jotaf **Journal of Tekirdag Agricultural Faculty**
Tekirdağ Ziraat Fakültesi Dergisi

Mayıs/May 2022, 19(2)
Başvuru/Received: 27/10/21
Kabul/Accepted: 06/01/22
DOI: 10.33462/jotaf.1015587

http://dergipark.gov.tr/jotaf
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ARAŞTIRMA MAKALESİ **RESEARCH ARTICLE**

Evaluation of Variation on Myostatin (MSTN) Gene of Turkish Donkey Populations in Thrace Region of Turkey

Türkiye'nin Trakya Bölgesindeki Türk Eşek Populasyonlarındaki Myostatin (MSTN) Genindeki Varyasyonun Değerlendirilmesi

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Abstract

The study aimed to determine the *MSTN* gene variation in 90 donkeys reared in the Thrace region of Turkey. Myostatin (MSTN), also named GDF-8 (growth differentiation factor 8) is a part of the transforming growth factor β (TGF-β) superfamily and it has a negative regulator role on muscle mass, growth and development in mammalian species. *MSTN* gene regulates the skeletal muscle growth in a negative way and has a significant role in homeostasis of skeletal muscles. Also, in muscle fibers balance of protein has been promoted by Myostatin factor. The total of 866 bp long partial intron 1 and 2, whole exon 2 regions of *MSTN* gene was amplified and PCR products analysed using DNA sequencing. In this study, a novel synonymous SNP was determined as g.4183919 G>A in the second exon region of the *MSTN* gene which does not cause an amino acid change in the protein. The G>A transition caused a silent mutation in leucine (leu) amino acid. Alterations in mRNA level and functionality of protein can occur due to synonymous mutations. Since leucine is an important amino acid that can avoid muscle mass loss and inhibits the expression of Myostatin, it can be said that silent mutation of Leu in donkeys may have altered the muscle mass and physical factor of donkeys in this study. Mutant leucine may have a lower efficient effect on preventing loss of muscles and causes more Myostatin protein expression. The identified SNP was firstly released and the DNA sequences of the *MSTN* gene in Turkish donkeys was revealed for the first time with recent study. Turkish donkeys lacked these mutations that were identified before in horses, which cause for the less might require for race ability of donkeys. The sequences of *MSTN* gene were submitted to the NCBI GenBank with the accession number: MW970078- MW970079. Further studies are needed to conduct, on protein and molecular levels, SNPs on the *MSTN* gene and their association with the morphological characters that may affect economic traits in donkey breeds.

Keywords: MSTN, Donkey, Thrace, SNP, Sequencing

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Atıf/Citation: IŞIK, R., ÖZDİL, F., MERAL, S. Evaluation of variation on Myostatin (MSTN) gene of Turkish donkey populations in Thrace Region of Turkey. *Tekirdağ Ziraat Fakültesi Dergisi,* 19 (2), 426-434.

