.

Keywords: Myostatin, Candidate gene, Polymorphism, Rabbit, Growth traits

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1. Introduction

The domestic rabbit (*Oryctolagus cuniculus*) is an animal species with several breeds differing with in their distinct characteristics and appearances. In fact, although it is not immediately visible, rabbit production plays several important roles in agriculture and food industry. Firstly, rabbit meat is rich in essential nutrients including protein, vitamins and minerals such as B12, iron and zinc. As a high-protein and low-fat meat source that rabbit production is more environment friendly than traditional livestock husbandry because they require less feed and water compared to cattle or pigs. Being satisfied with this little input makes it an efficient alternative source for meat production (Sarıçiçek, 1993). Compared to other livestock production, rabbit farming is a remarkable agricultural activity as a sustainable and low-cost source of meat production. In fact, the feeding and care costs, which are very high in other species, are quite low for rabbits. Feeding of fattening kits and labor as the major costs were calculated 26% and 18% of the total cost by Cartuche et al. (2014). Secondly, rabbits are raised for their fur and skin and contributes to the fashion and textile markets by providing materials for clothing and accessories. Additionally, rabbits produce manure that can be used as high-quality and valuable fertilizer, increasing soil fertility and promoting sustainable agricultural practices. Moreover, rabbit farming provides a source of income for many small farmers and rural communities. This requires relatively little investment and can be integrated into existing agricultural systems, thereby improving food security and livelihoods (Olawumi, 2014). The global rabbit meat production was estimated to be around 1.4 million tons in 2018 (Lukefahr et al., 2022). China is the main rabbit meat producing country with a share over 68% in the world followed by DPR Korea and some countries in Europe. Although Türkiye is a country where some rabbit breeds, including the long-haired Angora rabbit, originate, it is not among the top 30 in world rabbit production. The country has a very low rabbit meat production of 35.06 tons in 2022 for both local consumption and some export. Rabbit farming in Türkiye contributes little to the agricultural economy but provides a source of income for small-scale or low-input farmers. The low investment cost and high feed conversion efficiency make it an attractive option for rural

households. Although per capita rabbit meat consumption is still very low compared to other sources of meat, it is gaining popularity due to its health benefits in recent years (Nistor et al., 2013). As in other areas of animal husbandry, breeding practices based on the selection of individuals with high genetic merit are important for increasing efficiency and production in rabbit breeding. For this purpose, the advances in recent new generation sequencing technologies in genomic selection have come to the fore instead of traditional breeding. In this context, genomic selection based on Quantitative Trait Locus (QTL) has been one of the most studied topics for the last three decades (Dentine, 1992). QTL association studies aim to identify the specific regions of the genome that are associated with variation in traits. Candidate gene studies help to determine specific regions of genes that are hypothesized to influence a trait based on prior knowledge of their function, expression patterns, or involvement in related biological pathways. In this regard, myostatin (MSTN) or GDF-8 is a pivotal regulatory factor in muscle growth, belonging to the transforming growth factor-beta (TGF-β) superfamily (Langley et al., 2002; Thomas et al., 2000). It plays a critical role in regulating skeletal muscle growth and development across various animal species including rabbits (Bindu et al., 2011; Rasmussen, 2016). In livestock, understanding functions of MSTN has the importance due to its potential impact on meat production efficiency and overall growth traits. In their comprehensive review, Aiello et al. (2018) reports that MSTN acts at key points during pre- and post-natal life of amniotes that ultimately determine the overall muscle mass of an animal. Mutations have already demonstrated the impact of attenuating myostatin activity on muscle development. As a matter of fact, association analyses between the MSTN and IGF2 genes and phenotypes of farm animals, i.e., sheep (Osman et al., 2021); cattle (Esmailizadeh et al., 2008; Grisolia et al., 2009; Bagnicka et al., 2010; Berkowicz et al., 2011; Lin et al., 2022) have become one of the frequently studied research topics. Although it has not been studied as much as in other species, some studies focusing on the MSTN and IGF2 genes have been conducted in rabbits (Wallis and Wallis, 1995; Fontanesi et al., 2008; Sternstein et al., 2014; Abdel-Kafy et al., 2016; Hristova et al., 2017; Yang et al., 2019; Zhang et al., 2019; Ramadan et al., 2020; Helal et al., 2022; Safaa et al., 2023). Given the increasing demand for meat production and improved growth characteristics, identifying the polymorphic regions of MSTN on growth traits in rabbits could lead to enhanced breeding strategies and better management practices. For this purpose, this study aimed to determine the polymorphisms and their associations of MSTN with some important growth traits such as live weight, shoulder-to-tail length, front leg length, and chest circumference.

