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Original scientific paper

MOLECULAR CHARACTERIZATION OF *Myostatin* GENE IN MALPURA SHEEP OF RAJASTHAN

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Objective of study was to characterize the complete sequence of *Myostatin (MSTN)* gene in Malpura sheep and to study its genetic variability across population. This study is the first ever report of complete *Myostatin* gene characterization among the Indian sheep breeds. *MSTN* (GDF-8) is found to be a negative regulator of skeletal muscle growth which is responsible for double muscling. The MSTN gene sequence was partitioned in 18 different overlapping fragments (P1 to P18). We obtained a sequence of 8002 bp (MH025940) for complete *MSTN* gene. Phylogenetic analysis revealed that *MSTN* gene of Malpura sheep breed is in close relation with that of Texel sheep and far related to pig. A total of 16 nucleotide substitutions were identified, revealing rich genetic diversity. Out of these 16 substitutions, none was observed in exonic region, 2 were observed to be present at promoter, 3 in the 5'UTR, 6 in intron-1, 1 in intron-2 and 4 in 3'UTR. Two substitutions c.373+1189 A or G and c.*202 A or G were found in intron-1 and 3′UTR, respectively and were new. The putative mutation for double muscling in sheep (c.*1232A) was not present in Malpura population (c.*12132G).

Keywords: Characterization; GDF-8; Genetic variability*;* Malpura sheep*;* Myostatin gene.

INTRODUCTION

Sheep husbandry is a backbone to the rural livelihood in arid, semi-arid and mountainous regions in India. India has a huge genetic diversity of sheep resources with 43 sheep breeds and 74.26 million population (LIVESTOCK CENSUS, 2019). Malpura is a heavy and well adapted breed of sheep found in semi-arid region of Rajasthan, widely distributed in the

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Tonk, Jaipur and Sawai Madhopur districts and known for its adaptability to the harsh climate and potential for higher growth (GOWANE *et al*., 2010). The ICAR-Central Sheep & Wool Research Institute (CSWRI) is involved in genetic improvement and conservation of this sheep breed. For genetic improvement of sheep, faster growth and early maturity are important. The improvement of farm animals for meat production requires strategies to increase muscle growth and retaining leanness and meat quality at slaughter. Ideally, post-mortem carcass and meat characteristics need to be predicted and selected in the sheep when it is still alive, as this will allow the early selection of desirable breeding stock and thus increase genetic gain for desirable traits. The *MSTN* is located on the Ovine chromosome 2 (BOMAN *et al*., 2009). Quantitative trait loci (QTL) can affect the production traits. QTL studies have shown major effect of *MSTN* gene on growth and carcass traits in sheep.

MSTN gene (~ 8Kb) is made up of 3 exons and 2 introns. It negatively regulates the muscle growth in most animals and belong to transforming growth factor β (TGF-β) superfamily (MCPHERON *et al*.,1997; RIOS *et al*., 2002; MCMAHON *et al*., 2003). *MSTN* is responsible for inhibiting the proliferation of myoblast cells at different cell stages *viz*. G1 stage or G2/M stages of cell cycle (MATEESCU *et al*., 2002; RIOS *et al*., 2002). *MSTN* also activates cyclin-dependent kinase pathways that adversely affect prenatal and postnatal myogenesis (LANGLEY *et al*., 2002; MCCROSKERY *et al*., 2003; PASTE *et al*., 2004). During the last decade or so, a total of 77 *MSTN* SNPs were reported in various sheep breeds across the world (AIELLO *et al*., 2018).

