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NUCLEOTIDE SEQUENCE VARIATION IN LEPTIN GENE OF MURRAH BUFFALO *(BUBALUS BUBALIS)*

Sanjoy Datta, A.M.J.B. Adikari, Anuj Chauhan, Archana Verma, I. D. Gupta, Indrasen Chauhan, Mandeep S. Azad and Yathish H.M.

ABSTRACT : Leptin is a 16 kD protein, synthesized by adipose tissue and is involved in regulation of feed intake, energy balance, fertility and immune functions. Present study was undertaken with the objectives of sequence characterization and studying the nucleotide variation in leptin gene in Murrah buffalo. The leptin gene consists of three exons and two introns which spans about 18.9kb, of which the first exon is not transcribed into protein. In buffaloes, the leptin gene is located on chromosome eight and maps to BBU 8q32. The leptin gene was amplified by PCR using oligonucleotide primers to obtain 289 bp fragment comprising of exon 2 and 405 bp fragment containing exon 3 of leptin gene. The amplicons were sequenced to identify variation at nucleotide level. Sequence comparison of buffalo with cattle reveals variation at five nucleotide sequences at positions 983, 1083, 1147, 1152, 1221 and all the SNPs are synonymous resulting no in change in amino acids. Three of these eight nucleotide variations have been reported for the first time in buffalo. The results indicate conservation of DNA sequence between cattle and buffalo. Nucleotide sequence variations observed at leptin gene between *Bubalus bubalis and Bos taurus* species revealed 97% nucleotide identity.

Key words: Leptin, Sequence variation, Murrah.

INTRODUCTION

Leptin, a 16 kD a protein that is synthesized by adipose tissue is involved in regulation of feed intake, energy balance, fertility and immune functions (Fruhbeck *et al.* 1998). Milk production trait is a quantitative trait and polygenic in inheritance. Since milk production trait is directly related to feed intake and energy balance, it is obvious that there is effect of leptin on milk production. Several single nucleotide polymorphisms (SNP) have been reported in the coding region of the gene (Konfortov *et al.* 1999, Orrù *et al.* 2007, Liefers *et al.* 2002) found that some SNP are associated to production, live weight, energy balance, feed intake and fertility in Holstein heifers. Leptin is a 167-amino acid protein produced by the leptin gene (leptos means thin).

Molecular Genetics Lab, Dairy Cattle Breeding Division, National Dairy Research Institute, Karnal, Haryana, India, Pin - 132001.
** Corresponding author*

Leptin is also responsible for the regulation of body weight and energy homeostasis and is one of the potential biomarker for high performance leading to better adaptability and productivity. (Friedman and Halaas 1998). The Leptin gene consists of three exons and two introns which spans about 18.9 kb, of which the first exon is not transcribed into protein. Leptin gene has been mapped to chromosome 7 in human (Green *et al.* 1995) chromosome 4 (BTA4) in bovine (Stone *et al.* 1996) and chromosome 8 (BBU 8q32) (Vallinato *et al.* 2004) in buffalo. Several SNPs have been previously identified in introns and exons of leptin among different breeds of cattle.

Leptin plays a critical role in regulating and coordinating energy metabolism (Friedman and Halaas 1998). It regulates the metabolism of key tissues involved in the storage and dissipation of energy (Banks *et al.* 2000). Therefore, leptin may be important in regulating metabolic adaptation of nutrient partitioning during the energy-consuming processes of pregnancy and lactation (Moschos *et al.* 2002). Since buffalo contributes about 54 per cent of the total milk produced in India. Although the economic importance of buffaloes has always been known, yet very little work has been carried out to exploit the genetic potentials of this animal. Hence the present study was undertaken with the objective to identify sequence variation in leptin gene of buffalo.

MATERIALS AND METHODS

Sample Collection and DNA preparation

Blood samples were collected in vacutainers (Bacton-Dickinson vacutainer system) containing sodium EDTA as an anticoagulant from Murrah buffaloes maintained at National Dairy Research Institute, Karnal. Genomic DNA was isolated from blood samples following phenol-chloroform extraction method described by Sambrook and Russel (2001) with slight modifications. DNA was dissolved in TE buffer at 60°C in water bath for 2 hrs to dissolve pellet properly in buffer and was kept in refrigerator for further use. Quality of DNA was checked through spectrophotometry. DNA samples with O.D. ratio between 1.7 and 1.9 were considered as good and used for further study. The samples beyond this range were re-extracted by Phenol-chloroform extraction method. DNA quality was also checked by running the sample in 0.8 percent agarose gel electrophoresis. The quality of DNA was checked on 0.8% agarose and quantity by UV spectrophotometer at A_{260}/A_{280} nm.

