RESEARCH ARTICLE

Association of Myostatin Gene with On-field Sporting Traits of Indigenous Cattle Breeds (*Bos indicus*) of Tamil Nadu

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Abstract

Sports genomics is considered a branch of molecular genetics, which is used to identify the candidate genes associated with domestic animals' sporting traits, especially in horses, dogs, and cattle, to select animals with the best sporting ability. Blood samples were collected from indigenous bulls (n=233) used for a sporting event in Tamil Nadu for genomic DNA isolation. Molecular characterization of Myostatin gene, including its promoter region, was carried out to find the sequence variation. Compared with the Bos indicus sequence, two variations were detected in exon 1 and exon 3 and no variation in exon 2. The intronic regions were found to have single base pair variations, single base-pair insertions, and 12 to 16 bp insertions. In addition, nine types of variations found in the promotor region and the genotyping of SNP at position -644 revealed the predominance of genotype CC with the estimated frequencies of 0.81, 0.69, 1.00, and 0.80 in Pulikulam, Kangayam, Umblachery, and non-descript bulls, respectively. TT genotype had higher mean velocity and highest time spent within the boundary of bull tamers, and CC genotype had higher stride length. In conclusion, it could be inferred that the Myostatin gene would be used as a marker for sporting traits, but it needs extensive study to validate its role in regulating sporting traits.

Keywords: *Bos indicus*, Indigenous cattle breeds, *Jallikattu*, Myostatin gene, Sporting traits.

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Introduction

Nowadays, sports genomics is a newly emerging field to identify the candidate genes with known biological utility and directly or indirectly regulating the developmental processes of the sporting traits, confirmed by assessing the effects of the causative gene variations in molecular association studies in domestic animals intended for sporting events globally (Zhu and Zhao, 2007). There are many candidate genes used to assess the racing stamina or sprinting ability in horses and dogs. One of the top-rated sporting events in Tamilnadu is "Bull Baiting," in local vernacular language (Tamil) termed as "*Jallikattu,*" in which native indigenous cattle breeds such as Pulikulam, Kangayam, Umblacherry, and non-descript are being used. The genetic principle hidden in this sporting event was selective breeding of *Bos indicus* cattle of excellent draught quality for rural agriculture, thereby maintaining the genetic diversity of native cattle breeds and preventing them from extinction. Documentation of sporting events and their cultural association with the folk have been carried out extensively by anthropologists, scholars of ancient Tamil, and animal lovers.

In general, sporting is a heritable trait, and 66 percent of the variance is due to additive genetic components (Mosher *et al.*, 2007). Gene polymorphism study could be helpful if a particular genetic variant is associated with endurance, strength, and speed to exploit the genetic potential of domestic animals. But, many authors felt that polymorphism in the Myostatin (MSTN) gene would guide link to the athletic performance of domestic animals quantitatively. The good and potential genetic background of this famous sporting event had not been investigated so far. Hence, a study was undertaken to evaluate the molecular association of the MSTN gene with on-field sporting traits in the indigenous cattle breeds of Tamil Nadu.

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Materials and Methods

Blood samples were collected from sporting bulls (n=233) in Madurai, Trichy, and Pudukkottai districts of Tamil Nadu to isolate genomic DNA (Sambrook *et al.*, 1989). The purity and concentration of DNA samples were estimated by spectrophotometry (Nanodrop One C, ThermoScientific, USA) and stored at -80 °C till further processing.

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Details of myostatin gene of *Bos indicus* cattle	
Gene ID with accession number	: 109565565 NC_032651
Location	: Chromosome 2
Gene length (bp)	: 6647 bp
No. of exon	: Three (373, 374 nd 634 bp; five primer sets)
No. of intron	: Two (1828 and 2032 bp; six primer sets)
No. of primers for promoter region	: Two sets

The annealing temperature of each primer set was standardized by gradient PCR, based on the melting temperature of the primer set (Table 1). The amplification reactions were carried out in thermal cyclers (Eppendorf Master Cycler ep gradient S), and the amplified products were confirmed by a gel documentation system (Bio-Rad, USA). Upon confirmation of the amplified products, PCR was performed for 13 sets of primers and sequenced. Sequencing was done for eight samples per primer set containing winning and losing bulls (in equal numbers) in different genetic groups. The variations in sequences of MSTN gene among winning and losing bulls in different genetic groups were analyzed using the Editseq program of LASERGENE software. The sequence variation was analyzed among individuals within a genetic group and between genetic groups, which was helpful for the study of polymorphisms between two genetic groups.

