



SEQUENCE VARIATIONS AND HAPLOTYPIC DIVERSITY IN THE *MYOSTATIN* GENE OF NIGERIAN CATTLE BREEDS

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ABSTRACT

The myostatin gene (*MSTN*) encodes myostatin protein (MSTN), whose function involves regulating muscle growth and development. Using Polymerase Chain Reaction coupled with Single Strand Conformational Polymorphism analysis with nucleotide sequencing, a total of 161 animals from four different breeds and cross breeds of Nigerian cattle were genotyped at the introns 1 and 2 region, as well as exons 1, 2 and 3. Sixteen SNPs were identified, two (c.374-796C>T and c.374-762C>T) of which are novel and are reported here. The identification of new as well as previously reported genetic variations in *MSTN* within this species provides more insight into the polymorphic nature of this gene. Thus, providing an important resource for further studies around genomic selection and breeding.

Keywords: Myostatin gene, cattle, Nucleotide Sequence Variations, *Bos indicus*

INTRODUCTION

One of the major genetic challenges confronting the beef industry is how to improve the efficiency of beef production systems in terms of increasing growth and improving carcass value. To address this challenge, it is important to have an understanding on the gene(s) that control muscle growth and development, and one of such genes is the myostatin gene (*MSTN*).

MSTN is a circulating protein secreted by muscle cells, and whose function is to regulate the proliferation of muscle fibres as well as muscle regeneration. There has been a huge, documented evidence on genetic variations identified in *MSTN* across a variety of species such as sheep and cattle, mostly of European origin. In cattle, several studies have identified variations linked with increased numbers of muscle fibres, or what has been called double muscling, in a number of breeds (Kambadur *et al.*, 1997; McPherron and Lee, 1997, Grobet *et al.*, 1997; Grisolia *et al.*, 2009). Likewise in sheep, where these

genetic variations have resulted in a lack of biological function of the gene (Boman *et al.*, 2009; Clop *et al.*, 2006; Gan *et al.*, 2008; Heaton *et al.*, 2007; Hickford *et al.*, 2010; Kijas *et al.*, 2007; Sjakste *et al.*, 2011).

However, no studies on variation in *MSTN* have yet been conducted on the Nigerian *Bos indicus* cattle breeds. The Nigerian *Bos indicus* cattle belongs to the Zebu cattle and primarily comprises of the White Fulani (WF), Red Bororo (RB) and Sokoto Gudali (SG) breeds farmed for meat and milk purposes. They have a fatty thoracic hump on their shoulders and a large dewlap and are adapted to dry environmental conditions (Mattioli *et al.*, 2000).

The objective of this study was to analyse the nucleotide sequence across an extended region of *MSTN* in these cattle, aiming to identify polymorphisms unique to this species, or perhaps common with other cattle species which could be useful tool(s) for breeding programs.



MATERIALS AND METHODS

Number and Breed Of Cattle Used

A total of 161 animals from four different breeds and crossbred cattle were investigated. These included; Sokoto Gudali (n=3), Red Bororo (n=13), White Fulani (n=123) and crossbred White Fulani & Holstein Fresian (n=22) cattle.

Blood Collection and DNA Extraction

Blood samples were collected onto FTA cards by piercing the ear of each animal. The DNA extraction was carried out using a two-step procedure as described by Zhou *et al.* (2006).

Primer Design

Five pairs of primers *A- E*

(F 5' ggaagaagtaagaacaaggg 3' &

R 5' caccagcaggactactcac 3',

B-F 5' catggtactattgttgagag 3' &

R 5' aaggcaaatctattccagg 3',

C-F 5' gatatggaggtgtttgttcg 3' &

R 5' cagtaactacttaccagtcc 3',

D-F 5' ataagcaggaagacataagc 3' &

R 5' ggtgtgtctgttaccttgac 3'

E-F 5' ctcttcttcttccatac 3'

R 5' aagaccttccatgtttgag 3') were used to amplify five regions of bovine *MSTN* consisting of the exons and introns. These primers were designed using DNAMAN (Version 5.2.10, Lynnon BioSoft, Vaudreuil, Canada) software and were derived from bovine *MSTN* sequence with GenBank accession number AB076403.

Polymerase Chain Reaction (PCR)

Five different regions of *MSTN* were amplified using the PCR technique. The PCR was performed in 15- μ L reactions containing genomic DNA on a 1.2-mm diameter disc of

FTA card, 0.25 μ M for each primer, 150 μ M for each dNTP (Eppendorf, Hamburg, Germany), 3.0 mM Mg^{2+} , 0.5 U of *Taq* DNA polymerase (Qiagen, Hilden, Germany), and 1 \times the reaction buffer supplied with the enzyme. Amplification was undertaken in Bio-Rad S1000 thermal cyclers (Bio-Rad, Hercules, CA, USA). The thermal cycling conditions included an initial denaturation at 94 $^{\circ}$ C for 2 minutes, followed by 35 cycles of 94 $^{\circ}$ C for 30 seconds, annealing for 30 seconds at 58 $^{\circ}$ C, extension at 72 $^{\circ}$ C for 30 seconds, and a final extension step at 72 $^{\circ}$ C for 5 minutes.