MSTN is responsible for double muscling phenomenon initially documented in cattle (KAMBADUR *et al*., 1997). Double muscling increases the muscle mass and hence decrease weight of bones, fat and also elementary tract (MENISSIER, 1982). The reduction in the weight of other body components makes heavier carcasses and also higher percentage of leaner and expensive meat cuts (SHAHIN *et al*., 1985). The leaner and heavier carcasses are favorable for both farmers and processors as consumers require leaner cuts of meat (JONES *et al*., 2003). Mutation in 3'UTR (c*1232G>A) creates an illegitimate miRNA binding site that influences double muscling trait (CLOP *et al*., 2006). It was observed that the *MSTN* may also regulate the adipogenesis (LEE *et al*., 2001) via reduction in production as well as secretion of leptons (MCPHERRON *et al*., 2002). It was also found to be important for regulation of tendon structures (MENDIAS *et al*., 2008).

There is no information for complete *MSTN* gene characterization in any Indian sheep. Present study aimed at characterization of the full sequence of *MSTN* gene in Malpura sheep along with its genetic variability in the population.

MATERIALS AND METHOD

Experimental animals

In the current study a Malpura sheep flock (529 animals) was screened for genetic variability and representative samples of different genetic variable group were selected for final sequencing. The sheep flock was maintained at the ICAR-CSWRI Avikanagar, which is located in the semi-arid region at 75°28′ E Longitude and 26°17′ N Latitude. The location is at an altitude of 320 meters above mean sea level.

The approval of the Institute Animal Ethics Committee (IAEC) was accorded at serial number 17 of the proposal.

Genomic DNA isolation and Primer selection

For the study, the whole blood (1 ml) was collected from the jugular vein of lambs aseptically in a plastic tube containing 200 µl of Acid Citrate Dextrose (ACD) solution as an anticoagulant. DNA was extracted using Blood Genomic DNA extraction Kit (HiMedia) as per manufacturer's instruction. Quality and yield of genomic DNA was estimated by agarose gel electrophoresis (2%) and UV Spectrophotometer. Due to larger fragment size (~ 8kb), *MSTN* gene was broken in 18 small fragments and the amplification was performed for 18 different segments separately, however these segments had overlapping primers (Table 1).

Table 1. Primer sequences used for the amplification of Myostatin gene in Malpura sheep

Amplicon Number	*Region*	*Forward Primer*	*Reverse Primer*	*Annealing Temperature (˚C)*	*Size (bp)*
Amplicon 1	*5'UTR*	*ACACTTGTCTCATCAAAGTTG*	*TATGGCTTCTAGTCTTGAGG*	*60˚C*	*355 bp*
Amplicon 2	*Exon 1*	*GTAATCCATGCTTGTGGAGAC*	*ACACTAGAACAGCAGTCAG*	*57˚C*	*312 bp*
Amplicon 3	*Exon 2*	*CTAAATATGCACACATTATTCC*	*GCATGTTATTTTCAGTAATCACT*	*56˚C*	*513 bp*
Amplicon 4	*Exon 3*	*ACTCTTCTTTCCTT TCCATAC*	*CACAGCGATCTACTACCATG*	*57˚C*	*393bp*
Amplicon 5	*Exon 3*	*GTACTCCTACAAAGATGTCTC*	*AGAATTGCTTTCATTCACCTG*	*61˚C*	*405bp*
Amplicon 6	*3'UTR*	*AATTAGTTGATTAAATAGTGGT*	*ACAATTTGTAAGATACCATCAG*	*50˚C*	*273 bp*
Amplicon 7	*Promoter*	*ATCCCTGCCAGGAGTCTG*	*TACAAAGAGGATATTGTCAGC*	*54˚C*	*326 bp*
Amplicon 8	*Promoter*	*GTCATTCTAAGTTATTCTAAGATC*	*AGAAAATACAGATTTATTCAGG*	*48˚C*	*327 bp*
Amplicon 9	*Promoter*	*ATCACAATCTTTTCATTTAAGTC*	*TTGTTACAGTCAAGGGTGAG*	*53˚C*	*366 bp*
Amplicon 10	*Promoter*	*AAGAAGTAGTCAAATGAATCAG*	*CAACAAGCAGCATAAATAGGT*	*58 ˚C*	*370 bp*
Amplicon 11	*Intron 1*	*ACGGAAACGGTCATTACCA*	*ATTAAGCTGTGAAAACATAAAC*	*58˚C*	*328 bp*
Amplicon 12	*Intron 1*	*ATATGCTAATGAGACTGAAAG*	*ACACTGTCTTTAGGGTCAG*	*58˚C*	*1014 bp*
Amplicon 13	*Intron 1*	*ACTATTGTTGAGAGTACCTG*	*AAGTTTCAGAGATCGGATTC*	*60˚C*	*1259 bp*
Amplicon 14	*Intron 2*	*AGAGCATTGATGTGAAGAC*	*TCATT TGGGGAGTTAATAAC*	*58˚C*	*1185 bp*
Amplicon 15	*Intron 2*	*CTATGCTTGTATTTACTTCTG*	*CTAATCATTTTGTTTATGTCAC*	*59˚C*	*1068 bp*
Amplicon 16	*Intron 2*	*TGTCATCCATTAGTATATTCAG*	*ATTCACATTCTCCAGAGCAG*	*58˚C*	*497 bp*
Amplicon 17	*3'UTR*	*ACAGTATATGAACTAAAAGAG*	*ATTATACAGCCATCACGAAC*	*56˚C*	*1007 bp*
Amplicon 18	*3'UTR*	*TTAAATAGTGGTCTTAAAACTC*	*AGAACTTGTAACTCTAGGAC*	*54˚C*	*745 bp*

