

*Full Length Research Paper*

# Molecular characterization and novel genetic variability in leptin (obese) gene of mithun (*Bos frontalis*)

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Accepted 12 April, 2012

The present study was undertaken with the objectives of sequencing, characterization and single nucleotide polymorphisms (SNPs) identification of mithun leptin gene. The mithun leptin gene (3420 bp) was sequenced, compared with other species and phylogenetic tree were constructed. Single-strand conformation polymorphism (SSCP) showed three patterns in both exon-2 and exon-3 of the gene. Nucleotide sequences from all patterns revealed three novel SNPs. In exon-2 at 25<sup>th</sup> codon, one SNP (AGC/TGC) with amino acid changed from serine to cysteine, and at 37<sup>th</sup> codon one silent mutation (ACA/ACG) was detected. In exon-3 at 72<sup>nd</sup> codon, one silent mutation (CCG/CCA) was also identified. This preliminary study of existing gene variants can be used for further association studies, thereby establishing conservation of mithun germplasm.

**Key words:** Leptin gene, mithun, phylogeny, single nucleotide polymorphisms (SNPs).

## INTRODUCTION

Mithun (*Bos frontalis*), a semi-wild ruminant found in the hilly regions of northeastern India, Myanmar, Bhutan, Bangladesh, China and Malaysia. This bovine species is believed to be domesticated more than 8000 years ago (Simoons and FJ1984) from wild gaur (*Bos gaurus*). However, as per world conservation union, this species is on the verge of extinction (IUCN, 2002). This high-quality meat animal of the North-Eastern Hill Region (NEHR) normally lives in the areas of low oxygen tension prevails and thrives on very little conventional fodder. Mithun is closely associated with social, cultural, religious and livelihood of the tribes of north eastern Himalaya and is the main source of meat, milk and draft power (Basar, 2002). Mithun meat is considered to be more tender and better to the meat of any other species (Simoons and FJ 1984). Till date, only few studies have documented: its hormonal profile (Mondal et al., 2004, 2005), growth performance (Pal et al., 2004), carcass characteristics

(Heli et al., 1994) and milk composition (Mondal et al., 2001).

The selection of better animals in terms of production of good-quality meat and milk is of prime importance for a country like India where malnutrition rate is very high (World Bank Report, 2009). Application of molecular genetics for genetic improvement relies on the ability to genotype individuals for specific genetic loci. Single nucleotide polymorphisms (SNP) are the class of direct markers that locate the loci, coding for the functional mutation and thus have the edge over other markers. These markers can be potentially used for genetic improvement of livestock through within-breed selection (Dekker, 2004). Selection of suitable candidate gene, depending on the trait under selection and pleiotropic effects of the gene, are important. Analysis of marker-trait associations can bring a significant improvement for polygenic traits like milk yield, growth and meat production. Leptin, a 16 kDa protein, is synthesized by adipose tissue. It binds to a receptor mainly localized on neuropeptide Y neurons, which results in a reduction of feed intake and an increase of energy expenditure. Neuropeptide Y is also involved in the control of reproductive function (Magni et al., 2000). The leptin factor has

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**Table 1.** Primer used for sequencing and SNP identification.

Primer name	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Product size (bp)
LEPII	ACATCCGTTGTTCACTGTG	TGCAGGCATATCCCATAACC	340
LEPIII	AGGTACATTGTGGGGGATACA	GGGCCTGAAAACAGAAGAAA	378
LEPIV*	GTGCCACGTGTGGTTTCTTC	CCTCCCTACCGTGTGTGAGA	210
LEPV	ACACACGGTAGGGAGGGACT	GCTCAGTTACCAGGCAGGAA	345
LEPVI	GCATTCTTCCTGCCTGGTAA	ATGGGGTCTCAAAGAGTTGG	323
LEPVII	AAGCTAGTCAGGTTCCACAAGG	GGCACACACAGTTCTGGTTC	416
LEPVIII	GTGACCCTCCCTGAATACCC	GCCACCCACAGCAATCAAT	354
LEPIX	AGAGTGCCCCAACCTGTGT	ATCTTTCTGCCCTTCCCAA	361
LEPX	AGGGAGTCATGGCTGGTTCT	TTGGAGGAGACGGACTGCTA	310
LEPXI	GCTCTTGCTCTCCCCTTCT	GTTTTCTTCCCTGGACTTTGG	430
LEPXII	GGGTGCTGAAGCCTTGAA	CTTGCTTGATGGTCCAAAGG	346
LEPXIII *	CTGGGATTTTACAGCAGTCT	TCGAGATCCATTACAGAGCAA	431