Öz

Çalışma, Türkiye'de Trakya bölgesinde yetiştirilen 90 eşekte *MSTN* genindeki varyasyonunu belirlemeyi amaçlamıştır. GDF-8 (growth differentiation factor 8) olarak da adlandırılan miyostatin (MSTN), farklılaşma büyüme faktörü β (TGF-β) süper ailesinin bir parçasıdır ve memeli türlerinde kas kütlesi, büyüme ve gelişme üzerinde negatif düzenleyici rolü bulunmaktadır. *MSTN* geni, iskelet kası büyümesini olumsuz yönde düzenler ve iskelet kaslarının homeostazında önemli bir role sahiptir. Ayrıca, kas liflerinde protein dengesi Myostatin faktörü tarafından desteklenmektedir. *MSTN* geninin toplam 866 bç uzunluğunda kısmi intron 1, 2 ve ekzon 2 bölgeleri çoğaltılmış ve PCR ürünleri DNA dizilimi kullanılarak analiz edilmiştir. Bu çalışmada *MSTN* geninin ikinci ekzon bölgesinde proteinde amino asit değişikliğine neden olmayan yeni bir sinonim SNP g.4183919 G>A belirlenmiştir. G>A değişimi, lösin amino asidinde sessiz bir mutasyona neden olmuştur. Sinonim mutasyonlar nedeniyle mRNA düzeyinde ve proteinin işlevselliğinde değişiklikler meydana gelebilmektedir. Lösin, kas kütlesi kaybını önleyebilen ve miyostatin ekspresyonunu engelleyen önemli bir amino asit olduğundan, bu çalışmada eşeklerde Leu'nun sessiz mutasyonunun eşeklerin kas kütlesini ve fiziksel yapısını değiştirebileceği söylenebilir. Mutant lösin, kas kaybını önlemede daha düşük bir etkiye sahip olduğundan, daha fazla Myostatin protein ekspresyonuna neden olabilmektedir. Türk eşeklerinde daha önce atlarda bulunan bu mutasyonlar tespit edilememiştir, bu da eşeklerin yarış kabiliyeti için daha az gereksinime sahip olduklarını göstermektedir. Tespit edilen SNP ilk olarak ortaya konmuş ve mevcut çalışma ile Türk eşeklerinde *MSTN* geninin DNA dizileri ilk kez ortaya çıkartılmıştır. *MSTN* geninin dizileri, MW970078-MW970079 erişim numarasıyla NCBI GenBank'a girilmiştir. Eşek ırklarında *MSTN* genindeki SNP'lerin ve bunların ekonomik öneme sahip morfolojik karakterlerle ilişkisinin protein ve moleküler düzeyde belirlenmesi amacıyla daha ileri çalışmalara ihtiyaç vardır.

Anahtar Kelimeler: MSTN, Eşek, Trakya, SNP, Sekanslama

1. Introduction

Myostatin (MSTN), also named GDF-8 (growth differentiation factor 8) is a part of the transforming growth factor β (TGF-β) superfamily and it has a negative regulator role by increasing via glucocorticoids that caused the overexpression of MSTN gene on muscle mass, growth and development in most of mammalian species (Grobet et al., 1997; 1998; Gilson et al., 2007; Bertolini et al., 2015). Additionally, *MSTN* gene regulates the skeletal muscle growth in a negative way and has a significant role in homeostasis of skeletal muscles. Besides, in muscle fibers balance of protein has been promoted by Myostatin factor (Khaerunnis et al., 2016). Augmentation in the muscle size and fiber number in myostatin-null mice have led to containing considerably more muscle mass in contrast to normal mice models. Myogenic differentiation is autocrine and paracrine managed by growth factors such as insulin-like growth factors (IGF) and MSTN. The *MSTN* gene is expressed in the skeletal muscle early developmental stage and its expression continues to adult stage in skeletal muscle fibers (Khaerunnis et al., 2016). Moreover, *MSTN* gene has a crucial role in the regulation of adipose tissue development (Feldman et al., 2006). The *MSTN* gene located on chromosome 18, consists of three exons that coded 375 amino acids and two introns with a total of 7957 bp long (Accession number NW_014637147: 4181432 - 4189388) in equine (O'Hara et al., 2021). The studied *MSTN* gene sequences located on partially intron 1 and 2, spanning exon 2 regions in equine MSTN gene.

