



Sequence Variation and Polymorphism Analysis of the Myostatin (MSTN) Gene in Indigenous Donkeys

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ABSTRACT

Background: Myostatin (*MSTN*), known as growth differentiation factor 8 (GDF-8) is a member of the transforming growth factor superfamily. *MSTN* inhibits skeletal muscle development and growth. Myostatin related variations are extremely important for the regulation of skeletal muscle mass and apoptosis. However, polymorphism of *MSTN* gene is scanty in donkeys.

Methods: We sampled thirty-six donkeys, consisting of Halari, Spiti, Ladakhi and Indian-French (Poitou) genotypes and extracted genomic DNA from blood. PCR amplification and Sanger sequencing of the *MSTN* gene. Sequence analysis and SNP detection were done with UGENE software, followed by haplotype analysis. The phylogenetic relationships among the different breeds were determined using Maximum Likelihood method in MEGA X software.

Result: First time, we analyzed the single nucleotide polymorphisms (SNPs) of the *MSTN* gene in four Indian donkey breeds. In this study, two novel SNPs (T>C) at nucleotide positions 2396 (codon12) and 2398 (codon13) and one SNP (G>A) at position 2422 (codon 21) were identified in the second exon of the *MSTN* gene. From these two SNPs (T>C), one is a non-synonymous mutation the second is a synonymous mutation and the third (G>A) is a non-synonymous mutation. Two haplotypes (H1–H2) were analyzed, haplotype (H2) was the most dominant in all breeds. Ladakhi donkey represented the highest haplotype diversity. Maximum Likelihood Tree: The Ladakhi donkey breed is more genetically related to the horse and donkey reference; Similar to the donkey reference sequence, Halari donkey is also closely related. The sequences of the *MSTN* gene were submitted to the NCBI GenBank with Accession numbers OQ436746-and OQ447192-OQ447217. Finally, the *MSTN* gene and its SNPs require further studies, at protein and molecular level, in association with morphological traits which influence the economic traits of Indian donkey breeds.

Key words: Codon, Donkey, Haplotype, *MSTN*, Phylogenetic tree, SNP.

INTRODUCTION

Myostatin (*MSTN*), a member of the transforming growth factor- β (TGF- β) family, is a muscle development inhibitor. Myostatin (*MSTN*), also known as GDF-8 (Growth differentiation factor 8). This blocks cell cycle progression and thereby inhibits the proliferation of the myoblast during myokine differentiation (McPherron *et al.*, 1997; McCroskery *et al.*, 2003; Ayuti *et al.*, 2024).

In most mammals, *MSTN* gene expression is negatively regulated by glucocorticoids, causing overexpression of the gene, resulting in extra muscle mass, bone density and growth (Grobet *et al.*, 1998; Gilson *et al.*, 2007; Bertolini *et al.*, 2015; Szabó *et al.*, 2025). In addition, the *MSTN* gene negatively regulates skeletal muscle growth and plays an important role in maintaining skeletal muscle homeostasis. The myostatin factor is also responsible for promoting protein balance in muscles (McGivney *et al.*, 2012; Khaerunnis *et al.*, 2016). The *MSTN* gene regulates the development of adipose tissue (Feldman *et al.*, 2006) and therefore is crucial for the growth, complicated functions and development of these domesticated animals. In equine (O'Hara *et al.*, 2021), the *MSTN* is composed of three exons and two introns located on chromosome 18. Extensively studied *MSTN* gene insertion, deletion, or variation impacts muscle mass and growth traits in livestock (McPherron *et al.*, 1997; Grobet *et al.*, 1998; Marcq

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et al., 1998; Marshall *et al.*, 1999; Karim *et al.*, 2000; Tay *et al.*, 2004; Feldman *et al.*, 2006; Bignell *et al.*, 2010; Wang *et al.*, 2025). *MSTN* is inactivated by mutations of the *MSTN* gene, which are responsible for loss-of-function-

excessive muscle development in cattle (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; Miranda *et al.*, 2002), sheep (Walling *et al.*, 2004; Johnson *et al.*, 2005), humans (Schuelke *et al.*, 2004), mice (McPherron *et al.*, 1997; Szabó *et al.*, 1998), chickens (Gu *et al.*, 2002), pigs (Li *et al.*, 2002), dogs (Mosher *et al.*, 2007), horses (Sonali *et al.*, 2022; Miranda *et al.*, 2002) and donkeys (Liu *et al.*, 2017; Raziye *et al.*, 2022; Zhu *et al.*, 2025). The breed-specific studies of equine germplasm have explored the contribution of breeding to phenotypes and genotype-based SSR markers, but genome-wide scans are required for more profound investigations (Gupta *et al.*, 2012; 2014; Pal *et al.*, 2013, 2020, 2021).