\*Primers used for polymorphism study, LEPIV and LEPXIII correspond to exon-2 and exon-3 respectively.

gained much attention recently as a key regulator of biological processes that are related to important productive traits in beef and dairy cattle, such as feed intake, fat content, meat quality and milk production (Buchanan et al., 2002; Geary et al., 2003; Liefers et al., 2003; Lusk, 2007).

Genetic polymorphism in the bovine leptin gene has been described (Pomp et al., 1997; Fitzsimmons et al., 1998; Haegeman et al., 2000; Buchanan et al., 2002; Lagonigro et al., 2003) and an association with fat deposition in beef cattle has been reported (Fitzsimmons et al., 1998). In turn, polymorphisms in this gene have been proposed as predictors of relative differences among individuals for those traits (Nkrumah et al., 2005; Schenkel et al., 2006; Corva et al., 2009). In fact, markers in the leptin gene are already part of commercial genotyping panels designed for marker assisted selection (MAS) in beef and dairy cattle. The sequence information and polymorphism of leptin gene have been well documented in cattle, goat, buffalo, yak and pig, while in mithun the study is still not reported. This is the first attempt to sequencing, characterization and SNP identification of leptin gene in mithun.

## MATERIALS AND METHODS

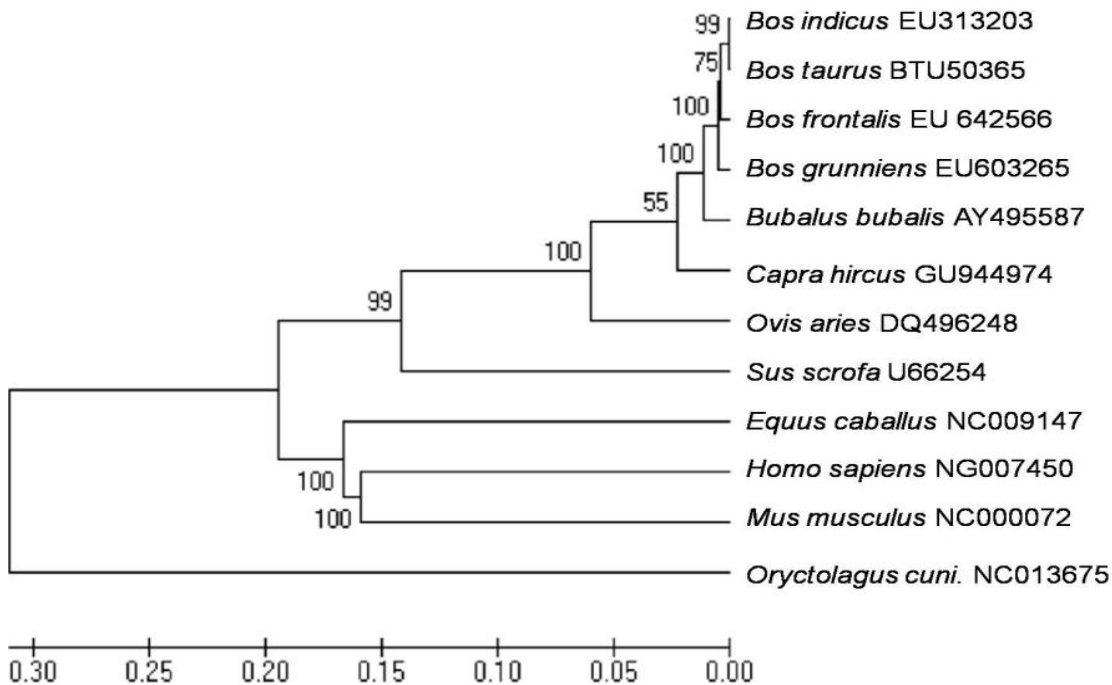
Blood samples (n = 50) were collected from genetically unrelated animals from Nagaland, India, representing the breeding tract of Nagami Mithun. Before collecting blood samples, animal owners were asked for their relatedness. Only one sample was collected from small herd (<10) and maximum of 2 to 3 samples from large herd were taken in the study. A random stratified technique was used to select the villages in the breeding region. Blood samples were stored at -20°C until use. Genomic DNA was isolated as per standard protocol (Sambrook and Russell, 2001) with minor modifications, checked for quality and quantity in agarose gel electrophoresis and nano-drop and was further diluted to a final concentration of 50 ng/μL. Primers (Table 1) and PCR conditions

