

# MOLECULAR CLONING AND CHARACTERIZATION OF RABBIT MYOSTATIN GENE

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## ABSTRACT

Myostatin is a member of the transforming growth factor- $\beta$  (TGF- $\beta$ ) superfamily, which is expressed specifically in vertebrate skeletal muscle and functions as negative regulator of skeletal muscle growth. In the present study, the three exonic regions of myostatin gene (MSTN) were resequenced in fifteen animals of three different rabbit (*Oryctolagus cuniculus*) breeds (White giant, Soviet chinchilla and Desi) with a view to make comparative study of it. Resequencing and comparison of three MSTN exonic region of these animals showed only one variation (a>G) in exon I of a soviet chinchilla (S11) and one variation (g>A) in exon III of a white giant breed (W12), respectively. The translated amino acid sequences of MSTN of these animals showed first variation at start codon (valine instead of methionine) and the second variation at amino acid position 267 (asparagine instead of aspartic acid). We conclude that the myostatin gene is highly conserved within these three different breeds of rabbit as well as within the different species of animals and is little affected by these variations.

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### KEY WORDS

myostatin; rabbit; exon; resequencing; codon

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## [1] INTRODUCTION

Rabbits are small mammals in the family Leporidae of the order Lagomorpha found in several parts of the world and used for many purposes mainly meat, fur production etc. Several DNA markers associated with production traits in livestock have been already identified through candidate gene approach [1], which is based on the fact that variability within genes coding for protein products involved in key physiological mechanisms and metabolic pathways directly or indirectly involved in determining an economic trait (e.g. feed efficiency, muscle mass accretion, reproduction efficiency, disease resistance, etc.) and might probably explain a fraction of the genetic variability for the production trait itself [2].

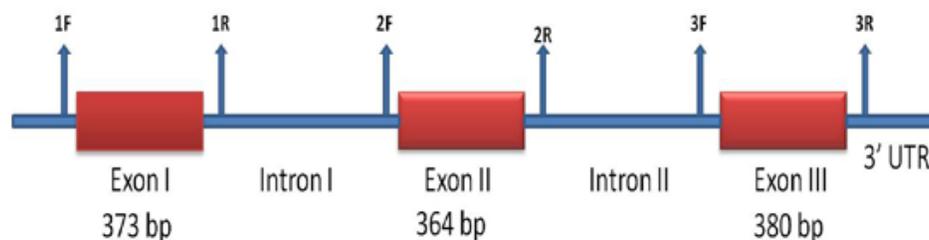
The myostatin gene, also known as GDF8 (growth differentiation factor 8), is specifically expressed during embryonic development, expressed at high level in adult skeletal muscle and controls skeletal muscle growth [3]. Molecular analysis of the myostatin gene in different species

has showed that it consists of three exons and two introns [Figure-1] and found to affect both the amount and composition of muscle fibers. For instance, the muscle mass of MSTN knockout mice is two to three times greater than that of wild-type mice [4], which is primarily due to an increased number of muscle fibers, followed by muscle cell hypertrophy and suppression of body fat accumulation [5].

Myostatin gene is one of the most conserved genes among vertebrate species [6]. However, there are many reports of mutations, disrupting the myostatin function, which cause double-muscle phenotypes in cattle and increase in body mass in mice [7]. 30% more muscle mass with less bone and fat is found in double-muscle cattle per animal on the same food intake as normal cattle [8]. Schuelke et al. [9] reported hyper muscularity and decreased fat mass in a child due to an inactivating mutation of the MSTN which further supports its role in the regulation of body composition. MSTN gene has

been cloned and identified from a wide variety of vertebrate species including human [10], mice [4], cattle [11], chicken

[12], yak [13], and zebrafish [14].



**Fig: 1. Schematic representation of the rabbit myostatin gene (exons, introns and untranslated region - UTR). (A) Gene position of nucleotide of the translated region of three exons, (B) Localization of 3 pairs of primers (1F, 1R, 2F, 2R and 3F, 3R**

The myostatin gene has been sequenced in other livestock and some fish to evaluate its commercial possibilities. Despite the fact that the rabbit is an animal of high economic value, myostatin has not been extensively studied and no mutation has been reported for the rabbit yet except one report by Fontanesi et al. [2]. Therefore, screening myostatin gene mutation may contribute to develop animal breeds with high meat performance. Hence, we resequenced this gene with the aim to compare myostatin gene among the three different rabbit breeds and also with other species.

## [II] MATERIALS AND METHODS

### 2.1. Animals and DNA Isolation

Fifteen rabbits (five animals, each from three breeds i.e. White giant, Soviet chinchilla and Desi, respectively) were used for resequencing of the MSTN. DNA was isolated from blood by phenol-chloroform method [15].