There are many studies conducted effect on the insertion/deletions (indels) or variation of *MSTN* gene on muscle mass and growth traits in livestock (McPherron and Lee, 1997; Grobet et al., 1997; 1998; Marcq et al., 1998; Marshall et al., 1999; Karim et al., 2000; Tay et al., 2004; Feldman et al., 2006; Bignell et al., 2010). The mutations or indels of *MSTN* have been shown to produce a double-muscling phenotype caused by muscular hyperplasia and hypertrophy in some of livestock species, such as cattle breeds (Belgian Blue, Piedmontese) (Grobet et al., 1997; McPherron and Lee, 1997; Kambadur et al., 1997; Miranda et al., 2002) and sheep (Texel sheep, East Friesian sheep) (Walling et al., 2004; Johnson et al., 2005; Clop et al., 2006; Bignell et al., 2010) and chickens (Gu et al., 2002 Furthermore, double-muscling is an autosomal recessive gene that can be inherited and it is occurred due to abnormal growth of muscles (Khaerunnis et al., 2016). A single nucleotide variation in intron region of the *MSTN* gene (g.66493737T>C) is using as a molecular marker for the breeding parameter such as race distance ability in horses and many morphological characters in livestock (Binns et al., 2010; Tozaki et al., 2010; 2011; 2012; Hill et al., 2010a; 2010b; 2012a; 2012b; Dall'Olio et al., 2010; 2014; Stefaniuk et al., 2014; 2016; Pereira et al., 2016; Işık et al., 2017; Tüten sevim et al., 2017; Cieslak et al., 2018). Many studies have been carried out *MSTN* gene polymorphisms in horses and its association with racing performances and body condition but in donkeys, there were a few published articles till now (Bertolini et al, 2015; Tozaki et al., 2010; 2011; 2012; Hill et al., 2010a; 2010b; 2012a; 2012b; Dall'Olio et al., 2010; 2014; Stefaniuk et al., 2014; 2016; Pereira et al., 2016; Cieslak et al., 2018; Dong-hua et al., 2017). Overall, in terms of growth, development, and performance in domesticated animals *MSTN* gene has a significant role. Thus, the *MSTN* gene is a preferable factor to take into consideration for animal breeding, and even specifically mutated animals for the *MSTN* gene can be opted in selective breeding (Dong-hua et al., 2017). The study aimed to determine the *MSTN* gene variation in donkeys reared in the Thrace region of Turkey using DNA sequencing.

2. Materials and Methods

2.1. Sampling and processing

In recent study, a total of 90 donkey individuals were taken from the Thrace Region of Turkey, Tekirdağ (20) and Kırklareli (70) Provinces that is a farm which collected donkeys from varied places of Turkey as Nevşehir, Mardin, Urfa and Iğdır (*Figure 1*). Blood samples were collected to 5 mL of vacuum tubes, including EDTA and preserved at −20 ◦C in the freezer to the DNA extraction procedure. DNA was extracted by using phenol chloroform method (Sambrook et al., 1989). The quantity and purity of DNA samples were checked by UV-Vis spectrophotometers (NanoDrop™ 2000/2000c, Thermo Fisher Scientific).