2. Materials and Methods

As the study material, phenotypic data and blood samples for genotyping were collected from a total of 60 randomly selected 2 and 3 years old animals (45 male and 15 female) belonging to the rabbit breed New Zealand White raised at the Çukurova University Faculty of Agriculture Research and Application Farm in Sarıçam district of Adana province. In this study, the following protocol was applied for DNA extraction from the blood samples collected. Using a pipette, 200 µl of uncoagulated blood and 600 µl of DP buffer were taken into a 1.5 ml Eppendorf tube and homogenized by pipetting. The prepared samples were centrifuged for 5 minutes at 7600 xg, and then the supernatant was discarded. 200 µl of DA buffer was added to the remaining in the Eppendorf tube and it was mixed by pipetting. 20 µl of Proteinase K and 220 µl of DB buffer were added and incubated at 65 oC for 20 minutes. 220 µl of pre-cooled ethanol was added to the incubated samples and vortexed. The mixture was transferred to filtered tubes and centrifuged at 10900 xg for 1 minute, and the liquid remaining under the filter was poured. 500 µl of DY buffer was added to the filtered tube and centrifuged for 1 minute at 10900 xg, and the liquid remaining under the filter was poured. This step was repeated once more, and then the filtered tube was centrifuged for 1 minute at 10900 xg as empty without adding any reagents to it. The collection tube remaining under the filtered tube was discarded. The filtered tube was placed in a new 1.5 ml Eppendorf tube, 70 µl DE buffer was added, and it was kept at room temperature for 1 minute, then centrifuged for 2 minutes at 10900 xg. The filtered tube part in the Eppendorf was discarded, and the obtained DNA product was placed in an Eppendorf tube.

Table 1. Protocol applied to the PCR instrument for MSTN gene amplification

Process	Temperature (°C)	Time (minute)	Number of cycles
First denaturation	95	5	1
Denaturation	95	0:30	
Annealing	44.7	0:40	30
Extension	72	0:50	
Last denaturation	72	10	1
Waiting	4	∞	1

The protocol given in Table 1 was applied to the PCR device for the synthesis of MSTN gene regions from genomic DNAs. Electrophoresis gel was prepared to visualize and isolate DNA (Gibbs, 1990). Due to the phosphate group contained in DNA, the prepared 5X stock Tris-Borate-EDTA (TBE) solution was diluted to 1X in order to have a negative charge and pH between 5-7 in the prepared gel. While preparing the stock solution, 108g Tris, 55g Boric Acid and 40 ml 0.5M EDTA (pH 8.0) were dissolved in 2 liters of pure water and transferred to glass bottles. The stock solution was diluted by adding 400 ml of pure water to 100 ml TBE 5X. While preparing the gel,

0.2440g agarose was weighed on a precision scale and placed in a 0.50 beaker. 30ml of ready-to-use 1X TBE was added to the agarose. The beaker was covered with stretch film and small holes were made on it. The solution prepared in the beaker was subjected to heat treatment in a microwave oven for 2 minutes at 600W. In order to make the DNA fluorescent under UV light, 1.0 µl ethidium bromide was added to the solution and it was mixed. The prepared solution was poured into the tank without creating air bubbles, and an 8-comb was placed and the gel was waited for to solidify. The comb on the solidified gel was gently removed without tearing it. The gel was placed back into the tank so that it would float in the tank and enough electrolyte solution was added to completely cover the gel. DNA fragments amplified by the PCR method were placed into the gel. After this process was completed, the gel was illuminated with UV light and examined. The PCR products obtained were stored at -20 ^{o}C to be sent for sequencing. The chain termination method developed by Sanger et al. (1977) is widely used in DNA sequencing analyses. In this method, which is based on enzymatic DNA synthesis, the DNA strand to be sequenced is used as a template for the newly synthesized strand. One of the enzymes Klenov, Taq DNA polymerase, reverse transcriptase or sequencing can be used to provide DNA synthesis. The basis of the method is that DNA polymerase uses dNTPs (deoxyribonucleoside triphosphate) as well as ddNTPs (dideoxyribonucleoside triphosphate) that do not carry an OH group at the 3' position of deoxyribose. The addition of a ddNTP to the synthesized DNA stops the synthesis because there is no OH group at the 3' position. Four separate reaction mixtures are prepared during sequencing. Each mixture contains the template DNA strand, a primer, four of the dNTPs and a small amount of one of the ddNTPs. A different ddNTP is present in each reaction for specific chain iv termination. Since very small amounts of modified nucleotides are used in each reaction, new chain synthesis is randomly terminated and a series of DNA fragments are formed (Klug and Cummings, 2000).

