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Molecular Assessment of Evolutionary Divergence of Myostatin Gene in Bikaneri Camel and Marwari Goat

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ABSTRACT

The growth regulating myostatin gene (*MSTN*) is considered to be conserved between species of vertebrate animals. Therefore, the present study was undertaken to explore within and between species genetic diversity for exon 2 of *MSTN* gene in Marwari goat (*Capra hircus*) and Bikaneri camel (*Camelus dromedarius*). Genomic DNA was isolated from whole blood (2ml) through spin column method. A common primer pair was used to amplify the exon 2 region of *MSTN* gene in Marwari goat (n=42) and Bikaneri camels (n=56). Restriction fragment length polymorphism (RFLP) was carried out with *Hae* III restriction enzyme to explore the genetic divergence. Representative (n=5) amplified fragments of *MSTN* exon 2 region of both the species were sequenced through Sanger dideoxy chain termination method. RFLP analysis in both species revealed similar restriction information (*mm* genotype) indicating the absence of intra and interspecies variation. Sequence analysis of *MSTN* exon 2 region within each species also revealed absence of within species variation. However, interspecies variation between Marwari goat and Bikaneri camel were observed in the form of nine SNP's in exon 2 of *MSTN* gene. Two SNP's at 70th and 118th position were found to be non-synonymous with predicted amino acids of tyrosine and glutamine in Bikaneri camel instead of histidine and lysine in Marwari goat, respectively. The results concluded that sequence comparison of myostatin gene of Marwari goat and Bikaneri camel could be used for better understanding of divergent evolution of species in similar agro climatic conditions.

Keywords: Camel, goat, myostatin, molecular analysis

The nucleic acid diversity is a historical indicator of inter and intraspecies population relationship (Zhang *et al*., 2013). Detection of single nucleotide polymorphisms (SNP's) and pair-wise nucleotide sequence identity values are useful to study the variation between species or individuals and in the identification of evolutionary pattern of divergent genomes (Wang *et al*., 2010). Myostatin (*MSTN*) or growth and differentiation factor (GDF 8), a member of the *Transforming Growth Factor*-β (*TGF*-β*)* superfamily, is considered as a candidate gene for muscular growth and development of domestic animals (Zhang *et al*., 2012) that normally acts to limit skeletal muscle mass through regulation of number and growth of muscle fibres (Hickford *et al*., 2010).

The investigation of three coding exons of *MSTN* gene (Bellinge *et al*., 2005) through gene sequencing and polymerase chain reaction (PCR) in *Capra hircus* (Bakerwal breed) and its comparison with *Ovis aries* revealed the presence of one T→C transition in exon 2 of *MSTN* gene, responsible for a leucine to proline amino acid substitution with no polymorphisms was detected in within individuals of the Bakerwal goat breed (Ahad *et al*., 2016). Dushyanth *et al*. (2016) highlighted the role of DNA sequencing in detection of polymorphism in the coding region of the myostatin gene in three chicken lines and observed varied levels of *MSTN* expression among lines. The high level of sequence conservation in myostatin gene of different vertebrate's species also suggests its importance in evolutionary studies (Sahu *et al*., 2016; Agrawal *et al*., 2017a; Agrawal *et al*., 2017b).

Scant efforts have been concerted to establish the evolutionary nature of *MSTN* gene in Bikaneri camel and

Marwari goat species that belongs to two different families of vertebrates. Therefore, keeping in view the polymorphic nature of *MSTN* exon 2 region, the present study was aimed to assess the molecular variation in *MSTN* exon 2 region in two diverse ruminant species, Marwari goat (*Capra hircus*) and Bikaneri camel (*Camelus dromedarius*) inhabitating in the similar semi-arid geographical region of Bikaner (Rajasthan) through restriction fragment analysis and single nucleotide polymorphisms (SNP's).

MATERIALS AND METHODS

Ethical approval

All essential procedures of sample collection were performed strictly as specified by Institutional Ethical Committee with minimal stress to animals.

Sample collection

Forty two goats of Marwari breeds and fifty six camels of Bikaneri breed were randomly selected from out bred populations in the similar geographical region of Bikaner district of Rajasthan having grid position of 28.02°N and 73.31°E for the collection of blood samples. About 2 ml blood samples were collected from jugular vein in sterile vacutainer tubes containing EDTA as an anticoagulant under aseptic conditions.

