Exploring the genetic influence of Myostatin (*MSTN*) gene on growth traits in Palla strain of Nellore sheep

K.E. Bhavani[1]*, S. Vani[1], K.S. Devi[1], P.P.R. Reddy[1], J.M. Bhumireddy[2], V. Manasa[3], S.M. Siraj[1]

[1]Department of Animal Genetics and Breeding, [3]Department of Veterinary Biochemistry, College of Veterinary Science, Proddatur-516360, Sri Venkateswara Veterinary University, Andhra Pradesh, India. [2] Department of Animal Genetics and Breeding, School of Veterinary and Animal Sciences, Centurion University of Technology and Management, R. Sitapur, Paralakhemundi, Gajapati, Odisha-761211.

*Corresponding author e-mail: eswarkillana97@gmail.com

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Abstract

The present study was undertaken in 96 number of Nellore Palla sheep of Andhra Pradesh maintained at farmer's flocks in the breeding tract of Nellore district to characterize *MSTN* gene through Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR- RFLP) genotyping. The exon 3 region of *MSTN* gene was amplified and the PCR product was digested with *Msp*1 restriction enzyme to check the presence of SNP at G5622C. The genetic variability in exon 3 region of *MSTN* gene showed (MM), (Mm), (mm) genotypes which was confirmed by sequencing. The gene frequency of 'M' and 'm' alleles is 0.453 and 0.546 respectively, whereas, the genotypic frequency of MM, Mm, mm genotypes are 0.03125, 0.84375, 0.125. The population significant deviation from the Hardy Weinberg equilibrium with respect to the gene under study. There is a significant difference ($P<0.05$) in the body weights having these genotypes. The result showed the suitability of PCR-RFLP technique for evaluating genetic variability in *MSTN* gene and its association with growth traits in Nellore Palla sheep.

Keywords: *MSTN* gene Polymorphism, Association, Nellore Palla sheep

Introduction

Despite being renowned for their adaptability and inexpensive maintenance, indigenous animals are accountable for their poor growth, delayed puberty and low productivity in-terms of their low body weight. Selection of superior animals that grow quickly with more muscle mass would be more beneficial to the sheep rearers and in-turn the economy of the country could be increased. Within India's tropical belt, especially in the south, small ruminants are dedicated to mutton production, aligning with the region's increased meat consumption.

Myostatin (*MSTN*), also known as growth differentiation factor-8 (GDF-8), is a protein whose expression is modulated by the myostatin gene (Nagahara *et al.,* 2025). It is located on long arm of chromosome 2 (2q32.2 locus) of sheep consisting of three exons and two introns. It acts as a negative regulator of muscle cell growth, differentiation and regeneration (Aiello *et al*., 2018); where the loss of functional myostatin known to cause muscling. (Archibald *et al*., 2010 and Lazar *et al.,* 2016). Association analysis using single nucleotide polymorphisms (SNPs) is the most effective approach to identify genetic markers potentially related to a trait of interest. Genetic variations (polymorphisms) within the *MSTN* gene, a key regulator of growth and carcass characteristics, have been identified as useful markers for genetic-assisted selection aimed at boosting meat quality and yield. (Mirhoseini and Zare, 2012).

Hence, the present study was undertaken to study the polymorphism of the myostatin (MSTN) gene in the Palla strain of Nellore sheep of Andhra Pradesh and to determine its association with growth traits, thereby facilitating selection of genetically superior germplasm and aiding formulation of improved breeding strategies for enhanced mutton production.

Materials and Methods

Ethical Approval

The Institutional Animal Ethics Committee (IAEC) approval has not been taken as study involves only the collection of blood samples aseptically from the jugular veins of live animals which was done under the supervision of a qualified veterinarian.

Blood Sample Collection

Whole blood (4ml) was collected aseptically from the jugular veins of 107 unrelated healthy Palla sheep using EDTA as an anticoagulant. After collection, the blood samples were gently mixed well, properly labelled and transported to the laboratory in an icebox and were stored at 4ºC until further processing.

DNA Isolation

The genomic DNA was extracted from blood samples using standard phenol-chloroform procedure with slight modifications using GITC buffer (Guanidine-Iso Thiocyanate buffer) instead of SDS and proteinase-K tailored to the lab needs. The concentration and purity of the extracted DNA were estimated by spectrophotometer (NanoDrop OneC of Thermo Scientific, USA). DNA samples with $OD_{260/280}$ ratio of 1.7 to 1.9 were considered to be good and used for further in the studies.