Table 2. Descriptive statistics of the studied growth traits according to MSTN genotypes

Traits		Mean.	SD	Min	Max	CV(%)	P-value
LW	TT	2.39	0.15	2.13	2.61	6.28	0.001*
	CT	2.38	0.10	2.08	2.52	4.20	
	Overall	2.39	0.12	2.08	2.61	5.22	
LNS	TT	11.21	1.20	9.00	13.00	10.70	0.850
	CT	11.36	0.89	10.00	12.50	7.83	
	Overall	11.29	1.04	9.00	13.00	9.24	
LST	TT	29.93	1.90	26.50	33.00	6.35	0.019*
	CT	31.02	2.23	28.00	35.50	7.19	
	Overall	30.48	2.13	26.50	35.50	6.99	
LFL	TT	12.64	0.56	12.00	13.50	4.43	0.015*
	CT	12.40	0.62	11.50	13.50	5.00	
	Overall l	12.52	0.60	11.50	13.50	4.77	
LRL	TT	11.32	0.85	10.00	12.50	7.51	0.115
	CT	11.14	0.75	10.00	12.50	6.73	
	Overall	11.23	0.80	10.00	12.50	7.14	
LE	TT	12.25	1.87	9.00	14.50	15.27	0.373
	CT	12.50	1.81	9.50	15.00	14.48	
	Overall	12.38	1.83	9.00	15.00	14.79	
CC	TT	29.00	1.78	25.50	32.00	6.14	0.039*
	CT	28.60	1.26	27.00	31.50	4.41	
	Overall	28.80	1.54	25.50	32.00	5.35	

* P<0.05, LW= live weight (kg), LNS= length from neck to shoulder (cm), LST= length from shoulder to tail (cm), LFL= front leg length (cm), LRL= rear leg length (cm), LE= ear length (cm), CC= chest circumference (cm).

The DNA fragments obtained as a result of the reactions are electrophoresed and run side by side on the gel. With the effect of the applied electric field, a staircase image is created on the gel, with the shortest DNA fragments at the front. According to the labeling method, the detected fragments on the gel are read according to the type of ddNTP added to the reaction mixture (Klug and Cummings, 2000). Before the association analysis, the descriptive statistics in Table 2 of the studied growth traits were calculated, and then a quality control was performed on the phenotypic data. In order to visualize the distribution of the traits and also to check probable outliers, the data distributions were examined by drawing the relevant plots seen in Figure 1. When the probability density function plots of the growth traits along the diagonal in Figure 1 are examined, it is seen that all traits except CC are not normally distributed. In fact, according to the results of the Shapiro-Wilk normality test performed with the shapiro.test function of R, it was determined that the traits LW, LNS, LST, LFL, LRL and LE

(LW: Live Weight (kg), LNS: Length from neck to shoulder (cm), LST: Length from shoulder to tail (cm), LFL: Front Leg Length (cm), LRL: Rear Leg Length (cm), LE: Ear Length (cm), CC: Chest Circumference (cm)) were not normally distributed (P<0.05). There are many methods such as arcsine, logarithmic, square root, Box-Cox etc. for normalizing the data, and which method to use depends on the distribution of characteristics of the data. For example, square root or logarithmic transformation can be recommended for normalizing right-skewed distributions; the methods of cube or square transformation can be better options for left-skewed distributions. However, in practice, it is seen that the traditional methods may not be efficient to normalize many types of data, recently, Inverse Normal Transformation (Shore, 2000; 2002), abbreviated as INT, has gained popularity and is offered as a tool in various software (Cebeci and Gökçe, 2023).