Source: Han et al., 2013; Mol. Biol. Rep. (40):6379-6384

PCR amplification of different regions of MSTN gene

Amplification protocol varied between the segments. For less than 500 bp amplicons, all the PCR reactions were carried out in 25 µl volume comprised of 5X PCR Buffer (5µl), 25mM $MgCl_2$ (3µl), 10mM dNTP (1µl), 10 pmol (1µl) of each primer, 1 units of *Taq* DNA Polymerase, template DNA (2µl) and nuclease free water (NFW) to make 25µl reaction mixture. The thermal profile was adjusted for amplification of the *MSTN* gene. Initial denaturation at 94ºC for 2 minutes, that was followed by 35 cycles of denaturation for 94ºC for 30 s, annealing at 48ºC to 61 ºC for 30s (varied between segments), extension at 72ºC for 30s and a final extension at 72ºC for 5 minutes on ABI thermal cycler (Applied BioSystem). PCR products were

resolved on 2% Agarose (SRL Pvt. Ltd.) gel with 100 bp DNA markers (SRL Pvt. Ltd.). The larger (>500bp) amplicons (amplicons 12-18) were amplified by using 0.5 IU proof reading (*Pfu) Taq* DNA polymerase (Thermo fisher scientific), 200 ng DNA, 1X phusion high fidelity buffer containing $MgCl_2$ (Thermo fisher scientific), 0.1 mM PCR nucleotides, 10 µM each primer having 25 µl reaction volume. PCR conditions were initial denaturation at 94˚C for 2 minutes followed by 35 repeated cycles of denaturation at 94˚C for 30 s, annealing temperature from 54˚C to 60˚C (varied for different amplicons) for 30 s, extension at 72 ˚C for 30 s and final extension at 72˚C for 5 minutes on ABI thermal cycler (Applied Biosystem). PCR products were resolved on 2% Agarose (SRL Pvt. Ltd.) gel with DNA markers (SRL Pvt. Ltd.).

The amplified and purified products were genotyped using Single Stranded Confirmation Polymorphism (SSCP) (GREEN *et al*., 2012). The samples were then grouped according to SSCP patterns. The representative samples were sequenced in both the directions by Sanger sequencing method that exploits di-deoxy chain termination principle.