PCR amplification

The genomic DNA was extracted from whole blood by spin column method following standard procedures as per manufacturer's protocol through commercial kit supplied by HiMedia Limited. The quality and the concentration of extracted DNA were checked on 0.8% agarose and Nano Drop Spectrophotometer, respectively. A common forward and reverse primer pair (F5'AAAAACCCAAATGTTGCTTCTTTA3'; R5'CAGTCCTTCTTCTCCTGGTCTGG3') as suggested by Agrawal *et al.* (2017a) was used for the amplification of 375 bp fragment of myostatin exon-2 gene. Satisfactory amplification was obtained by using the following constituents: in a final volume of 25µl containing 5X PCR buffer, 1 unit of *Taq* DNA polymerase, 0.2mM each of dNTPs, 1.5mM $MgCl_2$, 75pMol of each primer and 100ng of template DNA. Amplification was performed in a thermal cycler with the following program; after an initial denaturation step at 95°C for 5 min, 35 cycles were programmed as follows: 94 °C for 30s, 54°C for 60s, 72°C for 60s and final extension at 72°C for 10 min. The amplified DNA fragments were stained and visualized on 1.5% agarose gel under Gel Documentation System.

RFLP genotyping

PCR-RFLP protocols were designed to genotype to detect genetic divergence in the amplified fragment at specific restricted site GG CC. The restriction digestion of each amplified product (6µl) of 375bp of *MSTN* exon-2 gene was carried out for 3 hours with 10 units of restriction enzyme *Hae* III at 37ºC. Then the enzyme was inactivated by increasing the incubation temperature to 80°C for 20 minutes. The polymorphism in exon-2 region of *MSTN* gene in Marwari goat and Bikaneri camel was assessed on 2.5% agarose gel electrophoresis with 100 bp DNA ladder in a gel documentation system. Gene and genotypic frequencies were calculated through direct counting method.

Sequencing and sequence analysis

The five representative samples of amplified product of *MSTN* gene of both Marwari goat and Bikaneri camel were initially purified and then sequenced through Sanger dideoxy chain termination method in both directions. Forward and reverse sequences of each gene fragment was assembled against the 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/). Sequences were aligned and processed with the help of the BioEdit software v.3.6a 2.1. Polymorphisms were identified by visual inspection of the electropherograms and ClustalW2 program. The nucleotide sequence of the amplified fragment was submitted to NCBI database. The nucleic acid sequence maximum likelihood values were statistically estimated through Bioedit software (version 3.6a 2.1) to observe the evolutionary relationships between species.

RESULTS AND DISCUSSION

The present study focused on the identification of possible genetic divergence in exon 2 of *MSTN* gene within and between species of *Capra hircus* and *Camelus dromedarius*; and an evaluation of SNP's on the basis of predicted amino acid sequence. The genomic DNA of Marwari goat (*Capra hircus*) and Bikaneri camel (*Camelus dromedarius*) were successfully amplified with the common set of primer and were found to be similar in size, *i.e.,* 375bp for the exon 2 region of *MSTN* gene. The observation on amplified fragments for expected sizes demonstrate the applicability of common primer pairs for both Bikaneri camel and Marwari goat for genome analysis (Katzir *et al*., 1996).

RFLP analysis through restriction digestion of amplified fragment of exon 2 region of *MSTN* gene in Marwari goat and Bikaneri camel with *Hae* III restriction enzyme generated similar genotypic information with same restriction pattern 'mm'. The *Hae* III digestion of 'm' allele produced two fragments of 88 and 287 bp (Fig. 1).

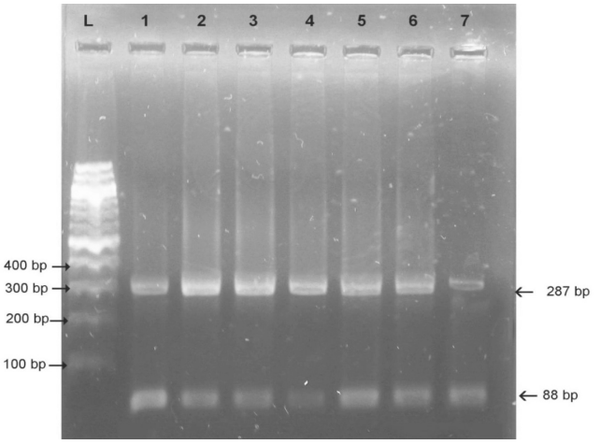

Fig. 1: RLFP genotyping of *MSTN* exon-2 gene in Marwari goat and Bikaneri camel

Lane 1: Molecular weight marker (1Kb ladder), Lane 2-7: Genotypic pattern 'mm'