Since ancient times, donkeys (*Equus asinus*) have been used as working animals, mainly as pack animals. In India, donkeys are used for transportation in mountainous regions. The latest 20th Livestock Census shows a startling decline in the donkey population of India from 3.2 lakh in 2012 to 1.2 lakh in 2019, losing approximately 1.0 Lakh donkey population (~61.2% loss) from base level values over seven years (Department of Animal Husbandry and Dairying, 2019).

Adult female donkeys have been enumerated as a mere 89,603 against which the population has declined by 37% over the last five years. The decline in number of donkey is due to mechanization of agriculture and transport (Bhardwaj *et al.*, 2020). Few studies have been performed on Indian donkey breeds. The MSTN gene encodes a negative regulator of muscle mass and is integral to growth,

development and performance in domesticated animals. To our knowledge, there is no characterization of sequence variants in genomic MSTN sequence of an Indian donkey.

In our study, we first examined the sequence variations of the second exon in the *MSTN* gene of Indian donkeys.

MATERIALS AND METHODS

Selection of animals

We have selected different donkey breeds namely, Halari, Spiti, Ladakhi and Indian French donkeys (Poitou) for our study (Fig 1). The average ages of donkeys was more than two years. All experimental procedures were approved by the institutional animal ethics committee (IAEC), National Research Centre on Equines, Hisar (Haryana) India. Experimental studies were carried out during three years at Animal Biotechnology Laboratory, ICAR-National Research Centre on Equines, Hisar (Haryana).

Donkey blood sample collection

Thirty-six donkeys were bled from their jugular veins using a vacutainer K2 EDTA with 5 mL of whole blood. Blood samples were received in an ice box and stored at a temperature of 4°C in the lab.

Genomic DNA extraction from blood

Genomic DNA extraction was performed from 200 µL blood samples of each of the donkey breeds studied using DNeasy blood and tissue kit (Qiagen, Germany) following the manufacturer's protocol. We stored the isolated DNA at

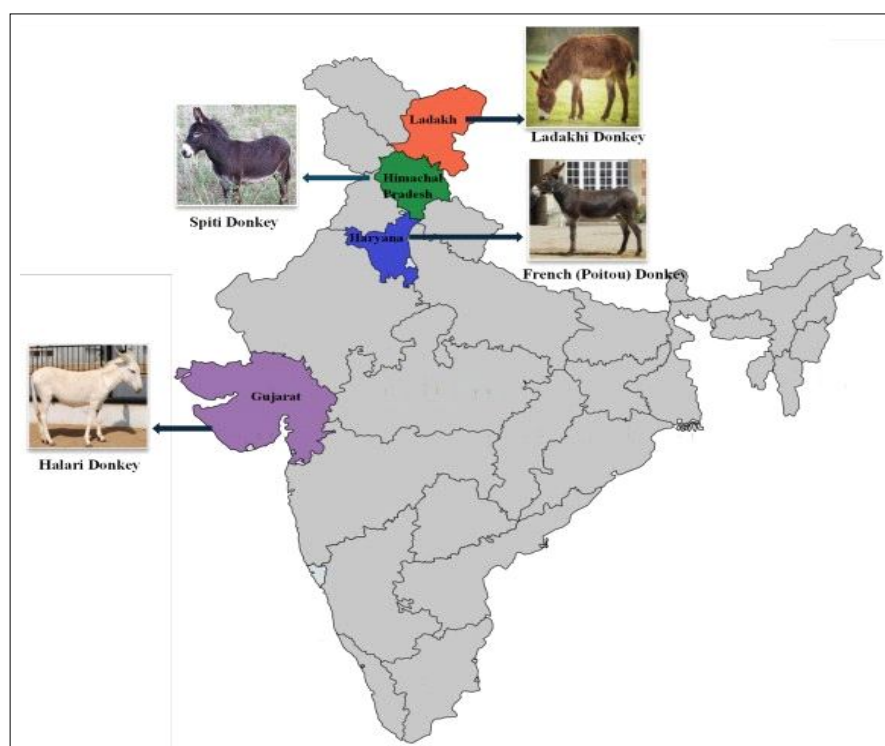


Fig 1: Selected donkey breeds and different states of India for blood sampling.

a temperature of -20°C to prevent its degradation and quantified it by agarose gel electrophoresis (1% agarose gel) followed by qualitative analysis and quantification using a Qubit4 fluorometer (260 nm/280 nm absorbance).