Characterization and functional analysis of MSTN variation of Malpura sheep

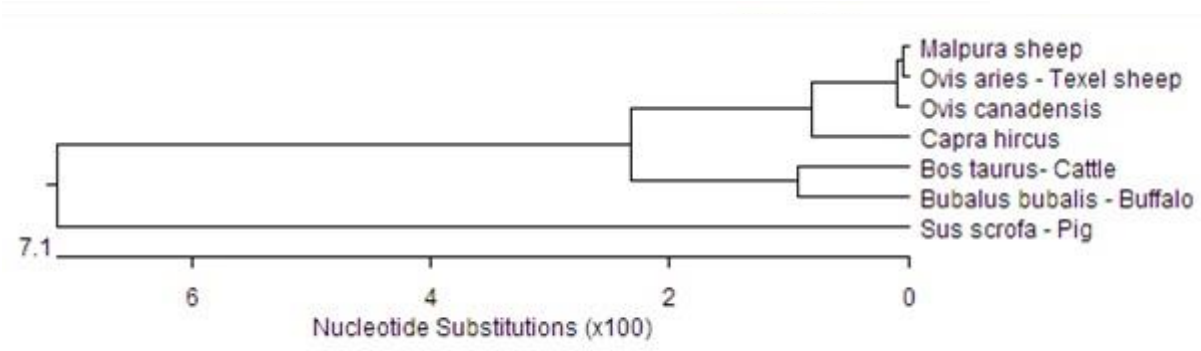
The obtained sequences were analyzed by Chromas Lite 2.0 software to read amplified segments of the sequence. All the amplicons in the population were studied by aligning them with the help of MegAlign algorithm (LASERGENE Software) to know the variability present in the population at each important polymorphic region. The final complete sequence of *MSTN* was obtained by aligning the entire amplicons one after the other with the help of EMBOSS Merger (RICE *et al*., 1999;2000). The Exonic regions were identified using Ensembl genome browser 91(FLICEK *et al*., 2010) and the peptide sequence were translated versions of obtained nucleotide sequences. BLAST for the obtained sequences was carried out for percent identity with other nucleotide and protein sequences of cattle, goat, sheep, pig, *etc*. MatInspector (CARTHARIUS *et al*., 2005) was used for the analysis of transcription factor binding sites (TFBS) at polymorphic loci of Malpura sheep. For TFBS identification, only matrix/core similarity >0.85/1.00 were taken into consideration.

RESULTS

In-silico analysis of MSTN gene nucleotide sequences

A complete sequence of *Myostatin* gene was obtained for the Malpura sheep (MH025940). The sequence included Promoter region, 5'UTR, all three exons, two introns and the 3'UTR region of Ovine *MSTN* gene in Malpura sheep. This study is the first ever report for characterization complete sequence of *Myostatin* gene in the Indian sheep.

BLAST analysis of the sequence was performed with *MSTN* gene of other domestic species *viz*. Pig (AY208121.1), Buffalo (DQ091762.1), Goat (EF591039.1), Cattle (JQ711180.1), as well as other sheep breeds such as Texel sheep (DQ530260.1) and also Bighorn sheep (*Ovis Canadensis)* (CP011887). Present study showed that the Malpura breed has close similarity for *MSTN* gene with Texel sheep (99.6%), Bighorn sheep (99.5%), goat (97.2%), buffalo (95.0%), cattle (92.1%) and also pig (86.9%). The phylogenetic analysis revealed that *MSTN* gene sequence of this breed is closely in relation to that of Texel sheep (Fig.1) and far away related to pig.



Nucleotide substitution and SNP identification

We observed 16 substitutions in Malpura sheep population in the present study (Table 2). The Nucleotide numbering nomenclature (http://varnomen.hgvs.org/recommendations/DNA/, dated 1 May 2016; based on NCBI GenBank accession number DQ530260) was followed for the naming of substitutions. Out of total 16 substitutions, 3 were located at the 5′UTR, 2 at the Promoter region, 6 were at intron-1, 1 at intron-2 and 4 at 3′UTR. One deletion was observed at 5′UTR region of Malpura sheep MSTN. Two substitutions were found to be new and not reported in any earlier study. These were c.373+1189 A or G and c.*202 A or G at the intron-1 and 3′UTR, respectively. Study revealed huge variability of *MSTN* gene in the current population.