reported in our previous publication (Dubey et al., 2008; Dongre et al., 2009), used for sequencing of *Bos indicus* and *Bos grunniens* leptin genes were used to amplify the complete gene sequence in mithun.

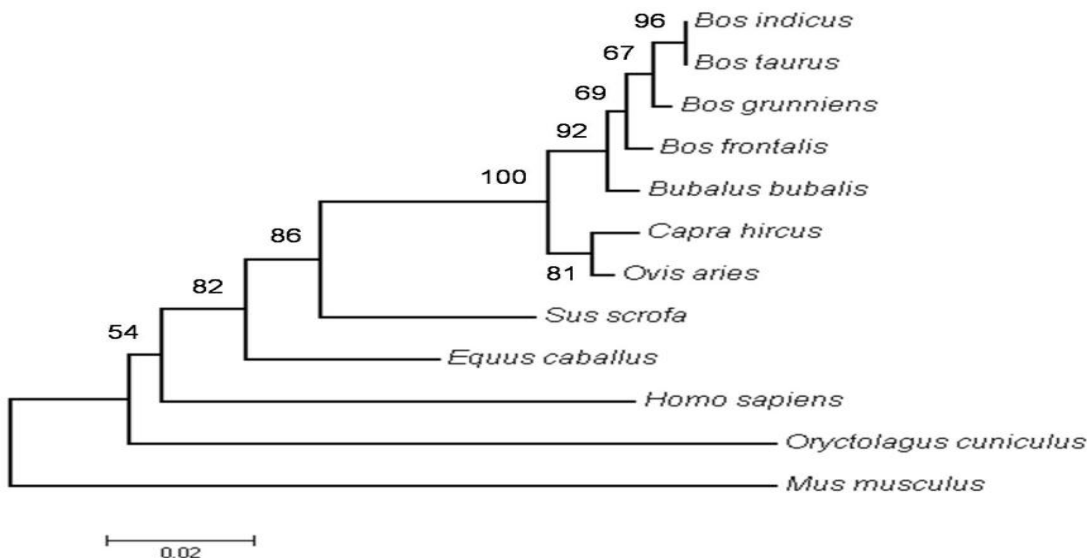
The BLAST algorithm was used to search the NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>) databases for homologous sequences. The sequence analysis was carried out using various modules of DNASTAR Version 4.0, Inc., Madison, USA, for protein translations by EDITSEQ, sequence alignments and contigs comparisons by MEGALIGN and chromatograph evaluation by SEQMAN. Novel sequences were submitted to the NCBI GenBank, and accessions were obtained, which are in public domain now. Comparison analysis of the gene of mithun to other species and phylogenetic tree were constructed with Mega 4.0 software (Phoenix, USA, Tamura et al., 2007). Genetic distances were calculated using the Kimura 2-parameter method and confidence of probability of each branch were assessed using the bootstrap of 1000 replications.