### 2.2. Polymerase Chain Reactions (PCR)

Three PCR primer pairs were used to amplify the rabbit MSTN as previously reported by Fontanesi et al. [2] [Table-1]. Polymerase chain reaction (PCR) amplifications using the three primer pairs were done separately, and carried out in a final reaction volume of 25  $\mu$ l containing 1X PCR Master Mix (Fermentas), 10 pmol of each primers and 50-100 ng template DNA. PCR profile was as follows: 5 min at 95°C, 35 amplification cycles of 45 sec at 95°C, 45 sec at 52°C, 45 sec at 72°C and 10 min at 72°C. The size of PCR products were of 499 bp, 570 bp and 523 bp of MSTN exon I, exon II and exon III, respectively. In every experiment, negative controls were performed containing all reagents except DNA, aiming to avoid contaminations. Assays were performed in a thermal cycler (Minicycler, MJ Research), and the amplicons were analyzed on 2% agarose containing 1  $\mu$ g/ml ethidium bromide in horizontal gel electrophoresis and visualized under UV light by gel documentation system (SynGene). After gel photo-documentation, remaining PCR products were purified to remove free nucleotides, primers and enzymes by Acroprep™ 96 filterplate protocol (PALL Corporation) as manufacturer's instruction.

### 2.3. Cloning and sequencing

These PCR products were then ligated in pTZ57R vector using the Insta T/A cloning kit (Fermentas). The vectors containing the insert were propagated in E.coli DH5- $\alpha$  host following manufacturer's instructions. Transformed colonies were screened by blue white screening and the recombinant plasmids, carrying correct inserts, were isolated from the representative clone using QIAprep® Spin Miniprep kit (QIAGEN). These plasmids were subjected to BigDye® Terminator v3.1 Cycle Sequencing reaction (Applied Biosystems) and products were resolved on automated ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) at Animal Biotechnology Laboratory, AAU, Anand, Gujarat, India. By using SeqScape Software (Applied Biosystems), forward and reverse sequences of representative sample of each gene fragment were assembled against most closely related reference sequence of respective gene to obtain total sequence length and similarity was looked in to the non-redundant database of GenBank with BLAST algorithms (<http://www.ncbi.nlm.nih.gov/BLAST/>).

### 2.4. Phylogenetic analysis

Sequence analysis was further validated by multiple sequences alignment of myostatin gene from different species in FASTA format using ClustalW (<http://www.ebi.ac.uk/Tools/clustalw/>) available at European Molecular Biology Laboratory (EMBL) website. Mutation detection was done by multiple sequence alignment of consensus sequences of rabbit in Bioedit (v 7.0.7.1) and phylogenetic tree was constructed using Neighborhood joining method of bootstrap test of phylogeny in MEGA4 [16].

## [III] RESULTS AND DISCUSSION

Myostatin gene polymorphisms were detected by the cloning and sequencing of protein coding region, spanning all three exons. To cover complete MSTN coding region, these three exons (499 bp, 570 bp and 523 bp) were amplified using primers as previously reported [2] [Figure-2]. On the whole, resequencing of the myostatin gene in three different rabbits (Fifteen animals) generated sequence information for 1692 bp. The first 499 bp fragment included part of the 5' UTR, exon I and part of intron I, second 570 bp covered part of intron I, exon II and part of intron II and last 523 fragment bp encompassed part of intron II, exon III and part of the 3' UTR, respectively.

Table: 1. Primers used for amplification in PCR reaction

Exon no.	Primes sequence (5'-3')	Amplicon size (bp)	Annealing temp. (°C)
I	F -AATTTTGCTTGCCATTACTGA R- TCAGCAGAACTGTTGACATACAC	499	52
II	F- TGCATGCATTATCCCAATAGA R- TCGGTAGTTGTTTCCCACTTT	570	
III	F- AAAGGTATTCCAAGCAAATGA R- GGGGAAGACCTTCATGTTT	523	