Amplification of *MSTN* gene

PCR reactions for the *MSTN* gene were carried out using 12.5 µL of Promega GoTaq Green master mix (1X), 2 µL of each 10 µM primer, 6.5 µL nucleases free water and 2 µL genomic DNA (100 ng/µL). Amplification of *MSTN* gene by PCR conditions included, an initial denaturation at 5 min at 95°C followed by 35 cycles with one cycle consisting of denaturation (45 sec at 95°C), annealing step (45 sec at 57°C) and extension or amplification (45 sec at 72°C) (Table 1). A final 5 minute extension was performed at 72°C and the products were analyzed on a 1.5% Agarose gel.

DNA sequencing of *MSTN* gene

According to the manufacturer's instructions, 36 *MSTN* gene PCR products were purified by a NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Purified PCR products were sequenced from AgriGenome Pvt Ltd, Kochi, Kerala.

Detection of SNP's in *MSTN* gene and haplotype analysis

The chromatograms of the *MSTN* gene sequences were carefully examined by using CHROMAS version 2.6.6 (Technelysium Pvt Ltd, Australia). The single nucleotide polymorphisms (SNPs) have been analysed *MSTN* gene through UGENE software.

The gene sequences were aligned with the international horse reference sequence [AY840554.2 and 3 international donkey (MZ169554.1, MW970078. 1 and

MW970079. 1)] by using MEGA-X (Kumar *et al.*, 2018). They were then processed with the DnaSP v6 tool (Rozas *et al.*, 2017) to create haplotype datasets. The representation of the haplotype dataset was number of haplotypes, haplotype diversity and nucleotide diversity monomorphic and polymorphic sites.

Phylogenetic analysis of *MSTN* gene

Phylogenetic analysis of *MSTN* gene was performed by maximum likelihood statistical method using MEGA-X software (Kumar *et al.*, 2018) with the Kimura 2- parameter model and a total number of 1000 bootstraps.

RESULTS AND DISCUSSION

Gene amplification of *MSTN*

The isolated genomic DNA of 36 studied donkey samples was analyzed in 1% agarose gel (Fig 2). An Agarose gel with 1.5% concentration was used to check the *MSTN* gene PCR products (250bp) as shown in Fig 3.

MSTN gene sequencing

The trimmed nucleotide sequence (221bp) of *MSTN* gene has been shown in Fig 5.

Analysis of SNPs in *MSTN* gene among studied donkey breeds

Total three SNPs has been detected in exon 2 of chromosome 18 after analysis with the Mongolian horse (accession number-AY840554.2) in 36 samples of four Indian donkey breeds. Out of three SNPs, two novel SNPs (T>C, transition) have been detected at nucleotide position 2396 (codon 12) and 2398 (codon 13) respectively and

Table 1: List of primers sequences used for genotyping in *MSTN* gene.

Primer of <i>MSTN</i>	Primer sequence	Primer annealing temp.	Primer annealing region	Amplified region (bp)	Reference
<i>MSTN</i> -F	5'-GACCCGTCAGACTCCTACA-3'	57°C	Exon 2	250	Dall'Oli <i>et al.</i> , 2010
<i>MSTN</i> -R	3'-TGGAAGGTTACAGCAAGA-5'				