The observed genotyping information suggested that both Bikaneri camel and Marwari goat lacks polymorphism in exon 2 region of *MSTN* gene and failed to demonstrate the occurrence of polymorphic site at restricted position of coding region of exon 2 of *MSTN* gene indicating the conserve status of myostatin gene within and between species of goat and camel (Cieslak *et al*., 2003). As a result, the polymorphic nature of 'm' allele was not established in either of the species through PCR-RFLP technique. The suitability of technique PCR-RFLP employed for the generation of genotyping information in the present study is in agreement with similar study conducted by Agrawal *et al*. (2017b) for the detection of carrier genotype in Kankrej cattle.

The nucleotide sequence information generated for Marwari goat and Bikaneri camel was submitted to NCBI GenBank database and has been assigned accession numbers of KX863743 and KX863740, respectively. Intra species sequence analysis of amplified products revealed absence of any base pair substitution within species of Marwari goat and Bikaneri camel population. Likewise, sequence comparison of exon 2 of *MSTN* gene of Marwari goat with already available *MSTN* sequence of Jamunapari (GenBank accession number AY032689), Sirohi (GenBank accession number HM462259), Black Bengal (GenBank accession number HM462261) goat breeds also revealed similar nucleotide information indicating that indigenous Marwari goat breed of Rajasthan is completely devoid of intraspecies gene diversity.

The *insilico* interspecies pair wise sequence comparison analysis of exon 2 of *MSTN* gene of Marwari goat with Bikaneri camel reflected the presence of 97.6 per cent nucleotide sequence similarity with occurrence of nine SNP's in exon 2 of *MSTN* gene (Fig. 2). The higher homology observed in the studied locus between the two species indicates that these two species have a smaller number of mutations than documented for camel and buffalo; and camel and horse (Agrawal *et al*., 2017a). Six out of nine detected SNP's were transition in nature whereas three SNP's were observed as transversion mutation (Table 1).

The nucleotide substitutions detected at 70th and 118th base position were found to be non-synonymous in nature with predicted protein sequence revealed presence of histidine and lysine amino acid in Bikaneri camel (Protein ID AQM 57930) instead of tyrosine and glutamine amino acid in Marwari goat (Protein ID AQM 57933) (Fig. 3).

The mean pairwise distance between Bikaneri camel and Marwari goat breed was found to be 0.0250 for exon 2 of *MSTN* gene through Tajima-Nei model. The co-variation analysis between Marwari goat and Bikaneri camel

Table 1: List of SNP's and their nature in *MSTN* exon 2 gene of Marwari goat and Bikaneri camel

Sl. No.	SNP	Base Position	Transition/ Transversion	Synonymous/ Nonsynonymous	Predicted Amino Acid Change
1	A>G	51	Transition	Synonymous	No change
2	G>A	63	Transition	Synonymous	No change
3	C>T	70	Transition	Non synonymous	Y>H (Histidine to tyrosine at 24th position)
4	C>T	94	Transition	Synonymous	No change
5	A>C	102	Transversion	Synonymous	No change
6	T>C	114	Transition	Synonymous	No change
7	C>A	117	Transversion	Synonymous	No change
8	A>C	118	Transversion	Non synonymous	Q>K (Lysine to glutamine at 40th position)
9	C>T	270	Transition	Synonymous	No change