Polymerase chain reaction (PCR) products of exon-2 and exon-3 of the gene were resolved by single-strand conformation polymorphism (SSCP) analysis. In order to optimize SSCP resolution on gel, several factors were tested for each fragment like amount of PCR product, denaturing solution, acrylamide concentration, percentage cross-linking, glycerol, voltage, running time and temperature. Each PCR product was diluted in denaturing solution (95% formamide, 10 mM NaOH, 0.05% xylene cyanol and 0.05% bromophenol blue, 20 mM ethylenediaminetetraacetic acid (EDTA)), denatured at 95°C for 5 min, chilled on ice and resolved on 12% polyacrylamide gel. The electrophoresis was carried out in a vertical unit (Biorad Protean II xi), in 1X TBE buffer. The gels were stained with silver nitrate as per the method described by Benbouza et al. (2006). DNA samples showing different banding patterns on SSCP gels were selected for sequencing. Primers for sequencing were the same as those used for the PCR-SSCP amplification. The PCR products were purified by PCR purification kit (Biogene, New Delhi, India) and cloned using instant cloning kit, (Banglore Genei, Bengaluru, India). Sequencing of cloned PCR product was carried out using ABI prism Big dye terminator sequencing kit version 3.1 as per the manufacturer's protocol on ABI PRISM® 3100-Avant Genetic Analyzer (Applied Biosystems, Foster City, USA). In order to identify the putative SNPs, nucleotide sequence alignments were carried out using the MEGALIGN software module of DNASTAR version 4.0 (software of DNASTAR, Inc., Madison, USA).





**Figure 2.** Phylogenetic relationship of the leptin nucleotide sequences from different species using Mega version 4.1 following the alignment of the complete nucleotide sequences using Clustal-W and neighbor-joining method (nucleotide p-distance). Numbers outside the branches indicate the bootstrap values obtained using 1,000 replicates. Scale bar at the bottom measures the nucleotide distance.

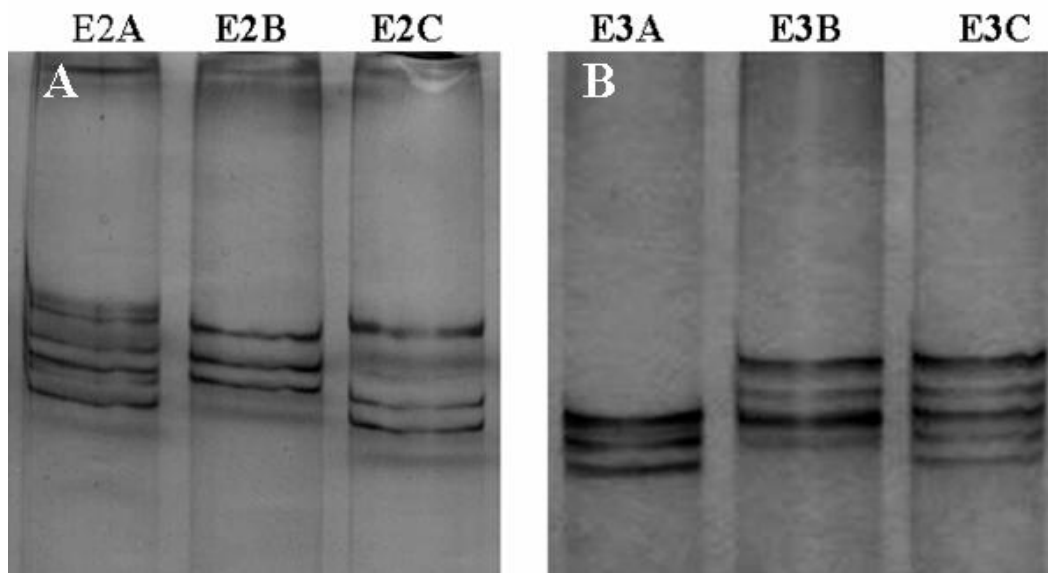


**Figure 3.** Phylogenetic relationship of the leptin coding nucleotide sequences from different species using Mega version 4.1 following the alignment of the complete nucleotide sequences using Clustal-W and neighbor-joining method (nucleotide p-distance). Numbers outside the branches indicate the bootstrap values obtained using 1,000 replicates and scale bar at the bottom measures the nucleotide distance.