Polymerase Chain Reaction

Mutation at G5622C of exon-3 of Myostatin as reported by (Sahu *et al*., 2017) was selected for amplification. The exon 3 (380 bp) of *MSTN*, along with part of intron 2 and 3 UTR on either side was amplified as 549bp fragment. Primers were procured from Europhins Genomic Pvt.Ltd., Chennai. The details of primers used in the present study along with their genomic locations is shown in Table 1.

Table 1. List of Primers for amplification of Myostatin Gene

Gene	Primers (5'-3')	Chromosome number	Product
MSTN Exon 3 with part of intron 2 and 3'UTR side	F: 5'- GAA GTC AAG GTA ACA GAC-3'	2	549bp
	R: 5'- GAT GGT TAA ATG CCA ACC-3'		

PCR for *MSTN* gene mutation was carried out in an Applied Biosystems thermal cycler with a final reaction volume of 10µl consisting 5µl of 2X PCR master mix (Ampliqon), 0.5µl of each of forward and reverse primers, 1µl of template DNA and 3µl of Nuclease free water. The amplifications were performed with an initial denaturation at 95°C for 5 min, followed by 32cycles at 95°C for 30 Sec, annealing at 53-60°C for 35 Sec and extension at 72°C for 30 Sec with the final extension at 72°C for 5 min. The PCR amplicons were checked on two per cent agarose gel and the size of PCR product was confirmed using a 100 bp DNA ladder (Genei Merck, Bangalore, India). The image of the gel was captured using a gel imaging system. (Omega Fluor™ Plus Documentation Systems, BioExpress, USA).

The PCR product (549bp) was digested with Msp1 restriction enzyme that have a recognition site for C/CGG at 37°C for 6hr in hot water bath with the restriction digestion mix of 10µl PCR product, 1.5 µl of 1× cut smart buffer, 0.1 µl of *MspI* enzyme (3units / µl) and 3.4 µl of NFW. The genotypes were assigned on the basis of band pattern (2.5 per cent agarose gel) of the PCR products as wild type, mutant or heterozygotes. The genotype and gene frequencies were estimated and the locus was tested for Hardy-Weinberg equilibrium. (Falconer and Mackay 1996). The amplicons of eighteen random samples were sent for sequencing at M/s.

Barcode Biosciences, Bangalore and sequences were analyzed using Lasergene software version 7.1.0(44) (DNASTAR Inc., USA). Expasy tool was used to know whether mutation is of synonymous or not.

Association of SNPs with growth data

The genotypic and gene frequencies of the identified SNP were estimated and then tested for hardy Weinberg equilibrium. Association between body weight at different ages and genotypes obtained for selected SNP in *MSTN* was performed by Least squares method of analysis using *lmer* test package in R software as follows.

$$Y\ ijkl = \mu + Gi + Sj + Ak + e\ ijkl$$

Where,

Y_{ijkl} = Body weight of j^{th} sex of k^{th} age and i^{th} genotype

μ = Overall mean

G_i = Random effect of i^{th} genotype

S_j = Fixed effect of j^{th} sex

A_k = Fixed effect of k^{th} age

e_{ijkl} = Random error normally and independently distributed with mean zero and variance $\sigma^2 e$.

Results

Quantity and Quality of Genomic DNA

Of the 107 blood samples collected, 11 were excluded because they did not meet the required DNA purity criteria. The remaining 96 samples yielded genomic DNA ranging from 131.80 to 5,198.2ng/µl and 1.70 to 1.91 with a mean concentration of 560.86 ng/µl. The OD260/280 ratio ranged from 1.70 to 1.91, with a mean of 1.78 respectively. indicating satisfactory DNA purity for downstream analysis. The integrity of the extracted DNA was confirmed by 1% agarose gel electrophoresis (Figure 1).

PCR-RFLP

A 549-bp fragment encompassing exon 3 of the *MSTN* gene was successfully amplified in the analyzed samples (Figure 2). Digestion with *MspI* revealed a polymorphic restriction site corresponding to the G5622C locus. Three genotypes were identified: MM (549 bp), Mm (549, 301 and 248 bp), and mm (301 and 248 bp) (Figure 3). Among the 96 animals, the Mm genotype was predominant (81; 84.38%), followed by mm (12; 12.50%) and MM (3; 3.13%). The frequencies of the M and m alleles were 0.453 and 0.547, respectively. The observed genotype distribution differed significantly from that expected under Hardy–Weinberg equilibrium ($\chi^2 = 47.06$, $P < 0.001$), indicating significant deviation from equilibrium at this locus (Table 2).