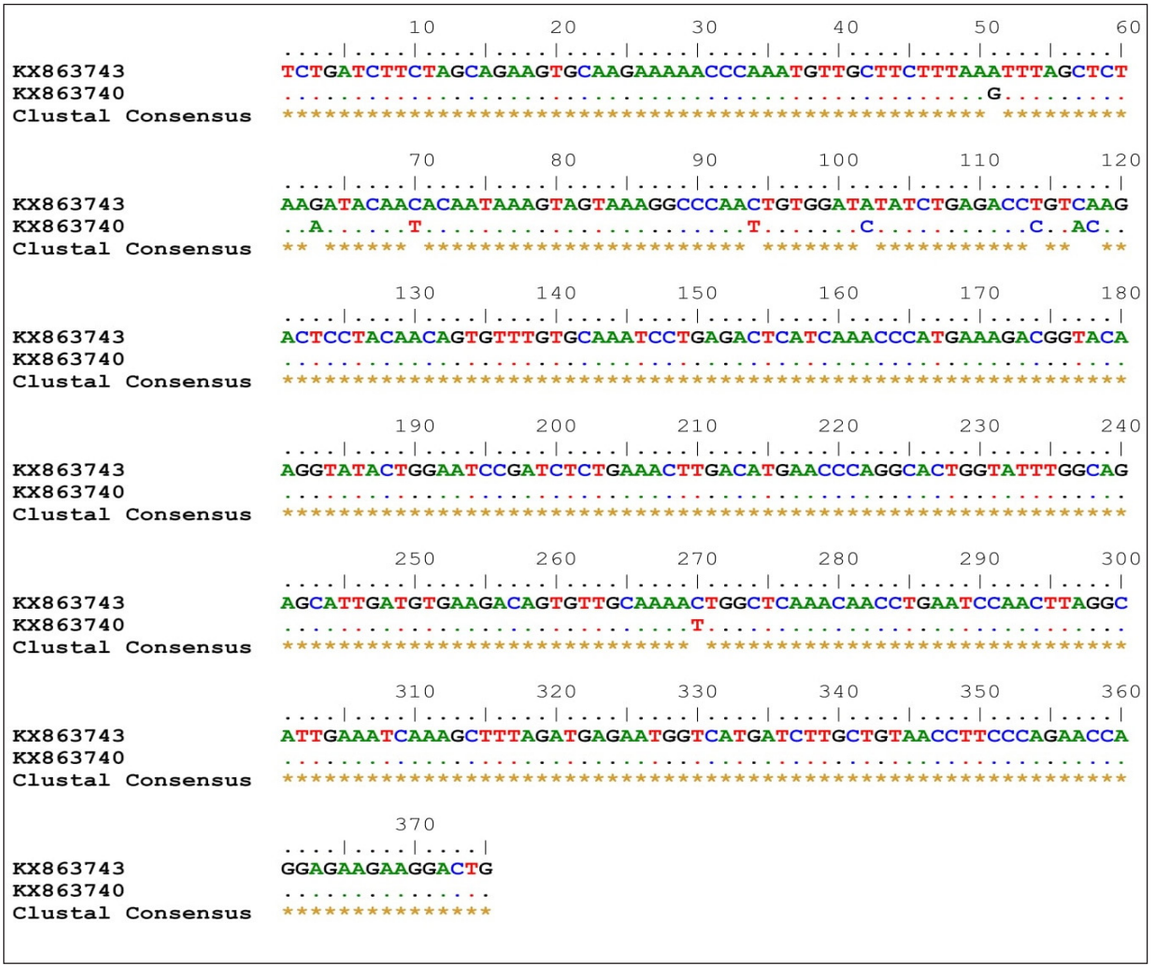

Fig. 2: Pair wise nucleotide sequence clustal analysis of exon 2 of MSTN gene of Marwari goat (KX863743) with Bikaneri **camel (KX863740)**