Transcription Factor Binding Site

The SNPs in the promoter region (-644) was screened for transcription factors binding site using free online software (www.generegulation.com). The SNPs detected were genotyped using the PCR-Restriction Fragment Length Polymorphism. The restriction site and the corresponding enzyme were analyzed using the NEB cutter (*http:/tools,neb.com/NEBuffer2*). The restriction enzyme (*Taq* I; New England Biolabs -20000 units/mL) with incubation (65°C for 60 mt.) and inactivation temperatures (85°C for 5 mt.) were used to digest the PCR product to identify SNPs. The MSTN gene sequences with SNPs were subjected to translation VaxaSoftware to look for the type (*http://rnat-rna-codons-to-amino-acids-translato.software.informer.com/*) of mutation and also to determine the protein structure.

Table 1: Designed primers and their annealing temperature for myostatin gene

Region	*Primer sequence (5' to 3')*		*Annealing temperature (°C)*	*Products size (bp)*
Promotor-1	Forward	GCTCAGAAGTGTATAGGGGCATA	61	861
	Reverse	TTTTCCTCAGCTTTCCAGTG		
Promotor-2	Forward	GGACTAGCACACTACTGAGAAGCA	62.	851
	Reverse	CCTGTCTGTCACAAGTCACCA		
Exon 1	Forward	TCGAATGTCACATACAGCCT	59	851
	Reverse	CCTTACATACAAGCCAGCAG		
Intron 1A	Forward	GGAAACGGTCATTACCATG	58	752
	Reverse	CATGGTCAGGGTATAAGTGG		
Intron 1B	Forward	CTGGGCATTTGCTGAACACT	63	847
	Reverse	ACGCTGGGACAGCCTTTTA		
Intron 1C	Forward	AGCCTGGCCCTAAAGACAAT	62	672
	Reverse	GGGTTTTCCTTCCACTTGC		
Exon 2	Forward	GGCTGCTCATAACAGCTGAA	62	780
	Reverse	GGATGTGAAATGGGACACCT		
Intron 2A	Forward	TTCCCAGAACCAGGAGAAGA	62	849
	Reverse	GCCAGAAGAGTGAGTAGCTCTAAAC		
Intron 2B	Forward	ACTTTGTGAATTACCCCTGGT	60	862
	Reverse	AGGGTTGGAAAGACTAACTCCA		
Intron 2C	Forward	GGTACAGGAGGAGGATTAGCAA	62	677
	Reverse	TCCCAAAACACTCTCCTACCTC		
Exon 3A	Forward	AGGGGAATCCCTATGGCTACT	62	864
	Reverse	TCACCTGCATGTGTTGTGA		
Exon 3B	Forward	GCTATGGTTGGCATTTAACC	60	678
	Reverse	AAGCTGCAGTATTGCAAAGG		
Exon 3C	Forward	TTCTGTAGCATACTTGGAGAAGC	61	862
	Reverse	CCAAACTTTTGTGCTCAGTCA		

Estimation of Gene and Genotype Frequencies

The genotypes were assigned based on the restriction digestion pattern of the PCR products. The gene and genotype frequencies were calculated by the standard formula (Falconar and Mackay, 1996).

Assessment of on-field Sporting Traits

Video footage of the sporting event was done personally as well collected from authorized videographers and the owners. A total of 857 sporting bulls were assessed for on-field sporting traits such as velocity, stride length, time spent within the boundary of bull tamers, and time taken to get tamed. Velocity was measured as time (in seconds) to travel a distance of 50 meters by the sporting bulls. Stride length was measured by counting the number of strides taken to cover a 50-meter distance. Time spent within the boundary of bull tamers was assessed by measuring the time spent (in minutes) by the bull (not restrained by the bull tamers) within the arena. It indicates the confrontational or pugnacious behavior of the sporting bull. Time taken for taming is assessed by measuring the bull tamer's time taken (in seconds) to get tamed by a bull tamer within the arena.

Least-squares analysis of variance for an association between genotypes and sporting traits

The data on different genotypes obtained through RFLP were subjected to least-squares analyses of variance to estimate the association between SNPs with the on-field sporting traits. Duncan's Multiple Range Test was employed to make pairwise comparisons of least-squares means by Kramer, (1957).