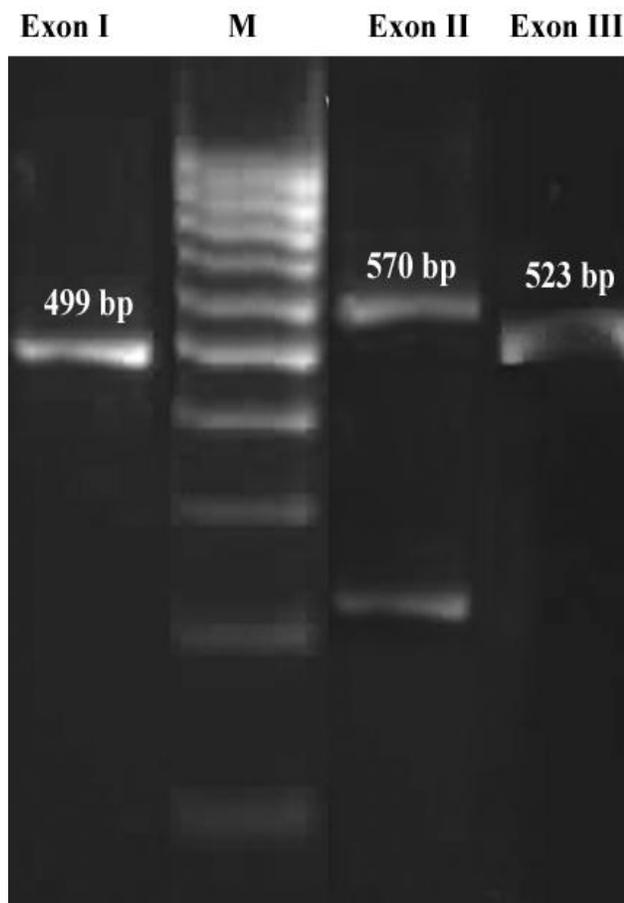


Fig: 2. 2% Agarose gel electrophoresis of exon I, II and III (499 bp, 570 bp and 523 bp) (Representative sample)

Comparing the sequences of all fifteen animals of the three rabbit breeds, only one mutation was found in exon I at nucleotide position 88 (a > G, initiation codon) in one animal of soviet chinchilla breed (S11) [Figure-3a], whereas no mutation was identified in the exon II, while in case of exon

III, one mutation was found at nucleotide position 147 (g > A) in one animal of white giant breed (W12) [Figure-3b]. In

eukaryotic systems, ATG is almost exclusive as this codon act as translational initiation, whereas in mammalian cells, ACG and CTG have also been found as initiator codons in a few mRNAs. Moreover, it has been reported that GTG acts as initiation codon in eukaryotic mitochondrial gene, bacterial ORF and yeast ORF [17]. These mutations are not always beneficial as Klein et al. [18] reported that A to G transition mutation in the ATG initiation codon of a protein coding gene may cause disease in humans.

The MSTN exon I, II and III of all the three breeds of rabbit were compared with homologous regions of Bos taurus (AAB81508), Sus scrofa (ABR08657), Capra hircus (AAR12161), Gallus gallus (ACY68210), Homo sapiens (ABI48372), Mus musculus (AAI05675) and Ovis aries (ABJ97058) using Bioedit (v 7.0.7.1), which revealed that the exonic regions are highly conserved in between species. All the three exons of reference sequence of rabbit (AM931155, AM931157 and AM931158), sample S11, sample W12 and above mentioned species were translated into protein using the Expsy Translation Bioinformatics tool. In term of amino acid, it was found that there was valine instead of methionine at start codon (a>G) and asparagine instead of aspartic acid at amino acid position 267 (g>A) [Figure-4]. When translated sequence were used for preparation of phylogeny, it was found that rabbit MSTN gene was nearest to M. musculus MSTN gene (AA105675) and S. scrofa (ABR88567) farthest from G. gallus MSTN gene (ACY68210) [Figure-5]. Apart from this, other variations were also observed in the intronic regions of myostatin gene. However, the nucleotide variations and the amino acid changes seen in the two animals of rabbits showing mutations did not affect the myostatin protein structure and conformation. Thus from this, it can be concluded that the Myostatin gene is highly conserved in between these three rabbit breeds as well as within the different species of other animals. The multiple sequence alignment of MSTN amino acid sequences of various species also showed many other variations.

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AM931155      10      20      30      40      50      60      70      80      90      100
D1            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
D2            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
D3            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
D4            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
D9            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
S4            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
S7            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
S8            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
S10           AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
S11           AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
W5            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
W7            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
W9            AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
W11           AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
W12           AATTTTGCT TGCCATTACT GAAAAGCAAA AGAAAAGTAA AAGGAAGACA TAAGAACAAG GGAAAGGATT GTATAGCTTT TAAATCATG CAAAACTGC
  
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**Fig: 3a. Multiple sequence alignment of all fifteen samples of *Oryctolagus cuniculus* MSTN Exon I showing mutation (a>G) at position 88 in one animal of Soviet Chinchilla breed (S11).**

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AM931158      60      70      80      90      100      110      120      130      140      150
D1            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
D2            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
D3            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
D4            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
D9            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
S1            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
S2            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
S3            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
S10           TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
S11           TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
W1            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
W2            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
W3            TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
W11           TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
W12           TTTAGAATAA ATTGATATAC TTATCGTTCT TTCCTTTTCA CACAGAATCC CTTTTTAGAG GTCAAGGTAA CGGACACACC AAAAAGATCC AGAAGAGATT
  