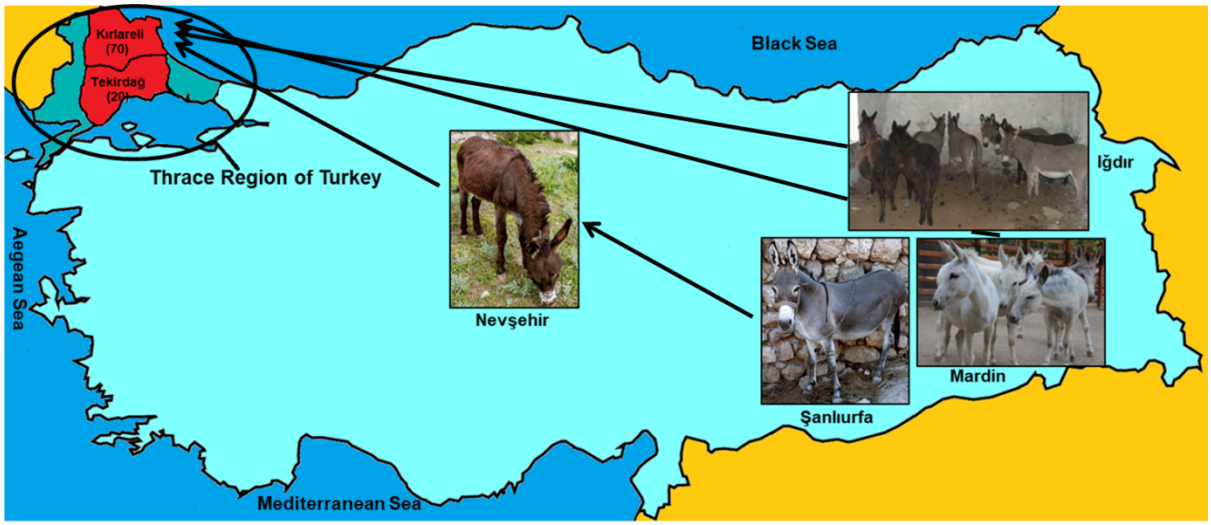

Figure 1. Location of sampling provinces of Turkey

2.2 PCR primers and PCR conditions

Primer sequences were designed using the donkey *MSTN* gene sequence in NCBI GenBank database (Accession number NW_014637147.1) using Primer3web (version 4.1.0) algorithm (https://primer3.ut.ee/). Primer sequences of *MSTN* gene are F: 5'-ATGTTCCTCCACGGTGTCTC-3', and R: 5'-GCTGCCATTGGGGTAAGATA-3'. The PCR amplification mixture was included 40 ng genomic DNA, 1 µM of each primer, 1× PCR Buffer ((NH4)2SO4), 200 µM dNTP, 2.0 mM MgCl2 and 0.1U i-Taq™ DNA polymerase (5U/ml) (iNtRON Biotechnology Inc., US) and finalizing to a final volume of 25 µL. The PCR reaction procedure prepares 4 min at 95°C for initial denaturation, 37 cycles of amplification; 95°C for 45 s, 60°C annealing for 60 s, 72°C for 40s and 10 min at 72°C for final extension. Then, the PCR products of *MSTN* gene region were run on 2.0 % agarose gel electrophoresis that stained with SafeView™ Classic (Applied Biological Material Inc. Canada). PCR products were checked under UV light in the Gel Documentation System.

2.3 Sanger sequencing and Phylogenetic tree

The amplified 866 bp fragment of the *MSTN* gene containing partial intron 1 and 2, whole exon 2 regions were sequenced directly by Applied Biosystems 3500XL Genetic Analyzer System (Applied Biosystems, USA) using the designed primers. The *MSTN* gene sequences were carefully checked the chromatogram by ChromasPro Version 2.1.8 (Technelysium Pty. Ltd. Australia). The checked sequences file including of *MSTN* gene region were controlled by the MEGA7 software (version 7.0, Molecular Evolutionary Genetics Analysis) (Kumar et al., 2016).

Evolutionary analyses were conducted and distances and groupings were defined using the MEGA 7 software by Neighbour Joining (NJ) method (Kimura 2) (Kumar et al., 2016). The bootstrap consensus tree is inferred from 1000 replicates. This analysis involved 13 published NCBI GenBank *MSTN* gene nucleotide sequences as *Bubalus bubalis, Bos Taurus, Capra hircus, Ovis aries, Equus asinus, Equus caballus.* The total of 851 positions have analyzed in the final data set.

3. Results and Discussion

In the current study, we have conducted the donkey *MSTN* gene in that were detected in relation with muscle mass and growth traits in cattle, sheep and goats. The studied donkey *MSTN* gene region has spanned between 2nd exon and partial 1st, 2nd intron and it includes 866 bp and 124 amino acid residues (*Figure 2*).

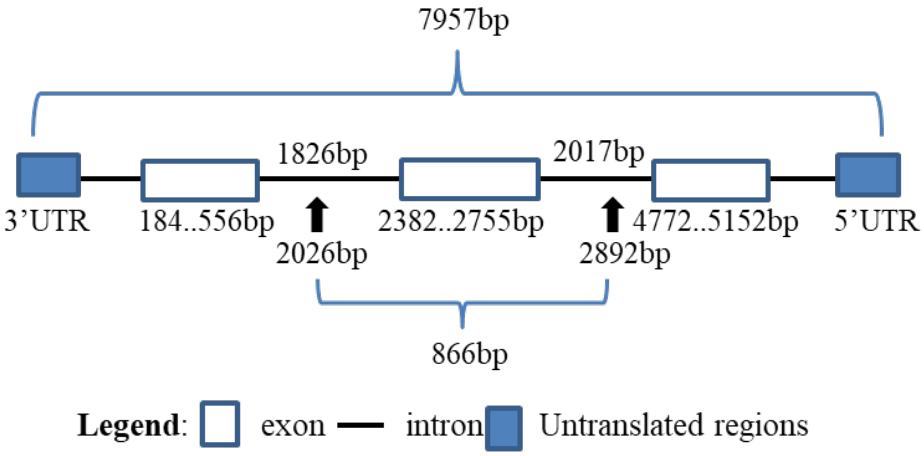

Figure 2. Localization of studied partially MSTN gene in donkey MSTN gene.