Single-Strand Conformational Polymorphism (SSCP) Technique for Identifying Genetic Variation(s)

The five amplicons were subjected to SSCP analysis. A 0.7- μ L aliquot of the amplicons was added to 7 μ L of loading dye containing 10 mM EDTA, 0.025% bromophenol blue, 0.025% xylene-cyanol, and 98% formamide prior to being loaded onto 16 cm x 18 cm, acrylamide: bisacrylamide (37.5:1) (Bio-Rad) gels. Electrophoresis was carried out using Protean II xi cells (Bio-Rad) for 19 hr at various voltages, gel concentrations and room temperatures. Different PCR-SSCP patterns were identified through silver staining (Byun *et al.* 2009)

In the intron 1 region examined, 10 genotypes were identified (AA=75, BB=2, CC=5, DD=2, BD=7, AB=24, AC=19, AD=22, BC=3 and CD=2), whereas in intron 2-exon 3 region, 137 cattle were investigated and 7 genotypes were detected (AA=77, AB=35, AC=30, BC=5, CC=5, AD=8 and BD=1).

Nucleotide Sequencing Technique Used in Detecting Genetic Variation(s)

Based on the identified PCR-SSCP patterns, homozygous samples with unique banding patterns in amplicons 1 and 2 were subjected to direct sequencing. For heterozygous

variants, the unique band was excised from the wet gel and incubated at 51 °C for 1 hr. A 1-uL aliquot of the incubated product was aspirated and mixed with 14 uL of PCR pre-mixture for amplification via PCR reaction. Using a MinElute™ PCR Purification Kit (Qiagen), DNA from the PCR products were purified and sequenced by the Lincoln University DNA Sequencing Facility.

Determination of Extended Haplotypes

In determining the extended haplotypes, cattle homozygous for one PCR-SSCP pattern in a region of the gene (e.g. *AA* for amplicon 1), were then genotyped in another region of the gene. If heterozygous in the second region (e.g. *AB* for amplicon 2), then two haplotypes (*A-A* and *A-B*), spanning the first and second regions could be defined.

RESULTS AND DISCUSSION

In this study, five pairs of primers were used to genotype 161 animals from four different breeds and cross breeds of Nigerian cattle. A total of five amplicons covering 1,967 bp were generated. The amplified regions included exons 1, 2 and 3 and parts of introns 1 and 2. A total of 16 nucleotide variations were identified, 7 of which were located in intron 1 (amplicon 1) and the remaining 9 were identified in the intron 2-exon 3 boundary region (amplicon 2) (figure 1). There were no genetic variations in the other regions examined.

In this investigation, 16 nucleotide variations were identified, 2 of which are novel and are reported here for the first time.

This study is in concordance with the previous report of Haruna *et al.*, (2020), which examined variations in *MSTN* across 10 NZ cattle breeds of *Bos taurus* origin. In the intron 1 region (amplicon 1) investigated here, variants *A*, *B* and *C* with the single nucleotide variations c.373+751G>T, c.373+803T>G,

c.373+877A>G, c.373+895G>C, and c.374-909C>T were the same with variants *A*, *B* & *C* with accession numbers MK353501, MK353502 & MK353503 respectively, as documented by Haruna *et al.*, (2020). Likewise in the intron 2 region, variants *A*, *B* & *C* with SNP's c.748-78del, c.748-195C>T, c.748-196C>T, c.748-281C>G, c.748-350C>T, and c.748-352C>T were the same with variants *A*, *B* & *C* with accession numbers MK353508, MK353509 & MK353510 respectively, carrying the same SNP's as documented in GenBank.

This result does not come as a surprise, mainly because *Bos taurus* and *Bos indicus* are thought to have a possible relationship in terms of origin, as the wild auroch, *Bos primigenius*, is the progenitor of all taurine and zebu (African *Bos indicus*) cattle (Edwards *et al.*, 2007).

The c.374-796C>T & c.374-762C>T were identified in the intron 1 region investigated and were exclusively found in the “*D*” variant. These are novel SNP's and are reported here for the first time. The discovery of these SNP's in this study partly contradicts the findings of Haruna *et al.*, (2020), whose report did not identify these SNP's even though the same region of intron 1 was examined in New Zealand cattle breeds. A possible explanation might be because of species differences and/or the number of animals investigated. While the previous study looked at 722 cattle from 10 different breeds of NZ *Bos taurus* origin, this study investigated 161 cattle from 4 different breeds and cross breeds of Nigerian *Bos indicus* cattle. What is interesting and worthy of note is that the *Bos indicus* breeds investigated had a unique variant “*D*” in the intron 1 region, this variant carries the variations c.374-796C>T and c.374-762C>T.