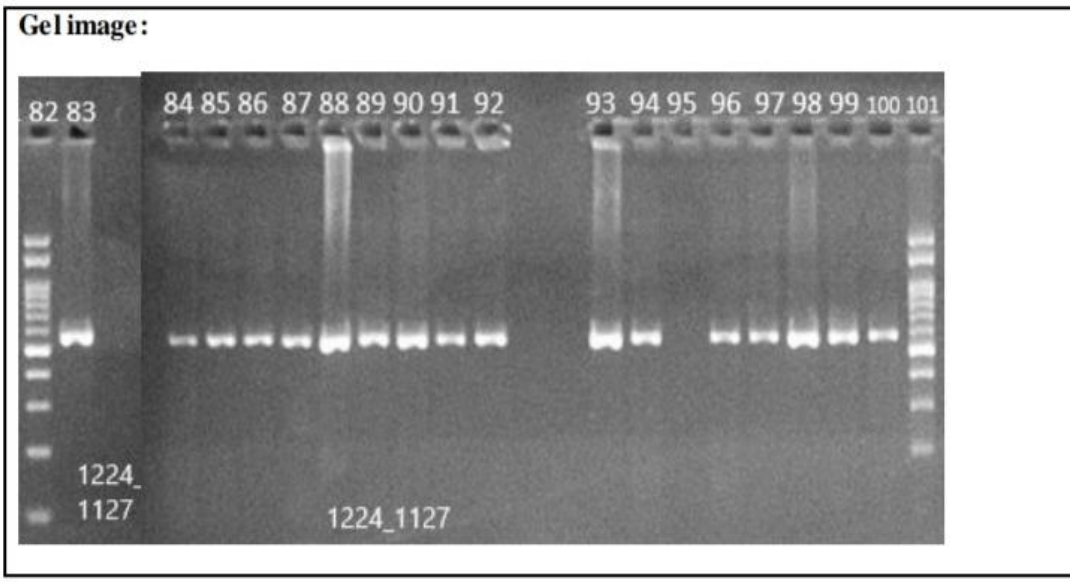

Figure 1: Gel image showing amplification of *MSTN* gene (549bp) (right and left indicates 100bp DNA ladder)

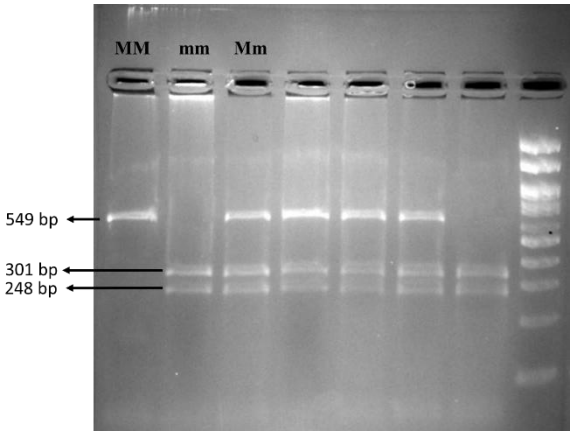

Figure 2: Genotypes of SNP for *MSTN* gene (Right side lane: 100 bp DNA ladder)

Table 2. Gene and genotype frequencies of *MSTN* gene

Locus	Genotype	Number of animals	Genotypic Frequency	Allelic Frequency	χ^2 value
5622(G>C)	MM	3	0.03125	M=0.453 m=0.546	47.06***
	Mm	81	0.84375		
	mm	12	0.125		

Characterization of SNP Mutation

Sequence analysis confirmed the presence of a G>C substitution at 304 position (*i.e.,* 67th codon; 14716th position in) at the identified *MSTN* locus. The three genotypes, GG (MM), GC (Mm), and CC (mm), were identified from the sequence chromatograms. Based on the reference sequence and sequence alignment, the identified nucleotide substitution resulted in an amino acid change from alanine to proline. The functional significance of this nonsynonymous substitution requires further validation.