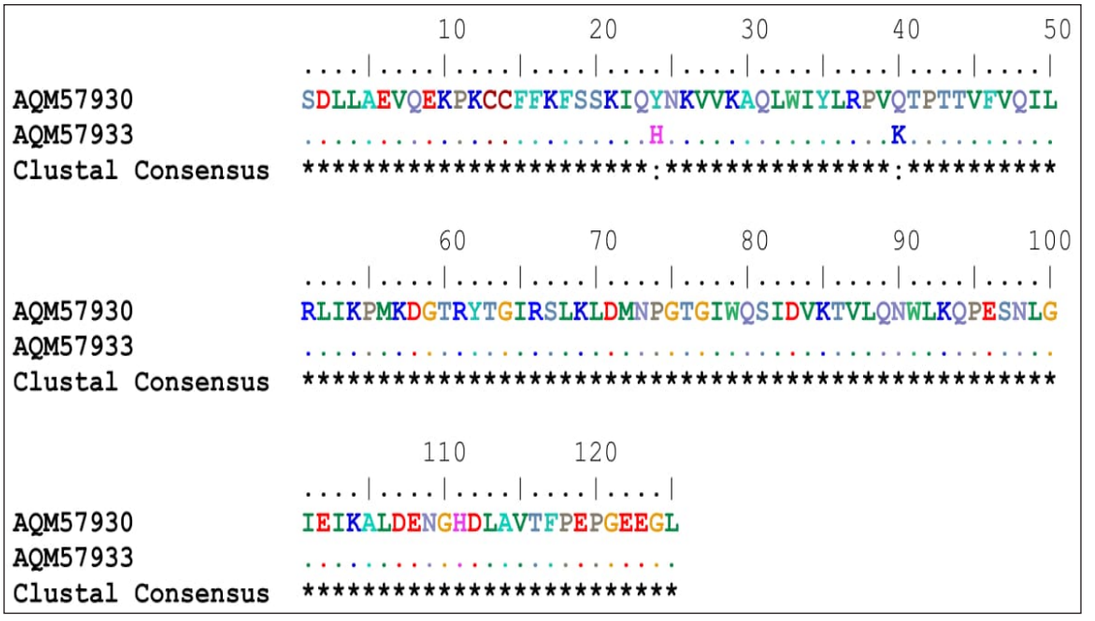

Fig. 3: Pair wise protein sequence clustal analysis of exon 2 of *MSTN* gene of Marwari goat (AQM57933) with Bikaneri camel (AQM57930)

Table 2: Nucleotide sequence maximum likelihood values between Marwari goat and Bikaneri camel for MSTN exon 2 gene

Between	And	Length	Approx. Confidence Limits	Transition/transversion ratio = 2.000000
1	Marwari	0.00006	(zero, 0.01614)	
1	Bikaneri	0.02435	(0.00834, 0.04044) **	Ln Likelihood = -560.94491
1	Clustal_Co	0.05814	(zero, infinity)	

** = significantly positive, P < 0.01.

indicated violation of Watson Crick rules at 70th and 118th base position. The nucleic acid sequence maximum likelihood values were found to be statistically positive (P<0.01) with Ln Likelihood value of -560.94491 and transition/transversion ratio of 2.00 (Table 2). The results are consistent with random amplified polymorphic DNA and microsatellite information (Li and Alessio, 2004), which indicated that there is no obvious difference on genetic diversity between goat breeds on the basis of geographical regions. The study is in agreement with Li *et al*. (2006) and DNA sequencing analysis reported by An *et al*. (2011) in 664 animals of four goat population through primers designed from the same *MSTN* gene sequence.

Similar low within population diversity was observed in three different populations of *Camelus dromedarius* in Africa for second exon of *MSTN* gene (Muzzachi *et al*., 2015) which suggest high level of within species sequence conservation in camel.

Absence of polymorphism in *MSTN* exon 2 gene of Bikaneri camel is in agreement with the reports on different *Camelus dromedarius* breeds (Shah *et al,* 2006; Muzzachi *et al,* 2015). The absence of *MSTN* diversity observed in Bikaneri camel reflects the evolution of camel from low variable wild ancestor population. Similar low within population diversity was observed in three different populations of *Camelus dromedarius* in Africa for second exon of *MSTN* gene (Muzzachi *et al*., 2015) which suggest high level of within species sequence conservation in camel. The computational analysis in the present study revealed a higher level of pair-wise nucleotide sequence

identity values between Indian goat and camel (97.6 per cent) than those reported by Muzzachi *et al*. (2015) for Algerian camel and *Capra hircus* (94.04 per cent) indicating a low evolutionary genetic divergence between Indian Marwari goat and Bikaneri camel. Agrawal *et al*. (2017a) also reported highly conserved nature of *MSTN* exon-2 region among different livestock species through NJ tree based phylogenetic analysis with more than 95% homology of Bikaneri camel with that of the buffalo, pig, goat, sheep and horse. Comparative sequence analysis of *MSTN* exon-2 gene of Bikaneri camel with goat species could also help in understanding the mechanism of diseases associated with emaciation in camels (Ma *et al*., 2003).

CONCLUSION

The exon 2 of *MSTN* gene was investigated in Bikaneri camel and Marwari goat to detect molecular variation through RFLP and sequencing methods to understand the divergent evolution of these two livestock species in similar agro climatic zones. The absence of within species variation in Bikaneri camel and Marwari goat suggest the conserve nature of studied region in both the species. Interspecies comparison of studied *MSTN* locus indicated the presence of nucleotide variation that could be useful in understanding of divergent evolution of species in similar agro climatic zones.

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