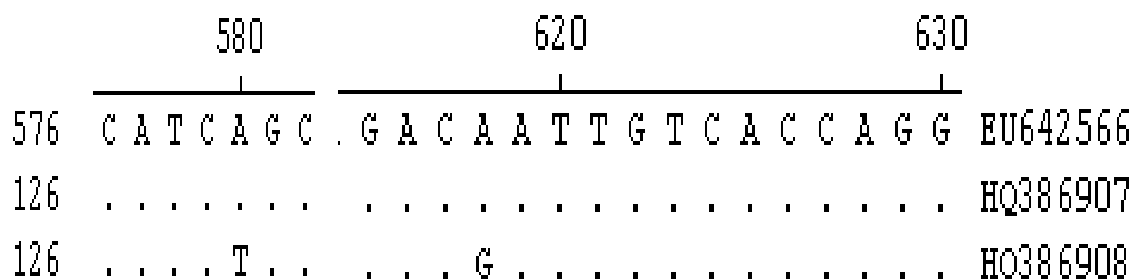
region (Figure 3) of the leptin gene of 12 species, showed very little difference except for horse, human, rabbit and mouse, indicating large differences in the non coding region of the gene.

**Single-strand conformational polymorphism analysis**

Genetic variability in the leptin gene was assessed by SSCP technique, which allows the detection of changes



**Figure 4.** PCR-SSCP patterns on a 12% non-denaturing polyacrylamide a) three patterns in exon-2 and b) three patterns in exon-3.



**Figure 5.** Comparative alignment of PCR-SSCP allelic variant E2A (HQ386907) and E2B (HQ386908) pattern of leptin gene exon-2 sequence in mithun with GenBank reference sequence EU642566 showing nucleotide positions 580/130 and 618/168 substitutions.

in the nucleotide sequence, and a PCR-product is affected by single base substitution (Orita et al., 1989; Hayashi, 1991). Three band patterns E2A, E2B and E2C with a frequency of 30, 16 and 54%, respectively were obtained in Exon-2 having part of intron-1 and intron-2. Exon-3 having part of intron-2 and 3' UTR region of the gene, also depicted three patterns E3A, E3B and E3C (Figure 4b) with frequencies of 22, 36 and 42%, respectively.

**Sequence analysis and SNP identification**

PCR Products of two samples of each SSCP pattern were cloned and sequenced. Nucleotide sequence comparison of three SSCP patterns of exon-2 revealed two SNPs (Figure 5; accession number HQ386907-08), first at 25<sup>th</sup> codon (AGC/TGC) leading to an amino acid change from serine to cysteine (Figure 6) and second at

37<sup>th</sup> codon causing a silent mutation (ACA/ACG). In exon-3 at 72<sup>nd</sup> codon one silent mutation (CCG/CCA) was also identified (Figure 7).

**DISCUSSION**

Mithun is an outstanding meat and draft power animal of north eastern region of India and adjoining countries in Southeast Asia (Gupta et al., 1999). Milk production in this animal is of little economic value to the local people directly but it plays a great role in the animals having a better growth rate for meat production. These animals are kept in small numbers by farmers, where pedigree records are impractical to maintain. It is widely accepted that the leptin gene affects the growth of body, feed intake, fat contents, immune function, milk production and reproduction in bovine and may thus be an important candidate gene for genetic improvement and to make

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21  Ala Val Pro Ile Ser Lys Val Gln Asp Asp EU642566
21  . . . . . . . . . . HQ386907
21  . . . . Cys . . . . HQ386908

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**Figure 6.** Comparative alignment of conceptualized protein sequences of exon-2 variants of leptin gene in mithun with GenBank reference sequence EU642566.

2620

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2611 G C C C C T T G C C A C A G G EU642566
235  . . . . . . . . . . HQ386909
235  . . . . . . . G . . . . HQ386910

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**Figure 7.** Comparative alignment of PCR-SSCP allelic variant E3A (HQ386909) and E3B (HQ386910) pattern of leptin gene exon-3 sequence in mithun with GenBank reference sequence EU642566 showing nucleotide position 2621/245 substitutions.

conservation strategy of mithun animal. Unlike other livestock, the complete leptin gene sequence is not available for mithun and, has been completed in this present study in order to characterize across species.