The variation of the *MSTN* gene was identified using DNA sequencing and novel single nucleotide variation as g.4183919 G>A determined in the second exon region of the *MSTN* gene (*Figure 3*). The G>A transition had caused a silent mutation (CTG>CTA) in leucine (Leu) amino acid. The novel single nucleotide polymorphism (SNP) of *MSTN* gene region was firstly revealed and the partial DNA fragments of the *MSTN* gene in donkeys were conducted for the first time with recent study. The sequences of the *MSTN* gene were submitted to the NCBI GenBank with the accession number: MW970078- MW970079.

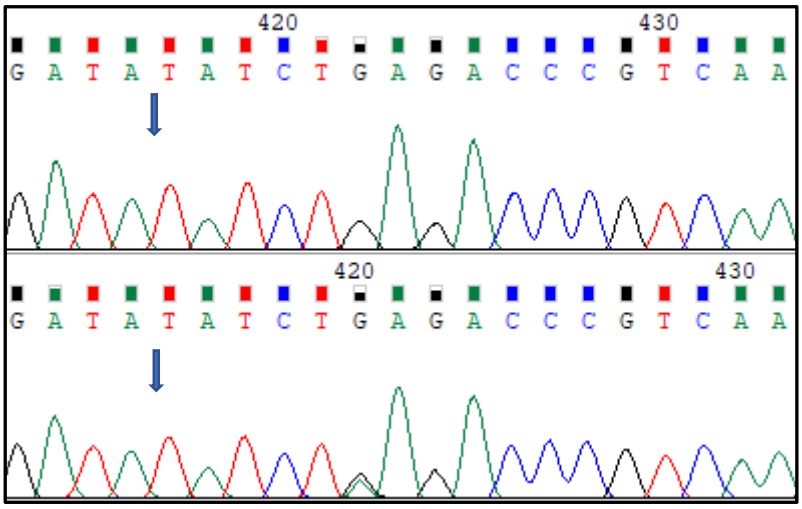

Figure 3. The silent mutation as g.4183919 G>A in the second exon region of the MSTN gene

The Neighbour Joining tree using the sequences of *MSTN* from donkeys and various species taken from NCBI GenBank is shown in *Figure 4*. For the NJ tree, which was characterized using the distance matrix; the fragment of studied *MSTN* gene region (*Equus asinus*) was clustered in the close clade with *Equus caballus* Pak Thoroughbred racehorses.