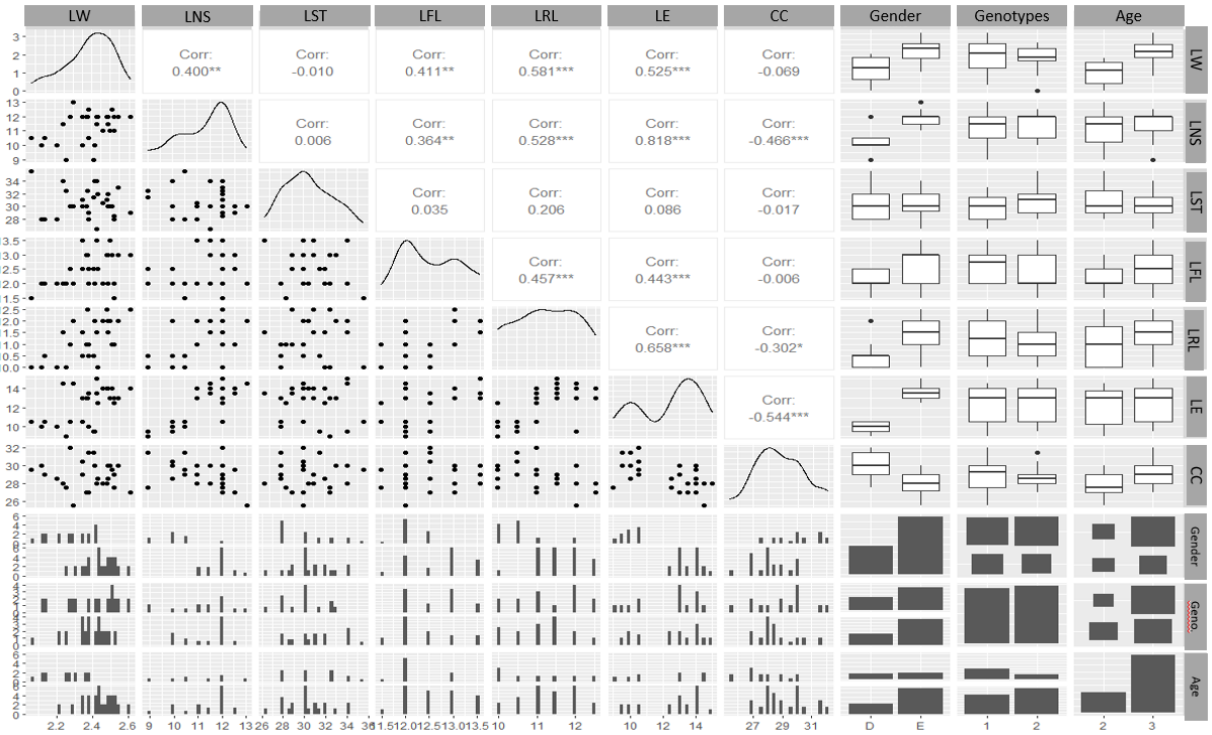

Figure 1. Distribution and scatter plots of the growth traits by the genotypes, genders and age groups.

In this study, thus, INT was applied to normalize the phenotypic data and the results are visualized in Figure 2. According to the post-normalization test results, INT was successful to normalize the phenotypic data. Following the normalization the data was also checked for possible outliers. For this purpose, a procedure based on Tukey's IQR method was run as described in Cebeci (2020), and no outliers found in the data for the all examined traits.

In the present study, a generalized linear model (GLM) shown in equation 1 was used to test the associations between the genotypes and phenotypes.

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijkl} \quad (1)$$

In equation 1:

Y_{ijkl}: Phenotypic value of the studied trait observed on the *l.* animal with *i.* genotype and *j.* gender and *k.* age group,

μ: Overall mean,

α_i: Effect of i. fixed genotype the MSTN mutation site (TT, CT),

β_j: Effect of *j.* gender (male, female),

γ_k: Effect of *k.* age group (2, 3),

ε_{ijkl}: Random error (or residual), $\varepsilon \sim N(0, \sigma^2)$.

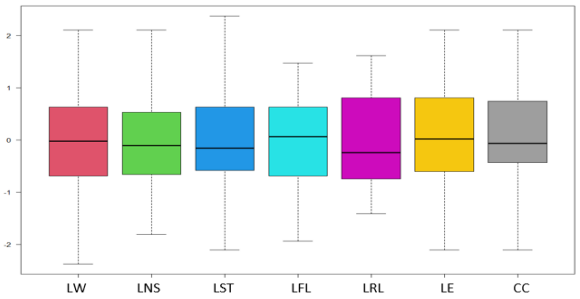

Figure2. Boxplots of the traits after normalization and outlier check. (LW= live weight (kg), LNS: length from neck to shoulder (cm), LST= length from shoulder to tail (cm), LFL= front leg length (cm), LRL= rear leg length (cm), LE= ear length (cm), CC: chest circumference (cm).