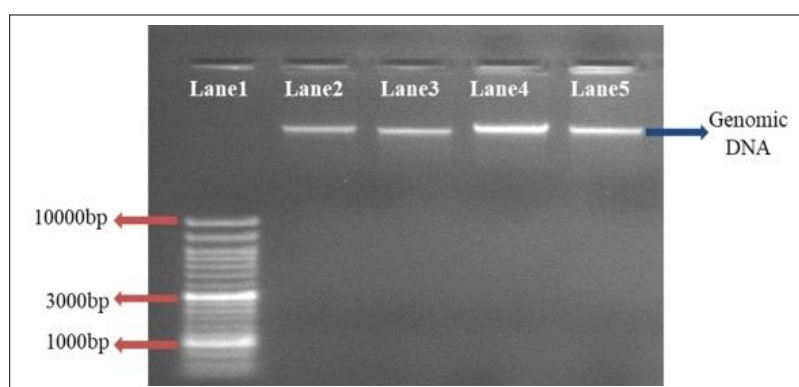


Fig 2: In 1% agarose gel, Lane 1 is showing 1kb ladder, lane 2 to 5 are showing DNA sample of Halari, Spiti, Ladakhi and French (Poitou) donkeys respectively.

one SNP (G>A, transition) has been found at nucleotide position 2422 (codon 21) as shown in Fig 4 and 5. The Phenylalanine (TTT) is converted into Serine (TCT) at codon 12 and Alanine (GCT) is converted into Threonine (ACT) at codon 21 so it is a non-synonymous mutation, while Leucine (TTG) is converted into Leucine (CTG) at codon 13 so it is a synonymous mutation (Table 2). Based on the analysis of all *MSTN* gene sequences with reference sequences, we found only mutant type mutations in all studied Indian donkey breeds (Table 3).

This study compared all 36 samples representing four Indian donkey populations with available international reference sequences of the Guangling donkey (GenBank accession no. MZ169554.1) and the Turkish donkeys (GenBank accession nos. MW970078.1 and MW970079.1). Sequence analysis showed that the Indian donkey populations had 100 % sequence similarity with these reference sequences.

In our study, novel SNPs of the *MSTN* gene have been discovered for the first time and partial DNA fragments of the *MSTN* gene have been obtained from Indian donkeys for the first time.

The sequences of *MSTN* gene were submitted to the NCBI GenBank with the accession number: OQ436746-OQ436755, OQ447192-OQ447217.

Phylogenetic analysis of *MSTN* gene

The phylogenetic tree has been constructed by using thirty-six sequences of four Indian donkey breeds and three sequences of two International Donkey breeds, along with horse reference sequence (AY840554.2) (Fig 6). Indian populations of donkey were Halari donkey, Spiti donkey and local donkeys from the Ladakh region and Poitou donkey used in this experiment.. The Guangling donkey was registered in NCBI as the international reference sequences (GenBank accession no. MZ169554.1) and Turkish donkeys (GenBank accession nos. MW970078.1 and MW970079.1).

A Neighbour Joining tree was constructed using MEGA-X software (Kumar *et al.*, 2018) as a maximum likelihood tree with 1000 bootstraps based on Kimura's 2-parameter model. Comparative analysis of phylogeny showed that *MSTN* gene is equally distributed among all the donkey breeds. The Ladakhi donkey breed is phylogenetically closer to horse reference and international donkey breeds.

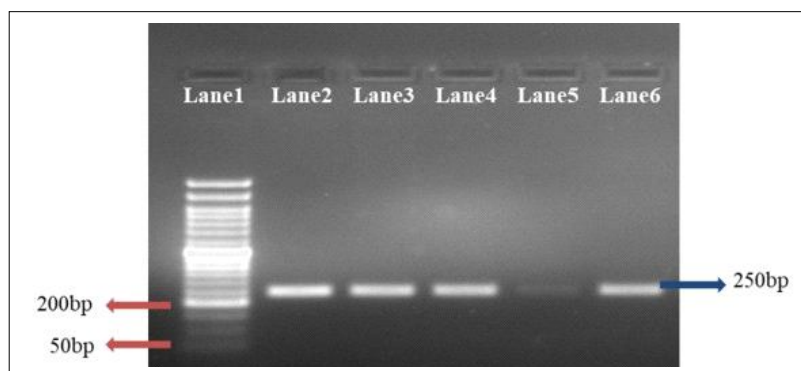


Fig 3: In 1.5% agarose gel, Lane 1 is showing 50 bp DNA ladder, Lane 2- Halari, Lane 3- Spiti, Lane 4- Ladakhi, Lane 5- Blank and Lane 6- French (Poitou) donkey are showing 250 bp PCR product of *MSTN* gene of Indian donkey breeds respectively.