Results and Discussion

Amplification of Myostatin Gene

In the present study, exons of the bovine myostatin gene were sequenced to assess the genetic variations as reported in Nellore cattle (Jeanplong *et al*., 2001 and Grisolia *et al*., 2009).

Exon 1

The regions included in exon 1 were -212 bp upstream of exon 1, 373 bp of the coding region, and the + 266 bp of part of intron one region. Two variations were detected at 263rd position with (C>G) transition and 419th position with (A>G) transition, and they were synonymous, replacing the same type of amino acids – glycine and glutamic acid respectively and the chromatogram showing SNP positions are given in Plate 1(a) and 1(b). The variation present in the 263rd position has already been reported in Nellore cattle (Canavez *et al*., 2012). On perusal of literature, genetic variation at 419th position was certainly not reported in *Bos indicus* bulls. It was concluded that the presence or absence of this mutation would not alter the functional property of the gene.

Exon 2

For exon 2 amplification, 232 bp from intron one, 374 bp of the coding region, and a part of intron two (174 bp) were included. The exon 2 region was highly homologous with the reference sequence and indicated that this region is highly conserved across different breeds.

Exon 3

The total amplified region was 864 bp, which comprised 230 bp of intron 2 and 634 bp of the coding region. The size of exon 3 differed due to variations in the polyadenylation site

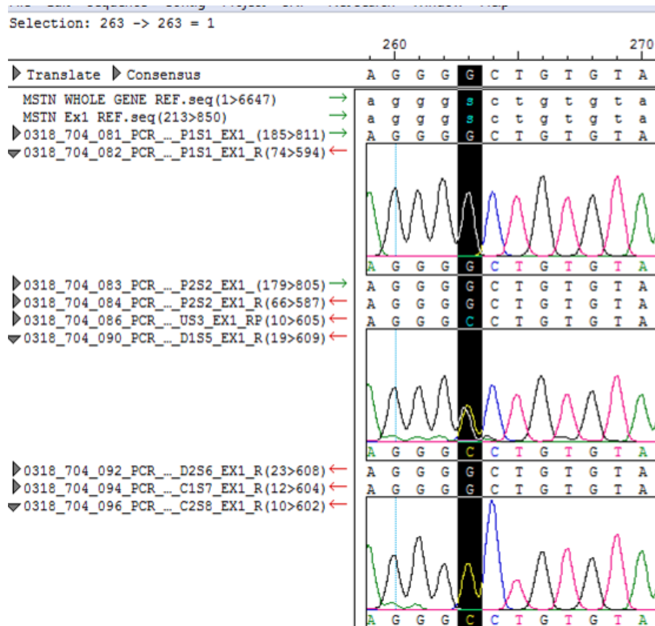

Plate 1(a): Chromatogram displaying SNP at position 263 (G>C) of exon 1

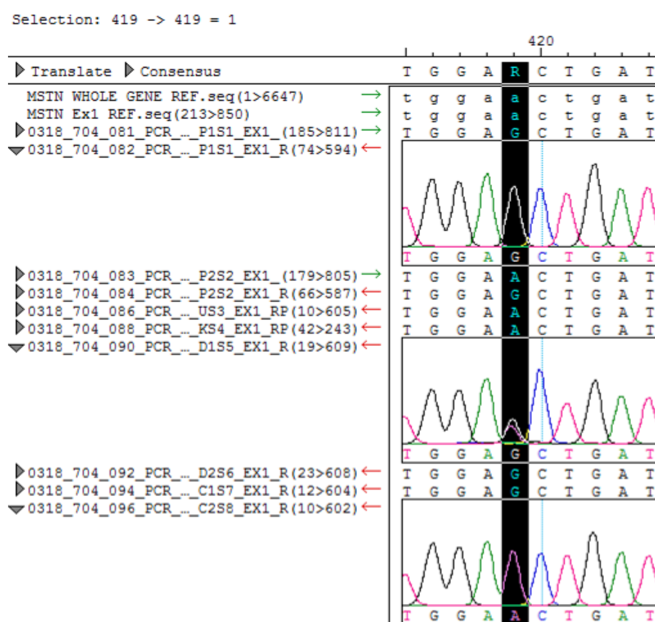

Plate 1(b): Chromatogram displaying SNP at position 419 (A>G) of exon 1

at the 3′ region. The third exon had two genetic variations, one at 5089th position with (C>A) transversion and another at 5095th position with (C>T) transition, and they were also synonymous and replacing with the same amino acids - isoleucine and tyrosine, respectively. These two genetic variations concurred in Nellore cattle (Canavez *et al*., 2012).