All the genomic and statistical analyzes including visualizations were done in the R version 4.3.2 (R Core Team, 2024). While the glm function in the basic stats package of R was used for GLM analysis, the functions plot and boxplot in the basic graphics package; the ggpairs function in the GGally package (Schloerke et al., 2024), and the functions in the corrplot package (Wei and Simko,

2021) were used to draw correlograms, boxplots, histograms and other plots. The R package genetics (Warnes et al., 2021) was used to calculate allele and genotype frequencies, and to test Hardy-Weinberg Equilibrum.

3. Results

In this study, a T>C substitution at 847 bp location of rabbit MSTN was identified by sequencing method as shown in Figure 3. This variant is a newly discovered variant in the intron region 2 of MSTN. According this finding, the associations between two genotypes (TT and CT) formed due to this mutation and the examined body measurements were analyzed. When the allele frequencies at this locus were examined, it was calculated that the T allele had a frequency of 74% and the C allele had a frequency of 26%; accordingly, the genotype frequencies were 47% for the TT genotype and 53% for the CT genotype. According to the χ^2 test, it was determined that the population deviated from the Hardy-Weinberg Equilibrium (HWE) in terms of genotype frequencies (χ^2=7.27, P=0.007). As seen in Table 2, the effect of genotypes was found to be significant for the traits of LW, LST, LFL and CC. The mean LW of the rabbits belonging to TT genotype was determined as 2.39±0.15 kg while the mean LW of rabbits from the CT genotype was found as 2.38±0.10 kg (P=0.001). This finding indicated that rabbits with the TT genotype at this loci might have higher LW compared to those with the CT allele, which could be valuable for breeding programs aimed at enhancing meat production. For the trait LST, the rabbits of the CT genotype were found to be longer with a mean of 31.02±2.23 cm than the rabbits with the TT genotype with a mean of 29.93±1.90 cm (P=0.019). For trait LFL, the rabbits with the TT genotype with an average of 12.64±0.56 cm were longer than rabbits the of CT genotype with a mean of 12.40±0.62 cm (P=0.015). The mean CC for the TT genotype was 29.00±1.78 cm while it was 28.60±1.26 cm with the animals have the CT genotype, and a significant difference was also observed between the genotypes for this trait (P=0.039). On the other hand, no statistically significant difference was determined between the TT and CT genotypes for the traits LNS, LRL and LE (P>0.05). These findings pointed out that MSTN gene polymorphism may affect some growth traits in rabbits. In particular, it indicates that genotypic differences observed for the growth traits such as LW, LFL, LST and CC should be taken into consideration in genetic breeding studies.

4. Discussion

Fontanesi et al. (2008) identified a single nucleotide polymorphism (C>T) in intron 2 of rabbit MSTN gene using a PCR-RFLP protocol designed to investigate this mutation in a larger number of rabbits (15 Checkered Giant, 9 Giant Grey, 6 Dwarf, 4 Burgundy Fawn, 3 Giant White, 3 Lop, 2 Belgian Hare, 1 New Zealand White). Allele frequencies across breeds were 0.51 for allele C and 0.49 for allele T. C. Bindu et al. (2011) reported that CT genotypes were associated with higher body weight but the difference in body weight among different genetic groups was not statistically significant in a sample of 60 animals from New Zealand, Soviet Chinchilla and crossbred rabbits subjected to PCR-based RFLP. Sternstein (2014) identified three SNPs (c.–125T>C, c.373+234G>A, c.747+34C>T) related with carcass composition traits in F_2 animals of the cross Giant Grey × New Zealand White. Hristova et al. (2017) also confirmed the presence of the polymorphisms in MSTN gene in the a80 bp fragment of the intron 2 of MSTN gene of New Zealand rabbits. Navratilova et al. (2018), revealed significant positive effect of allele T (c.747+34C>T) and allele G (c.194A>G) on meat performances in tested rabbit population. In one of more recent studies conducted by El-Sabrout and Aggag (2018), the effects of four novel SNPs identified in the MSTN and MC4R genes on carcass quality traits were examined. It was found that rabbits with the BB genotype had higher live weight, carcass weight, fleece and carcass fat than the rabbits with the AA genotype in their study. They revealed that genetic polymorphisms play an important role in both growth traits and carcass quality. Yang et al. (2019) also found the significant associations between MSTN genotypes and carcass traits, and reported that in the Exon 1 region of MSTN gene, CC genotype rabbits had significantly higher performances than the rabbit of TT genotype. For the genotypes in the Exon 2 region of the gene, AA genotype rabbits were heavier than TT genotype rabbits. Peng et al. (2013) indicated that the association between the genotypes and live weight gain on the 35th and 70th days were insignificant while the CT genotype had higher live weight than individuals with the CC genotype on the 84th day. In the present study, a T>C substitution was found at the 847th base of the second intron of rabbit MSTN gene. The frequencies of the genotypes of TT and CT were determined as 47% and 53% respectively. The frequency of the T allele was 74% and the C allele was 26%. This finding is similar to the findings by Fontanesi et al. (2008) and Hristova et al. (2017) because they reported polymorphisms in the second intron of MSTN gene of New Zealand rabbit breed. In this study, it was also detected that the studied population deviated significantly from the Hardy-Weinberg Equilibrium (HWE) in terms of genotype frequencies as stated in some research studies, i.e. Hristova (2017). Several factors may explain the deviations from HWE. Firstly inbreeding can increase homozygosity and alter allele frequencies. For example, if a small flock is kept in closed stock for generations, deviations from HWE can occur due to an increase in certain alleles. Secondly, artificial selection for certain traits, such as daily weight gain, can favor certain alleles and disrupt HWE. Moreover, random fluctuations in allele frequencies can occur in small populations. This can lead to HWE violations for rare alleles, which can be difficult to detect, especially in small flocks. The introduction of animals or genes into a flock can affect allele frequencies.