Therefore, it is very likely that this unique variant is species specific. This could suggest

that the “D” variant in intron 1 is unique only to the Nigerian *Bos indicus* species, and it will be interesting to see what phenotypic trait this variant may be influencing. Such variations exclusive to a species are to be expected, partly because they form the basis for species differences, irrespective of whether they occur in the coding or non-coding sequences. This is supported by a previous report by (Matic 2001), which suggests variations within the coding and noncoding sequences produces phenotypic variations between both

individuals in a species and between different species.

Considering a previous report by Hey *et al.*, (2004), which showed that SNPs that are close to each other tend to show strong linkage disequilibrium. This phenomenon was observed with the *MSTN* extended haplotypes, as a total of 10 extended haplotypes (H1-H10) spanning intron 1 (amplicon 1) to the intron 2 - exon 3 boundary region (amplicon 2) of *MSTN* were resolved (Table 1) and some of them could be separated into broad groups based on the location of the SNPs (Table 1).

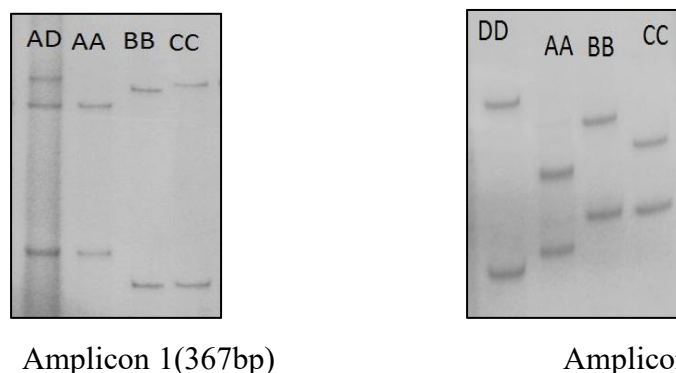


Figure 1. PCR-SSCP patterns for two amplicons across bovine *MSTN*. Amplicon 1 (intron 1) produces four banding patterns (A, B, C & D). Amplicon 2 spans intron-2 /exon 3 boundary with four banding patterns (A, B, C & D).

Table 1: SNPs identified in the 10 extended haplotypes (H1-H10) defined across the two regions of *MSTN* investigated. Some of the extended haplotypes cluster into groups based on the proximal occurrence of SNP’s.

Extended Haplotypes [#]	Intron 1								Intron 2- exon 3 boundary region							
	c.373 +751	c.373+ 803	c.373 +877	c.373 +895	c.374 -909	c.374 -796	c.374 -762	c.748- 78del1	c.748 -195	c.748 -196	c.748- 202	c.748 -243	c.748 -281	c.748- 341	c.748 -350	c.748- 352
H1	G	T	A	G	C	C	C		C	C	G	G	G	T	C	T
H2	G	T	A	G	C	C	C		C	C	G	G	C	T	C	C
H3	G	T	A	G	C	C	C	T	T	T	G	G	C	T	T	C
H4	G	T	A	G	T	C	C		C	C	G	G	G	T	C	T
H5	G	T	A	G	T	C	C		C	C	G	G	C	T	C	C
H6	G	T	A	G	T	C	C	T	T	T	G	G	C	T	T	C
H7	T	G	G	C	T	C	C		C	C	G	G	G	T	C	T
H8	T	G	G	C	T	C	C		C	C	G	G	C	T	C	C
H9	G	T	A	G	C	C	C	T	C	C	A	C	C	C	C	C
H10	G	T	A	G	C	T	T		C	C	G	G	G	T	C	T

* The symbol (-) represents a deletion and the highlighted nucleotides indicate the variations.

Some of the extended haplotypes cluster into groups based on the proximal occurrence of SNP's. In the intron 1 region, H4, H5 & H6 carrying c.374-909T (named Group 1), while H7 & H8 with SNP's c.373+751T, c.373+803G, c.373+877G, c.373+ 895C and c.374-909T cluster as group 2, whereas H10 carrying the novel SNP's c.374-796T & c.374-762T occurred independently. In the intron 2/exon 3 region, H3 & H6 carried c.748-195T, c.748-196T & c.748-350T and could be clustered as group 3, while H1, H4, H7, & H10 with SNP's c.748-78del, c.748-281G and c.748-352T could cluster as group 4 and finally, H9 carrying SNP's c.748-202G>A, c.748-243G>C and c.748-341T>C occurred independently.

CONCLUSION

In summary, this investigation reports the first characterization of *MSTN* in Nigerian *Bos indicus* cattle. This study provides more insight into the conserved and polymorphic nature of the coding and non-coding sequences of *MSTN* in Nigerian cattle, thus, providing important resource for further studies around genomic selection and breeding.

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