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**Fig: 3b. Multiple sequence alignment of all fifteen samples of *Oryctolagus cuniculus* MSTN Exon III showing mutation (g>A) at nucleotide position 147 in one animal of White Giant breed (W12).**

Fontanesi et al. [2] sequenced the entire MSTN gene in the four different breeds of rabbits (Belgian hare, Burgundy fawn, Checkered giant and Giant grey) in order to identify DNA markers useful for association studies with economic traits and reported that only one single nucleotide polymorphism (C>T) in intron II of rabbit MSTN gene. Apart from this, no significant study has been done regarding the characterization of MSTN gene in different breeds of rabbits. Similarly Tay et al. [19] reported 38 nucleotide differences between the myostatin sequences in cattle and that of the goat. They found that there were 25 non-synonymous changes and 13 synonymous changes and identified three SNPs, two in exon II and one in exon III. Grisolia et al. [20] found 37 polymorphisms in the untranslated region segment, and also one SNPs in intron I and three SNPs in intron II of Nellore cattle breed. They concluded that this high degree of allelic heterogeneity in the myostatin gene could be related to its high mutation rate, and it also could be the result of a long history of artificial selection for meat production, which has probably favored such modifications and maintained them in cattle

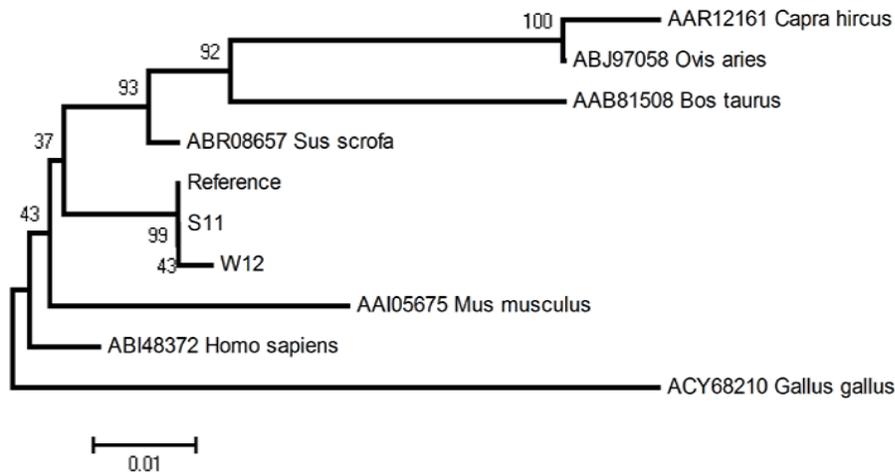
populations. Moreover, in yak myostatin gene, Jianquan et al. [13] reported one variation at encoding nucleotide position 641 in exon II which resulted into one amino acid changed between the yak and cattle, and also reported that there were 98% and 99% similarity in 5' UTR and 3' UTR respectively.

#### [IV] CONCLUSION

As some breeds of rabbits are significant meat producers, the double-muscling phenotype can be potentially exploited for economic gain. In summary, we have characterized the myostatin coding regions of rabbits and the identification of two nucleotide changes in the presumed myostatin protein sequence as compared to other species myostatin sequences. From an applied point of view, the identification of the myostatin gene polymorphisms in rabbits, have to be carried out in other meat species to find out DNA markers useful for association studies with economic traits.



**Fig. 4. A multiple sequence alignment of the translated myostatin protein sequences has been shown.** The consensus sequences for reference myostatin compiled (AM931155, AM931157 and AM931158) and aligned with S11, W12, *Homo sapiens* (ABI48372), *Sus scrofa* (ABR08657), *Bos Taurus* (AAB81508), *Capra hircus* (AAR12161), *Mus musculus* (AAI05675), *Ovis aries* (ABJ97058) and *Gallus gallus* (ACY68210). The nucleotides in the different species which differed are written.



**Fig. 5. Phylogenetic analysis** of the amino acid sequences of MSTN gene between rabbit, human, mouse, pig, cow, goat, sheep and poultry

## AUTHOR CONTRIBUTION

Experiment was performed by A.S. Kurkute, N. Shabir, C.V. Jawale and U.V. Ramani. Analysis and manuscript writing was completed by A.K. Tripathi. Moreover, Dr. C.G. Joshi, Dr. D.N. Rank and Dr. A.M. Pande are gratefully acknowledged for many helpful discussions while manuscript preparation.

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