A high homology between all four *Bos* species was found as they are tightly related. The nucleotide sequence comparison of mithun leptin gene with other members of *Bovidae* family was made. The mithun leptin gene shows 97.8% homology with zebu and taurus cattle, 96.3% with buffalo, 97.5% with yak, 99.4% with sheep (partial), 95.3% with pig, 94.4% with horse and 94.1% with human, which indicates a close evolutionary relationship. The genetic distances between mithun, yak, taurus cattle and zebu cattle were relatively small and they formed the first cluster (four *Bos* species). Only partial sequence of sheep leptin gene was available in GenBank database but being a species related to goat, the sheep (partial) sequence was used in the phylogenetic analysis, and it clustered in a group for coding region and co-clustered with completed nucleotide sequence while buffalo and pig, remain in the same position for both trees. The phylogenetic clustering based on leptin gene sequence of cattle, mithun, yak, sheep, goat, buffalo and pig were found to be comparable and reliable with the zoological classification as well as phylogenetic clustering constructed using lacto protein, mitochondria and growth hormone gene sequences (Fan et al., 2001; Gu et al., 2007; Zhong et al., 2007). The small differences in human, mouse, rabbit and horse observed in the molecular phylogenetic tree could possibly be due to the

alternative evolutionary rate of this gene during evolution. Thus, our data indicated that the leptin gene was suitable for studying species classification and phylogenetic relations.

Polymorphism study using SSCP technique shows genetic variability in mithun leptin gene. The number of SSCP patterns observed in this breed was lesser than in Sahiwal breed of Zebu cattle (Dubey et al., 2008), while the same region was found to be monomorphic in Yak (Dongre et al., 2009). In the present study, we reported novel genetic polymorphism in exon-2 and exon-3 of the Mithun leptin gene in contrast to the yak (*B. grunniens*), which has been found monomorphic at the nucleotide level in the similar regions of the gene (Dongre et al., 2009). However, studies in different cattle breeds have shown a correlation between leptin gene polymorphism and body weight, daily live weight gain, back fat thickness, feed intake, feed efficiency and some carcass characteristics (Zwierzchowski et al., 2002; Buchanan et al., 2002; Oprzadek et al., 2003; Lusk, 2007; Kulig et al., 2007; Kulig and Kmiec, 2009). SNPs at codon 25 of exon-2 and codon 80 of exon-3 have been reported to influence fat yield, meat production and other production traits (Giblin et al., 2010). Buchanan et al. (2003) reported that the T allele of the bovine leptin gene resulted in higher milk production. Komisarek and Dorynek (2005) reported that Arg4Cys TT genotype at codon 25 of exon2 has a highly significant effect on increasing milk yield. Similarly, Buchanan et al. (2002), Lusk (2007) and Kulig and Kmiec (2009) reported that TT

homozygote animals were found to have a higher daily weight gain than CC and CT genotypes. Thus, pleiotropic effects of leptin gene have a high potential to include in the quantitative trait loci studies.

In this study, the SNP at 25<sup>th</sup> codon is common for cattle and mithun but interestingly with different nucleotide change at the same position, resulting in amino acid change Ser4Cys (AGC/TGC-transition mutation) of mithun and Arg4Cys (CGC/TGC-transversion) of cattle leptin gene. However, SNP found at 37<sup>th</sup> codon (ACA/ACG) and at 72<sup>nd</sup> codon (CCG/CCA) has not been previously reported in other mammalian species and hence, these two SNPs can be considered exclusive in mithun leptin gene. All the three SNPs could be used for screening of different genotypes in mithun population for future association studies.

## ACKNOWLEDGEMENTS

The authors would like to thank Director, National Bureau of Animal Genetic Resources, Karnal, India, for providing facilities. This work was supported by grants from Indian Council of Agricultural Research (ICAR), New Delhi, Government of India.

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