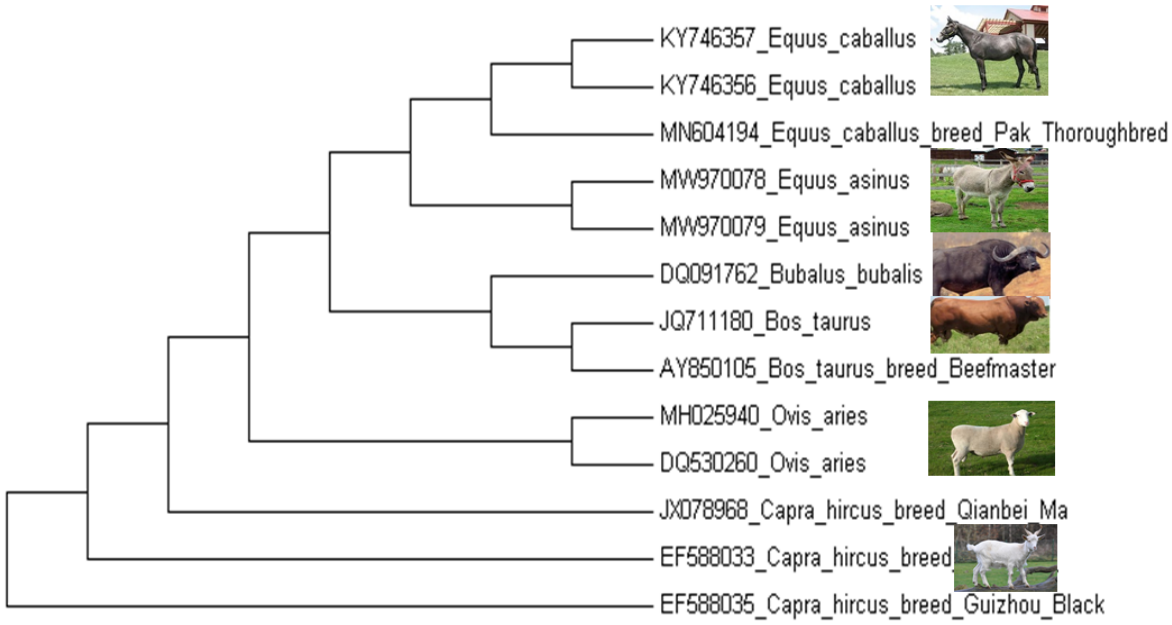

Figure 4. The phylogenic tree is estimated using the NJ method

Li et al. (2014) were identified six SNPs as g.26 T>C and g.156 T>C in promotor region, g.587A>G and g.598C>T in the 5′-UTR, g.1485C>T and g.2115A>G in first exon region of *MSTN* in 15 Chinese horse breeds. The SNPs as g.587A>G and g.598C>T in the 5′UTR region of the *MSTN* gene were detected by Li et al. (2014) for the first time. Baron et al. (2012) detected ten SNPs (2275C>T; 2279A>C; 2287G>A; 2389T>A; 2405T>C; 2407T>C; 2431G>C; 2478G>C; 2524G>A; 2525A>G in AY840554) which eight of them cause amino acid sequence change in the second exon of *MSTN* gene in horses. In this study, an SNP as g.4183919G>A (NW_014637147) which is located at g.2313G>A in the horse genome (AY840554) was identified in the second exon of the *MSTN* gene in Turkish donkeys.

Synonymous (silent) mutations in amino acids do not alter the amino acid coding but its sequence differs. So, although the mutant amino acid type is identical with the wild-type it cannot be counted as exactly the same. (Kristofich et al., 2018). It has been announced that silent (synonymous) mutations can affect several aspects at the molecular level (Kristofich et al., 2018). As known, mRNA is a template using for protein synthesis in the cell and thus has a vital role in translation processes. Synonymous mutation can differentiate the stability and efficient translation of mRNA (Kristofich et al., 2018). Besides, since the mutant amino acid not identical with the wild-type, the translation rate can differ which may lead to a change in protein functionality, protein folding, and its pathways (Kristofich et al., 2018). As mentioned, silent mutations of G>A in leucine (Leu) amino acid were found in this study. The investigation about the Leucine effect on Myostatin expression on skeletal muscles of Wistar rats (Cruz et al; 2020) demonstrated and confirm the importance of Leucine. They have found leucine prevents the loss of skeletal muscle mass and causes a decrement in the Myostatin expression, even block it in some circumstances (Cruz et al; 2020). Since leucine alters the Myostatin expression and synonymous mutations can affect the amino acid and lead to several alterations as mentioned above, it can be interpretable that silent mutation in leucine in this study may affect the *MSTN* gene expression. Mutant leucine may have a lower efficient effect on preventing loss of muscles and causes more Myostatin protein expression. Hence, donkeys that contain silent mutated leucine may have had more loss of muscles, and the difference in physical factors such as containing less muscle mass, lower tempo on physical activities can also be interpreted. These findings can be considered in the breeding of donkeys.