Table 2: Novel single nucleotide polymorphism in donkey's myostatin gene.

SNPs present in codon	Nucleotide change	Codon position	Mutation type	Mutation position (nucleotide)	Codon no.	Change in amino acid	Mutation
T <u>T</u> T12T <u>C</u> T	T>C	Second	Transition	2396	12	Phenylalanine to serine	Non synonymous
I <u>T</u> G13 <u>C</u> TG	T>C	First	Transition	2398	13	Leucine to leucine	Synonymous
G <u>C</u> T21 <u>A</u> CT	G>A	First	Transition	2422	21	Alanine to threonine	Non synonymous

Table 3: Genotypes of wild type, mutant type and different breeds within donkey at positions 2396, 2398 and 2422.

Donkey breeds	No. of samples	Wild	Mutant at 2396 position	Mutant at 2398 position	Mutant at 2422 position
Halari	10	0	10	10	10
Spiti	10	0	10	10	10
Poitou	10	0	10	10	10
Ladakhi	6	0	6	6	6

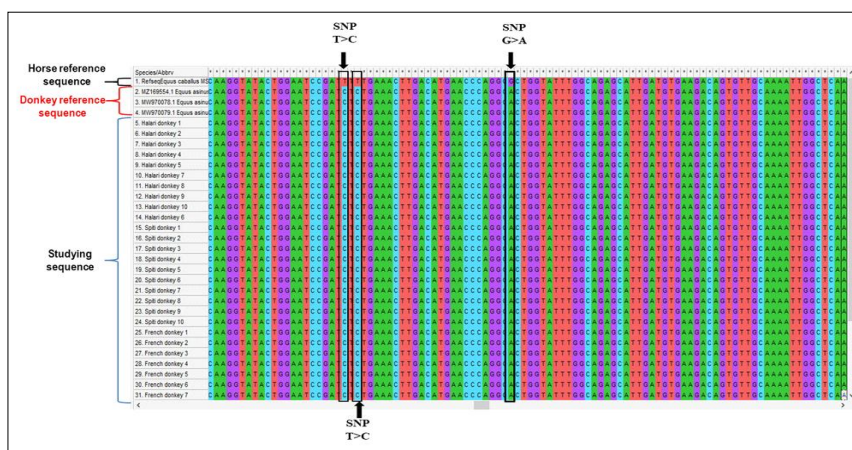


Fig 4: SNP's of T>C (Nucleotide positions 2396 and 2398) and G>A (Nucleotide position 2422) have been detected in MEGA software in the donkey breeds.

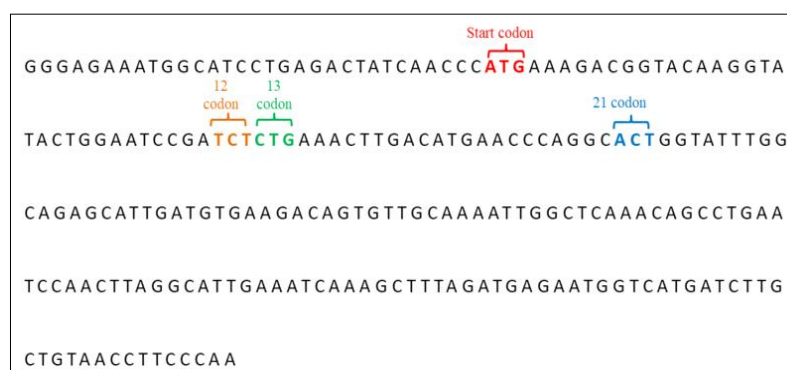


Fig 5: T>C SNP (12 and 13 codon) and G>A SNP (21 codon) has been detected.