Table 2. Nucleotide substitution found within ovine Myostatin gene in Malpura sheep

S No.	Nucleotide Change	Position from Start Sequence	Region
1	C or T	c.-1128	Promoter
2	C or T	c.-958	Promoter
3	C or A	c.-40	5’UTR
4	C or T	c.-37	5’UTR
5	Del of T	c.-31	5’UTR
6	G or T	c.373+18	Intron 1
7	A or G	c.373+243	Intron 1
8	C or T	c.373+249	Intron 1
9	G or T	c.373+259	Intron 1
10	T>C	c.373+323	Intron 1
11	**A or G**	**c.373+1189**	**Intron 1**
12	T>C	c.748-54	Intron 2
13	**A or G**	**c*202**	**3’UTR**
14	C>A	c*709	3’UTR
15	G>A	c*1232	3’UTR
16	A or G	c*1316	3’UTR

Nucleotide substitutions based on NCBI GenBank accession number DQ530260. Substitutions that are shown in bold were new. (Nucleotide nomenclature: *http://varnomen.hgvs.org/recommendations/DNA/, dated 1 May 2016)*

Functional significance of MSTN gene variation

A total of 3800 TFBS were found in the reconstructed 8002 bp *MSTN* gene sequence of Malpura sheep, out of which only polymorphic loci were analyzed in the present study. Figure 2 illustrates specific TFBS of the detected nucleotide distinctions in the *OvineMSTN* gene in Malpura breed. TF family/matrix description in detail has been given in the supplementary Table.

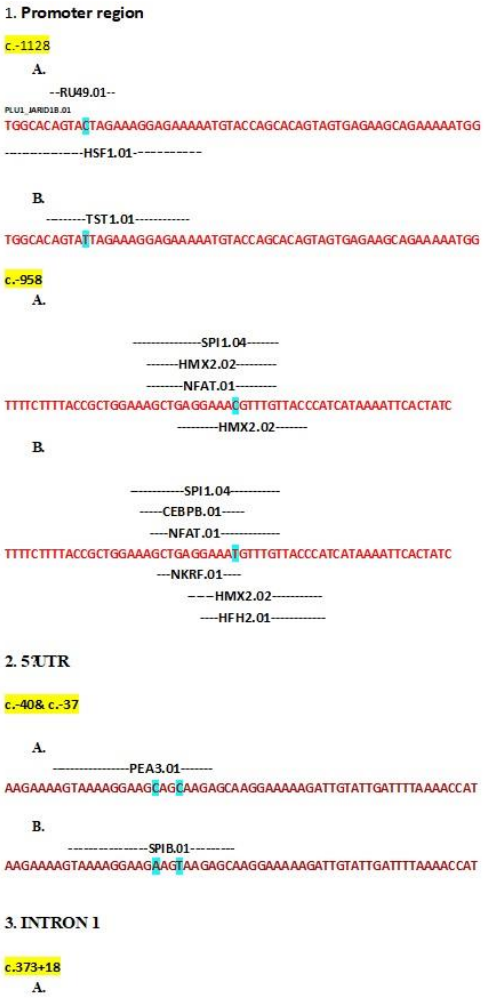


Fig 2. Transcription factor binding site (TFBSs) analysis of variable region of MSTN gene of Malpura sheep by MatInspector (https://www.genomatix.de/cgi-bin/matinspector_prof/). Highlighted portion are the variable position (with respect to start position) of MSTN gene in Malpura sheep with their respective TFBSs

Protein sequence identification

Open reading frame or cDNA of *MSTN* gene was 1128 bp in current study in Malpura breed. The derived protein sequence of 375 amino acid was obtained (*In-silico*) and compared for variability with the earlier reported complete and partial sequences of Texel sheep (DQ530260.1) and Bandur sheep breed (KM371731). Similarly, the sequence was compared with other species such as goat (EF591039.1), buffalo (DQ091762.1), cattle (JQ711180.1), horse (NM_001081817.1) and pig (AY208121.1). Phylogenetic analysis showed that Malpura sheep, Bandur sheep, Texel sheep, NZ sheep and goat have similar primary protein structure of *MSTN*.