On the contrary, 11 nucleotide deletions were identified in the third exon of the myostatin gene, leading to double muscling in cattle (Mcphenon and Lee, 1997). In addition, three, seven, and four polymorphisms in exon 1, exon 2, and exon 3 of MSTN gene respectively in Nellore cattle compared with *taurine* breeds and concluded that these variations were directly linked to double muscling (Grisolia *et al*., 2009). In Whippet dogs, three exons of MSTN gene identified that 2 bp deletion in the third exon, which caused a premature stop codon at amino acid at 313th position and associated with athletic performance (Mosher *et al*., 2007).

Nucleotide Sequencing of Introns

The reasons for including the intronic regions for amplification were to get the entire sequence of the exonic regions and avoid variations in the primer binding sites. The existence of intron in the genome was believed to affect mRNA maturation, transcription initiation, transcription regulation, transcription termination, polyadenylation, and mRNA stability (Lynch, 2007). Hence, the polymorphism in intronic regions of the gene will be helpful to assess gene expression.

Intron 1

The region was 1828 bp, ranged from 526 to 2353 nucleotides. The overlapping primers were designed and divided into three regions viz. intron 1A (752 bp), intron 1B (847 bp), and intron 1C (672 bp). The intron 1 region was found to have 12 types of single base pair variations. In addition, two single base pair insertions at 2273 to 2274 (A insertion) and 2331 to 2332 (T insertion) positions; and 16 bp and 12 bp insertions were also noticed at 1217 to 1218 and 2166 to 2167 positions. The results agree in Nellore cattle except for two single base pair insertions, 16 bp, and 12 bp insertions, and they are unique in this region, and none of the earlier studies reported (Zhu and Zhao, 2007).

Intron 2

The intron 2 was of 2032 bp size, starting from 2728 to 4759 nucleotides. Three sets of overlapping primers were designed, such as intron 2A (849 bp), intron 2B (862 bp), and intron 2C (677 bp). Twenty single base pair variations and one 'T' insertion at the 4171st position were observed in the present study. The variations found in this study have already been reported in Nellore (*Bos indicus*) cattle except for T insertion at 4171st position in MSTN gene (Canavez *et al*., 2012). However, only one polymorphism in intron 1 and three in intron 2 was found in Brazil's Nellore cattle (Grisolia *et al*., 2009).

Nucleotide Sequencing of Promoter

The promoter region of the myostatin gene was compared with *Bos indicus* genome and two overlapping primers designed (promoter 1 and 2, which consists of 861 and 851 bp sizes, respectively). There were nine type of variations found in the promotor region of *MSTN* gene were -1052, -946, -911, -907, -872, -791, -653, -644, and -106 positions. The promoter region was analyzed using free online software (www.generegulation.com), and it revealed that many binding sites for transcription factors and one transcription factor binding sites at SNP position of -644th (Plate 2; C>T) were COMP-1.

Genotyping of SNP at position-644

The SNP found in the -644th position of the promoter region was genotyped using restriction enzyme *Taq* I, which cleaved the PCR product into three fragments of 433, 310, and 117 bp in lengths if C allele was present and unrestricted when T allele was present. The bulls with CC genotype produced three bands of length 433, 310, and 117 bp; bulls with TT genotype showed only one band at the 860 bp region (no cleavage), and those with CT genotypes showed all the four bands as indicated in Plate 3. The genotypes and gene frequencies of SNPs are given in Table 2. The allele C was