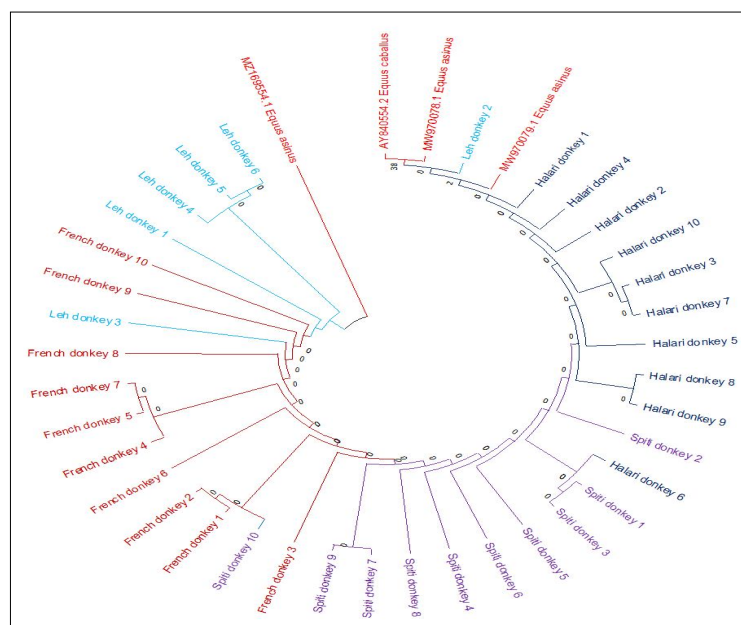


Fig 6: Phylogeny and maximum likelihood tree representing 36 sequences of Indian donkey breeds (Halari, Spiti and Ladakhi donkey) characterized by kimura 2 distance parameter model with 1000 bootstraps along with french donkey (Poitou).

Table 4: Haplotype, haplotype diversity, nucleotide diversity, monomorphic and polymorphic site.

Donkey breed	No. of samples	Haplotype	Haplotype diversity	Nucleotide diversity	Monomorphic	Polymorphic
Singleton result						
Halari	10	2	0.143	0.003	180	3
Spiti	10	2	0.143	0.004	178	3
Poitou	10	2	0.143	0.004	178	3
Ladakhi	6	2	0.200	0.004	178	3
Overall result						
All breeds	36	2	0.050	0.0008	178	3

The Halari donkey has close resemblance to the elite international breeds of donkeys.

Haplotypic analysis of *MSTN* gene

Alignment of all the sequences used in this study with the reference genome (AY840554). 2) identified in total two haplotypes (H1-H2) based on principally from 36 sequences of four Indian donkey breeds. This shows that the haplotype diversity of samples analysed in relation to evolution (the total) is 0.050. Notably, amongst the four breeds, both haplotypes observed for Ladakhi donkey when analysed separately showed maximum haplotype diversity. Halari, spiti donkey and poitu donkey exhibited identical haplotype diversity (0.143) with an equal number of haplotypes. Two haplotypes (H1-H2) were evaluated, the second haplotype (H2) was detected as most prevalent across all breeds. Based on haplotype and nucleotide diversities, population genetic analyses suggested that the genetic variability is reached at the highest level in Ladakhi donkey. From our study we found total of three polymorphic sites (Variable) and 178 monomorphic sites (Invariable) via out overall analysis of four Indian donkey breeds (Table 4).

Genetic polymorphism studies in livestock specially those focusing on myostatin (*MSTN*) and other functional candidate genes influencing growth and productivity, have been widely documented, underscoring their importance in molecular breeding and genetic improvement programs (Nugroho *et al.*, 2017; Liu *et al.*, 2023a; Liu *et al.*, 2023b).

The near 2.0 lakh fall in donkey numbers, between 2012-19 underscores extreme genetic dissolution among native stocks. The Myostatin (*MSTN*) gene, a major regulator of muscle development, is therefore pivotal in this regard. Specifically, the variations discovered in the donkey breed investigated could be linked to qualities such as muscle mass, endurance and ability to carry heavy loads and hence offer a molecular foundation for enhancing selection and conservation of genetically superior individuals.

In Indian donkey breeds the *MSTN* gene has been studied for the first time. When comparing 36 sequences of the *MSTN* gene with the reference sequence (AY840554.2), three SNPs were found in the second exon of chromosome 18. Two of these SNPs (T>C) were novel. One of the SNPs (T>C) was detected at the 2396 nucleotide position (codon 12), resulting in the conversion of