DISCUSSION

The present study investigated variation in the complete genomic region of *MSTN* gene in Malpura sheep breed of semi-arid tropical region of Rajasthan. The complete genomic region of 8003 bp (CLOP *et al*., 2006; HAN *et al*., 2013) was targeted for the present work which consists of lengthy genomic region across c.-1199 to c*1813, from the promoter to 3' UTR of sheep *MSTN*. We obtained 8002 bp long *MSTN* gene in Malpura sheep. There was one bp deletion (c.-31delT) at 5'UTR in this population as compared to previous references (CLOP *et al*., 2006; HAN *et al*., 2013). Out of total observed substitutions, 14 were reported in earlier studies in different populations. These include c.-1129C>T (HEATON *et al*., 2007) and c.-959C>T (HAN *et al*., 2013; WANG *et al*., 2016) in the promoter; c.-41A>C (14,24-26), c.-38C>T (CLOP *et al*., 2006; SJAKSTE *et al*., 2011) and c.- 31delT (ZHOU *et al*.,2008; BOMAN *et al*., 2009(a); 2009(b); SJAKSTE *et al*., 2011; WANG *et al*., 2016) in the 5'UTR; c.373+18T>G (CLOP *et al*., 2006; KIJAS *et al*., 2007; GAN *et al*., 2008; ZHOU *et al*., 2008; HICKFORD *et al*., 2010; SJAKSTE *et al*., 2011), c.373+243A>G (CLOP *et al*., 2006; GAN *et al*., 2008; HICKFORD *et al*., 2010; SJAKSTE *et al*., 2011), c.373+249C>T (CLOP *et al*., 2006; HICKFORD *et al*., 2010; SJAKSTE *et al*., 2011), c.373+259T>G and c.373+323T>C (CLOP *et al*., 2006; GAN *et al*., 2008; HICKFORD *et al*., 2010; SJAKSTE *et al*., 2011); c.748-54 T>C (HAN *et al*., 2013) in the Intron 2; and c.*709 C>A (HAN *et al*., 2013), c.*1232G>A (JOHNSON *et al*., 2000; CLOP *et al*., 2006; KIJAS *et al*., 2007; GAN *et al*., 2008; HADJIPAVLOU *et al*., 2008; HICKFORD *et al*., 2009; BOMAN *et al*., 2009(b); BOMAN *et al*., 2010; TAKEDA *et al*., 2010) and c.*1316A>G (HEATON *et al*., 2007) in the 3'UTR. Result revealed that, Malpura population is not highly variable for *MSTN* gene. This variation in MSTN may affect the functional activity of *MSTN* gene expression. The amino acid sequence (*In-silico*) in current study had little variation at position 274 (Val to Glu) from the previously reported amino acid sequence in Bandur breed. Val is a hydrophobic, non-polar aliphatic molecule, whereas Glu is hydrophilic, polar aliphatic molecule. This change in ORF may affect the *MSTN* protein and also may lead to changes in the meat traits, however this needs to be investigated further.