For example, the introduction of several high genetic merit males into a breeding stock can significantly alter the genetic make-up of the flock. If animals are mated preferentially for certain traits, this can lead to skewed allele frequencies. For example, if larger genetic merit animals are mated, this can lead to an increase in individuals homozygous for certain alleles. As another factor, new mutations can introduce variation and disrupt HWE. For example, if a new mutation that affects feed conversion efficiency emerges in the herd and provides a survival advantage, it can rapidly change allele frequencies. In this study, potential reasons for deviation from HWE could not be examined because sufficient data were not provided to understand these factors and better examine genetic diversity. However, it is thought that allele frequencies can be calculated more stably by increasing the sample size to increase the power of the analysis and possibly test HWE better. The findings from this and previous studies suggest that the polymorphisms in MSTN gene may affect some growth traits in rabbits. In particular, the genotypic differences observed in the studied growth traits of LW, LST, LFL and CC should be taken into account in genetic breeding studies. If the detected T>C variant affects regulatory elements within the intron, it may lead to changes in the expression levels of the myostatin protein, potentially leading to increased muscle growth. This finding is consistent with previous studies showing that certain mutations in the MSTN gene are associated with the 'double muscling' phenotype in cattle. As a matter of fact, Lv et al. (2016) generated the MSTN knockout (KO) rabbits by co-injection of Cas9 mRNA and sgRNA into zygotes, and they observed the typical phenotype of double muscle with hyperplasia or hypertrophy of muscle fiber in MSTN KO rabbits. Recently Zhang et al. (2019) also studied on double-muscling and pelvic tilt phenomena in rabbits with the cystine-knot motif deficiency of myostatin on exon 3. In rabbits, if the T>C variant is associated with improved muscle or growth rates, it also may be beneficial to increase meat yield. In fact, if there is a mutation in MSTN gene, its negative regulation function is disrupted leading to double muscle phenotype. However, more advanced association analyses such as GWAS or functional analyses can be performed to investigate the specific effects of this intronic variant on muscle traits.

5.Conclusion

A novel mutation T to C was detected at position 847 in the second intron region of the MSTN gene of rabbit. The study revealed the significant differences between the TT and TC genotypes for the growth traits of LW, LST, LFL and CC. For these traits, the animals with TT genotype significantly outperformed than the animals of TC genotype. These findings pointed out that MSTN gene polymorphisms should be considered in rabbit breeding programs targeting the meat and growth related traits. Applying genomic selection programs rabbit breeding based on selection could provide a rapid increase in meat yield. The genomic selection focusing on MSTN gene can contribute to the effectiveness of breeding programs and improve the development of more sustainable rabbit populations. Rabbit breeders could benefit the genetic polymorphisms in genetic breeding programs.

Author Contributions

The percentages of the authors' contributions are presented below. All authors reviewed and approved the final version of the manuscript.

	H.B.D.	Z.C.
C	50	50
D	90	10
S	10	90
DCP	100	0
DAI	50	50
L	90	10
W	100	0
CR	10	90
SR	100	0
PM	10	90
FA	100	0

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no conflict of interest.

Ethical Consideration

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to. The experimental procedures were approved by the Local Animal Care and Ethics Committee of Çukurova University in Adana, Türkiye (Approval date: March 28, 2024 and protocol code:3).

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