Phenylalanine (TTT) to Serine (TCT), which is a non-synonymous mutation. Another synonymous mutation (T>C) was found at the 2398 nucleotide position (codon 13), where Leucine (TTG) was converted to Leucine (CTG). Additionally, a mutation (G>A) was identified at the 2422 nucleotide position (codon 21), leading to the conversion of Alanine (GCT) to Threonine (ACT), making it a non-synonymous mutation (Table 2). Li *et al.* (2014) also identified six SNPs in the *MSTN* gene in 15 breeds of Chinese domestic horses. They are located in the promoter (g.26 T>C and g.156 T>C), 5'-UTR (g.587A>G and g.598C>T) and first exon region (g.1485C>T and g.2115A>G). Polymorphism in the *MSTN* gene has previously been associated with racing performance and other growth characteristics when compared between thoroughbred horses. Binns *et al.* (2010); Tozaki *et al.* (2011); Hill *et al.* (2012); Dall'Olio *et al.* (2014); Stefaniuk *et al.* (2016); Cieslak *et al.* (2018) and Pira *et al.* (2021). Notably Cieslak *et al.* (2018) made two particular associations between individual SNPs in the 5'-flanking region of *MSTN* including g.66495696T>C and g.6649 5826T>C and a 272 bp SINE insertion which appeared to be associated with biometric traits. They concluded that "CC genotypes" are fast and "TT genotypes" great in stamina. Four SNPs (g.229T>C, g.872A>G, g.2014G>A and g.2395C>G) from 13 Chinese donkey breeds were finally detected by Liu *et al.* (2017). The SNPs identified are in the promoter region (g.229T>C), first exon (g.872 A>G) and first intron region (g.2014 G>A, g.2395C >G). Also, Liu *et al.* (2017) discovered 1 SNP (g.4183 919 G>A) in the second exon of *MSTN* gene from Turkish donkey that has not been previously found in Chinese donkeys.

TT and TC haplotypes in promoter region of *MSTN* gene were detected in Polish Konik, Thoroughbred, Hucul, Arabian and Polish Heavy Draft by Stefaniuk *et al.* (2014). Mongolian horse breeds sample with 0.0084 nucleotide diversity of the exon-1 in the *MSTN* gene was reported by Sergelen *et al.* (2019). They also found 233 invariable sites and 5 variable sites.

In our study, in the phylogenetic analysis of Indian donkey breeds, the Ladakhi breed is more closely related to the horse (AY840554.2) and donkey (MZ169554.1, MW970078.1, MW970079.1) reference sequence while the Halari donkey breed is closely related to the donkey reference sequence. The remaining breeds were evenly distributed among equine breeds (Fig 6).

CONCLUSION

In this study, we have first time analyzed the *MSTN* gene in four Indian donkey breeds: Halari, Spiti, Ladakhi and Poitou donkey. We have identified three SNPs, out of which two SNPs (T>C, at 2396, 2398 nucleotide position) were novel in Indian donkey breeds. We have also analyzed two haplotypes (H1-H2), from which the second haplotype (H2) was most dominant among all breeds. The Ladakhi donkey breeds represented the highest haplotype diversity. A lot of studies had been done on the influence of *MSTN* gene in race performance of horses. The mutations previously discovered and associated with racing performance in horses were also found for these Indian donkey breeds and might improve the race ability of donkeys. Additional studies should be performed to investigate this SNP's effect on the protein and molecular levels in the *MSTN* gene among Indian donkeys.

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Data availability

All data used in this paper were generated by ICAR-National Research Centre on Equines, Hisar and are available from the corresponding author on request.

Author's contributions

Anuradha Bhardwaj, Shiv Kumar Giri developed and designed the study. Blood sample collection Sonali, Yashpal and Anuradha Bhardwaj. Sonali, Shiv Kumar Giri, Anuradha Bhardwaj conducted experiments. Bioinformatics and Data analysis Sonali, Varij Nayan and Anuradha Bhardwaj. Drafting the manuscript: Sonali, Anuradha Bhardwaj, Shiv Kumar Giri. The data analysis and manuscript preparation were informed by the contributions of Yashpal, Varij Nayan³ and Bhupendra Nath Tripathi. Also to inform you that, Anuradha Bhardwaj and Shiv Kumar Giri will be acting as a corresponding author for this research paper who have done equal contribution in all aspects. Corresponding authors confirm that all authors (including those listed in the Acknowledgment) have read and approved of this submission.

Conflict of interest

We declare that this manuscript is original, was not published before and it is not submitted for publication elsewhere. There are no conflicts of interests associated with this publication.

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