Many studies were conducted on *MSTN* gene polymorphism and its association with racing performances and growth traits in horses (Binns et al., 2010; Tozaki et al., 2010; 2011; 2012; Hill et al., 2010a; 2010b; 2012a; 2012b; Dall'Olio et al., 2010; 2014; Stefaniuk et al., 2014; 2016; Pereira et al., 2016; Cieslak et al., 2018). In previous studies, it was revealed that the SNP as g.2115A>G (g.66493737T>C) in the first exon region of the *MSTN* gene were associated with racing performances in Chinese domestic horses and elite Flat races are known as Group

(Europe and Australasia) or Stakes races (North America) (Hill et al. 2010b; Li et al. 2014). Cieslak et al. (2018) have identified SNPs as g.66495696T>C, g.66495826T>C, and 272 bp SINE insertion in the 5'-flanking of the *MSTN* gene and their associations with biometric traits. They have revealed that the association g.66495696-CC genotypes with differences in cannon bone circumference and height at the withers ($P<0.05$). Hill et al. (2010a) have detected an SNP as g.2115A>G (g.66493737T>C) is strongly related with best race distance in Thoroughbred horses. They have identified that the horses with CC genotype are fast; besides TT genotype horses have a great stamina condition. Tozaki et al. (2011) have investigated that the relations of four SNPs as g.65809482T>C, g.65868604G>T, g.66493737C>T, and g.66539967A>G with body composition traits (mass to height ratio) in Thoroughbred racehorses. Similar with Hill et al. (2010a), Tozaki et al. (2011) have revealed that g.66493737-CC genotype animals had advantage for short-distance racing while CT genotype animals had high body weight/withers height. Hill et al. (2010b) confirmed that the SNP (g.66493737C>T) in *MSTN* gene was a useful predictor of racing distance and speed indices for Thoroughbred racehorses using the EquineSNP50 Bead Chip (Hill et al., 2012a). Binns et al. (2010) investigated the effect of *MSTN* gene variations to athletic ability and racing distance parameters in the Thoroughbred horse. They found that among 54 600 SNPs, only two SNPs as BIEC2-417274 and BIEC2-417495 in *MSTN* gene had genome-wide significance using Equine SNP50 bead chip (Illumina).

Dong-hua et al. (2017) have found four SNPs (g.229T>C, g.872A>G, g.2014G>A, and g.2395C>G) in 13 Chinese donkey breeds. They detected an SNP as g.229T>C in the promotor region, one SNP (g.872A>G) in the first exon and two SNP (g.2014G>A, g.2395C>G) in the first intron region of *MSTN* gene. In this study, an SNP (g.4183919 G>A) was found in the second exon of the *MSTN* gene in Turkish donkeys that Dong-hua et al. (2017) have not identified before in Chinese donkeys. Some researchers conducted that silent mutations might change the secondary structure of proteins (Ramamurthi and Schneewind, 2005).

4. Conclusions

Novel single gene variation of g.4183919 G>A in the 2nd exon region and a synonymous mutation G>A (CTG>CTA) in leucine amino acid in the *MSTN* gene were found in this study. Alterations in mRNA level and functionality of protein can occur due to synonymous mutations. Since leucine is an important amino acid that can avoid muscle mass loss and inhibits the expression of myostatin, it can be said that silent mutation of Leu in donkeys may have altered the muscle mass and physical factor of donkeys in this study. Thus, further studies on protein and molecular levels are needed. Many studies were conducted to investigate the effect of the *MSTN* gene on the race performance of horses. Turkish donkeys lacked these mutations that were identified before in horses, which cause for the less might require for race ability of donkeys. To explore the potential effect of this SNP of the donkey *MSTN* gene, further studies on protein and molecular levels are needed.

Acknowledgment

Data curation: R. Işık. Formal analysis: R. Işık, F. Özdil. Investigation: R. Işık. Methodology: R. Işık, F. Özdil. Software: R. Işık. Validation: S. Meral. Writing-original draft: R. Işık, S. Meral, F. Özdil. Writing-review & editing: R. Işık, S. Meral, F. Özdil.

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