Wild type Sequence (32nd codon; 5622nd position-GCG): Met-Pro-Gly-Ile-phe-Pro-tyr-Ile-Ile-Cys-Ser-Leu-Pro-Leu-Lys-Tyr-Ser-Ile-Leu-Iso-Gly-Asp-Ile-Phe-Val-Gly-Val-Glu-Glu-Gly-Pro-**Ala**-Gln-Pro-Leu-Gly-Phe-Ala-Trp-Cys-Thr-Arg-Trp-Val***

Mutant Sequence (32nd codon; 5622nd position -CCG): Met-Pro-Gly-Ile-phe-Pro-tyr-Ile-Ile-Cys-Ser-Leu-Pro-Leu-Lys-Tyr-Ser-Ile-Leu-Ile-Gly-Asp-Ile-Phe-Val-Gly-Val-Glu-Glu-Gly-Pro-**Pro**-Gln-Pro-Leu-Gly-Phe-Ala-Trp-Cys-Thr-Arg-Trp-Val***

Association of SNPs with Body Weights Data

The association analysis revealed a significant effect of *MSTN* genotype on body weight at 6 months and at the two-teeth stage (P<0.05), whereas no significant association was observed at birth, 3 months, 4-teeth, 6-teeth, or 8-teeth stages (Table 3). Animals with the mm genotype had significantly higher body weights at 6 months and two-teeth age than animals carrying the other genotypes. At 6 months, the mean body weights of mm, Mm, and MM animals were 22.88 ± 0.406, 21.76 ± 0.506, and 20.45 ± 0.655 kg, respectively. At the two-teeth stage, corresponding values were 33.45 ± 0.512, 32.76 ± 0.432, and 32.18 ± 0.457 kg, respectively. Sex did not have a significant effect on body weight (P > 0.05).

Table 3. Estimated marginal mean values (±SE) of body weights (kg) for genotypes of *MSTN* gene

Age	Significance	Body weight of Genotypes		
		mm	Mm	MM
Birth	NS	3.642±0.023	3.61±0.056	3.59±0.034
3 months	NS	14.52±0.235	14.23±0.545	14.19±0.437
6 months	*	22.88±0.406^{a}	21.76±0.506^{b}	20.45±0.655^{b}
2T	*	33.45±0.512^{a}	32.76±0.432^{b}	32.18±0.457^{b}
4T	NS	37.66±0.692	36.87±0.564	36.45±0.698
6T	NS	40.89±0.789	40.05±0.478	39.87±0.458
8T	NS	42.84±0.774	42.13±0.598	42.09±0.932