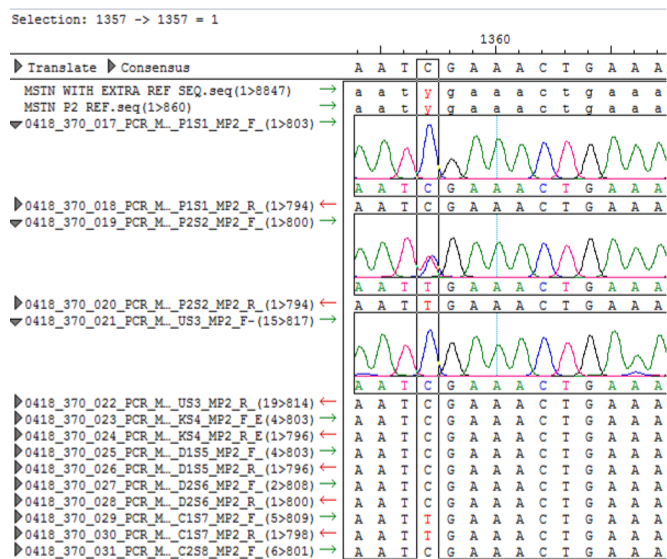

Plate 2: Chromatogram displaying SNP at position -644(C>T) of promoter

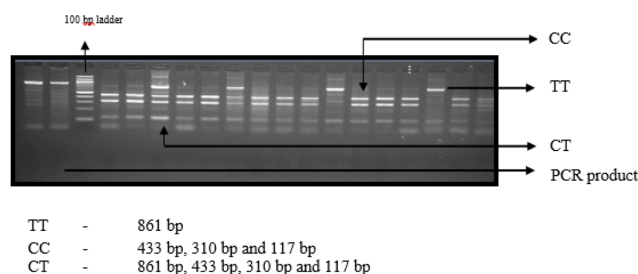

Plate 3: Genotyping of SNP (C>T) at -644th position upstream of myostatin gene

Table 2: Genotype and gene frequency of SNPs found in promoter region of myostatin gene in different genetic groups of sporting bulls

		Genetic groups			
Locus (Position in bp)	*Genotypes*	*Pulikulam*	*Kangayam*	*Umblachery*	*Non-descript*
Genotype frequency					
	CC	0.81	0.69	1.00	0.80
-644	CT	0.08	0.14	0.00	0.11
	TT	0.11	0.17	0.00	0.08
Gene frequency					
-644	C	0.85	0.76	1.00	0.86
	T	0.15	0.24	0.00	0.14

Table 3: Least-squares means for the effects of genotypes on on-field sporting traits

	Velocity(m/s)		*Stride Length(m)*	*Time spent within the boundary (sec)*		*Time taken to get tamed (sec)*	
	n	*NS*	*NS*	*n*	*NS*	*n*	*NS*
Overall	28	11.57 ± 0.58	7.95 ± 0.32	12	21.25 ± 2.96	11	6.82 ± 2.21
CC	20	11.26 ± 1.59	8.24 ± 0.36	8	20.25 ± 3.00	9	7.11 ± 2.69
CT	6	11.25 ± 1.25	7.06 ± 0.58	1	12.00	2	5.50 ± 2.50
TT	2	11.25 ± 1.25	7.78 ± 2.22	3	27.00 ± 8.74	-	-

n - number of observations and NS-Non-significant

predominant with the estimated gene frequencies of 0.85, 0.76, 1.00, and 0.86 in Pulikulam, Kangayam, Umblachery, and non-descript bulls, respectively. In Umblachery, only CC genotype was present. The genotype CC was predominant with the estimated genotype frequencies of 0.81, 0.69, 1.00, and 0.80 in Pulikulam, Kangayam, Umblachery, and non-descript bulls.

Association of Genotypes with on-field Sporting Traits

The least-squares analyses of variance for the effects of genotypes on sporting traits are furnished in Table 3. There were non-significant differences among genotypes CC, CT, and TT for velocity, stride length, time spent within the boundary of bull tamers, and time taken to get tamed. However, it was observed that bulls with TT genotype had a higher mean velocity (11.25 ± 1.25 m/s) and the highest time (27.00 ± 8.74 sec.) spent within the boundary of bull tamers, and CC genotype had a higher stride length (8.24 ± 0.36 m). There was no published literature about the direct association between polymorphism of the MSTN gene and sporting traits in bulls of indigenous origin.

Conclusion

Given the above discussion, it could be inferred that further study is required to validate the MSTN gene as a molecular marker for sporting traits with a large number of samples to confirm whether the MSTN gene or some other combination of genes is responsible for sporting traits. Since the MSTN gene is the most obvious target among livestock species to assess the sporting ability, this SNPs or insertions and deletions are subjected to association studies to declare that it could be used as a candidate marker for selection bull calves for sporting ability in the future.

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