Intronic region variation may influence secondary structure of mRNA, its splicing and also function of the *MSTN* gene, skeletal muscle mass and sheep meat quality (HILLER *et al*., 2007, SJAKSTE *et al*., 2011). Mutation c.373+18T/G found in this study plays a significant role in determination of stability of pre-mRNA secondary structure as it is present near donor splice site. This may affect the mRNA splicing and transcriptional efficiency. These mutations were also reported in different sheep breeds e.g. Latvian dark-sheep (SJAKSTE *et al*., 2011), in New Zealand sheep breeds (HAN *et al*., 2013) and in Suffolk and Texel breeds (KIJAS *et al*., 2007). Whereas,

the allele c.373+18G shows binding affinity for NACA.01 that is involved in regulation and differentiation of myoblast cells and myogenic lineages (YOTOV *et al*., 1996).

In the present investigation, nucleotide variations c.373+243G or A, c.373+249 G or T, c.373+259 G or T binds with E4BP4, NANOG and VBP which during muscle differentiation acts as transcriptional repressor (LANG *et al*., 2009). Whereas, c.373+323 T>C position was marked for GATA2 TF that regulate muscle growth through calcineurin signaling pathway (MICHEL *et al*., 2004; SJAKSTE *et al*., 2011). It is known that the SNP c.*1232A which is also known as g+6723 G-A, g+6223G>A is a selection marker for muscle traits (CLOP *et al*., 2006; KIJAS *et al*., 2007; JOHNSON *et al*., 2009). The c.*1232A is responsible for creation of an illegitimate mRNA binding site which affects the double muscling phenotype. Malpura sheep population has mutant allele guanine at this position. The last variable region observed in *MSTN* of Malpura sheep was c*1316 A/G in 3'UTR, the variable position c*1316A binds with *Six1* (Sine oculis homeobox homolog 1), which can control the expression of MRF by regulating the activity of *MEF*2, *MEF*3 and *MYF*5/MyoD proteins (CHENG *et al*., 1993; KAWAKAMI *et al*., 1996; SPITZ *et al*., 1998).

CONCLUSION

Current study is the first ever report of characterization of full sequence of *MSTN* gene (MH025940) in Indian sheep. The studied gene was observed to be highly variable in the studied population. In this study different nucleotide substitutions were observed in Malpura *MSTN* sequence along with two newly identified substitutions (c.373+1189 A or G in intron-1 and c.*202 A or G in 3′UTR). The variations observed in the *MSTN* gene of Malpura sheep may be significant for gene function and hence they should be studied in future for unraveling their influence on carcass characteristics.

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MOLEKULARNA KARAKTERIZACIJA *Myostatin* GENA KOD MALPURA OVACA U RADŽASTANU

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Izvod

Cilj ovog rada je bio da se karakteriše kompletna sekvenca gena *Miostatin* (MSTN) kod ovaca Malpur i da se prouči njegova genetska varijabilnost u populaciji. Ova studija je prvi izveštaj o potpunoj karakterizaciji gena *Miostatin* među indijskim rasama ovaca. Nađeno je da je MSTN (GDF-8) negativan regulator rasta skeletnih mišića koji je odgovoran za dvostruko uvećanje mišića. Sekvenca gena MSTN je podeljena u 18 različitih fragmenata koji se preklapaju (P1 do P18). Dobili smo sekvencu od 8002 bp (MH025940) za kompletan MSTN gen. Filogenetska analiza otkrila je da je MSTN gen rase ovaca Malpura u bliskoj vezi s genima Tekel ovaca, i daleko udaljen od svinja. Identifikovano je ukupno 16 nukleotidnih supstitucija, otkrivajući bogatu genetsku raznolikost. Od ovih 16 supstitucija nije primećena nijedna u regiji egzona, 2 su bile u prisutne u promotoru, 3 u 5'UTR, 6 u intronu-1, 1 u intronu-2 i 4 u 3'UTR. Dve supstitucije c.373 + 1189 A ili G i c. * 202 A ili G pronađene su u intron-1 i 3'UTR, i bile su nove. Pretpostavljene mutacije za dvostruko uvećanje mišića kod ovaca (c. * 1232A) nisu bile prisutne u populaciji Malpura (c. * 12132G).

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