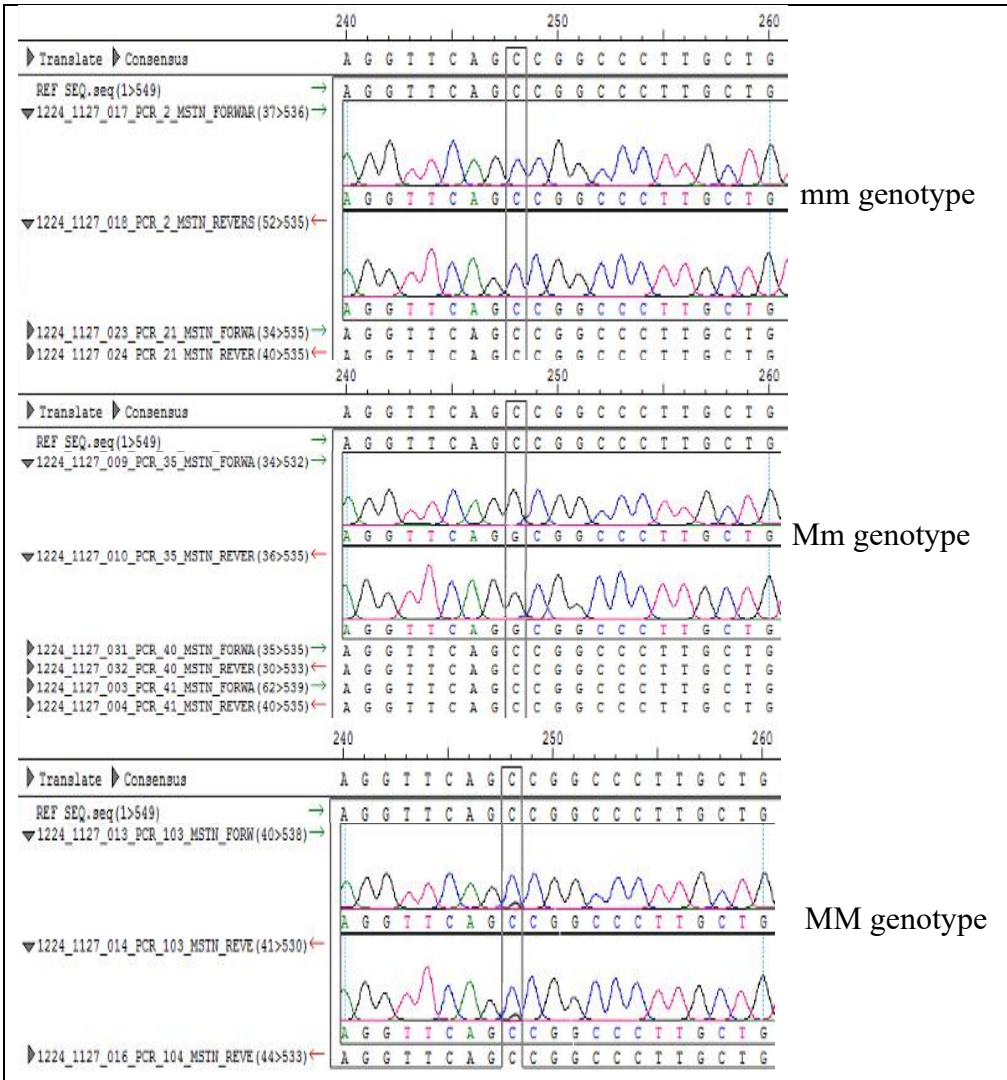

Figure 3: Chromatogram of identified SNP (5622G>C) in exon 3 of *MSTN* gene in Nellore Palla sheep

Discussion

Polymorphism of Myostatin gene

Myostatin, encoded by the *MSTN* (*GDF8*) gene, is a negative regulator of skeletal muscle development and growth. Genetic variation in *MSTN* has therefore been investigated as a potential molecular marker for growth-related traits in livestock (Hadjipavlou *et al*., 2008).

The present study demonstrated polymorphism at the G5622C locus of exon 3 of the *MSTN* gene in Palla sheep. The mutant m allele (0.547) occurred at a slightly higher frequency than the M allele (0.453), while the heterozygous Mm genotype was predominant (84.38%). The relatively high heterozygote frequency and low frequencies of the MM and mm genotypes resulted in a significant departure from Hardy–Weinberg equilibrium. The deviation from Hardy–Weinberg equilibrium should not be attributed solely to selection, as factors such as non-random mating, population structure, genetic drift, migration, and sampling effects might also contribute. Further population-level studies are therefore required to determine the factors responsible for the observed genotype distribution.

The polymorphism observed in the 5622G>C locus of exon 3 of *MSTN* gene in the present study is in harmony with the reports of Sahu *et al.* (2017) in Madras Red and Mecheri sheep breeds. Whereas, it is in contrary to the reports of Masoudi *et al.* (2017) in Irani Baluchi sheep, Gan *et al.* (2008) in Chinese breeds of sheep and Zhou *et al.* (2008) in New Zealand Romney sheep where exon 3 was monomorphic.

Association of SNPs with growth data

The present study revealed a significant association between the *MSTN* G5622C genotype and body weight at 6 months and the two-teeth stage. Animals with the mm genotype showed higher body weight than MM and Mm animals at these ages. These findings suggest a possible association between the homozygous mutant

genotype and enhanced growth during the later growth phase. Although the mm genotype showed superior body weight at 6 months and two-teeth age in the present population, the genotype occurred at a relatively low frequency (12.5%). Therefore, the observed association should be interpreted cautiously and validated using a larger independent population before recommending the G5622C locus as a selection marker. If confirmed, this polymorphism may have potential utility in marker-assisted selection for growth performance in Palla sheep. The absence of significant associations at the other ages indicates that the effect of the *MSTN* genotype may be age-dependent and should be further explored.

Despite the tendency of the sex of the animal to influence the growth traits in sheep, these effects were not significant in our results for body weight which are in concurrent with the Morghani sheep breed (Lavvaf *et al.,* 2007). On contrary, Othman *et al*. (2016); and Esenbuga *et al*. (2009) reported the significant effect of SNP on body weight of ewes and rams of Egyptian sheep; and Awassi, Morkaraman sheep breeds respectively. Differences among studies might reflect breed differences, management, nutritional conditions, age structure, and sample size.

Conclusion

The present study demonstrated that the *MSTN* G5622C locus is polymorphic in Palla sheep, with significant deviation from Hardy–Weinberg equilibrium. The *MSTN* genotype was significantly associated with body weight at 6 months and the two-teeth stage, with animals carrying the mm genotype showing higher body weight than the other genotypes. These findings suggest that the G5622C polymorphism may have potential as a candidate marker for growth-related traits in Palla sheep. However, the low frequency of the mm genotype and the relatively limited sample size warrant validation in larger and independent populations before its application in breeding programmes. The findings may contribute to the development of suitable breeding strategies for improving growth performance and market weight in Palla sheep.

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