DNA Amplification

The PCR amplification using primers (Adikari 2006) for exon 2 and 3 of leptin gene was carried out in a programmable thermal cycler (PTC 200, MJ Research) using the following program: For the amplification of 2nd exon, 25 µl of PCR reaction mix contained 3.000 µl genomic DNA (50 ng/µl), 0.600 µl primers each (100 pM/µl), 12.5 µl Fermentas Master Mix™ (2X) and 8.3 µl double distilled H_2O. , Initial denaturation at 93°C for 1 min, denaturation at 93°C for 1 min, annealing at 59°C for 30 sec, extension at 72°C for 1.3 min were carried out for 36 cycles.

For the amplification of 3rd exon, 25 µl of PCR reaction mix contained 3.000 µl genomic DNA (50 ng/µl), 0.600 µl primers each (100 pM/µl), 12.5 µl Fermentas Master Mix™ (2X) and 8.3 µl double distilled H_2O, Initial denaturation at 93°C for 1 min, denaturation

at 93°C for 1 min, annealing at 56°C for 30 sec, extension at 72°C for 1.3 min were carried out for 36 cycles. The PCR products were loaded on 1.5% agarose to confirm the amplification of target region using 100 bp ladder as a marker and were visualized by ethidium bromide staining, which was added to the agarose gel @ 1 µl /100 ml of gel. The agarose gel electrophoresis was performed in 1X buffer at 100 volts for 45 minutes and PCR products were visualized on UV transilluminator and photographed with gel documentation system. The primers designed, regions amplified, annealing temperatures and product sizes are given in Table1.

Nucleotide Sequencing

The PCR products were concentrated to 50 ng/µ by pooling several tubes to precipitate by the isopropanol procedure. In order to obtain clean fragment for sequencing, the PCR products were separated by electrophoresis in a TAE agarose gel containing ethidium bromide using standard protocols. The desired PCR product band was excised using a clean, sterile razor blade or scalpel (band was visualized in a medium or long wavelength (*e.g.* 300 nm) UV light, and excised quickly to minimize exposure of the DNA to UV light). The minimum agarose slice was transferred to a 1.5 ml microcentrifuge or screw cap tube and then purified by using commercially available gel extraction kits (Qiagen). Quantification was done by loading one µl of eluted sample in 1% Agarose gel and comparing with standard molecular marker (Phi X 174 DNA ladder or 100 bp DNA ladder). Only samples with good concentration (>50 ng/µl) were selected and subjected to sequencing. Amplified PCR products from each set of primers were subjected to custom DNA sequencing from both ends (5' and 3' ends). from M/s. Chromous biotech, INDIA.

Sequence Data Analysis

Sequence data were analyzed by using Chromas (Ver. 1.45, *http://www.technelysium.com.au/chromas.html).* Sequence data from variants of different regions were subjected to multiple alignments (DNASTAR, Clustal W) for identifying the SNPs.

Database Search

The database search of sequences for a possible match to the DNA sequence of leptin gene was conducted using the BLAST algorithm available at the National Center for

Table 1:Description of primers used and the amplified product of different loci studied

Primer	Primer sequence 5'-3'	T_m	Amplified product length
I	F-5'- GGT GGT AAC GGA TCA CAT GG -3' R-5'- CCA CGG TTC TAC CTC GTC TC -3'	59°C	289 bp fragment containing exon II
II	F-5'- GCA TAG CAG TCC GTC TCC TC -3' R-5'- TTC CCT GGA CTT TGG GAA G -3'	56°C	405 bp fragment containing exon III

Biotechnology Information (NCBI). Translated protein sequences of leptin genes in different species namely *Bos taurus, Bos indicus, Ovis aries, Capra hircus* and *Homo sapiens* were also subjected to BLAST algorithm.

RESULTS AND DISCUSSION

Sequence data were analysed using chromas (Ver.1.45, *http://www.technelsium.com.au / chromas.html*). ClustalW multiple alignments with Bubalus bubalis sequence reference sequence NCBI GI 46254758) revealed nucleotide changes at 5 positions in the gene. These variations are found in nucleotide sequences at positions G983A in intron 1 which is heterozygous; at C1083T-homozygous , T1147G- heterozygous, A1152G-homozygous and C1221T- heterozygous mutations in exon 2; at T1371C in intron 2 homozygous mutation; (Fig.I). However, all these nucleotide changes are synonymous *i.e.* there is no change in amino acids. (Table 1) Nucleotide sequence data and annotation details are given in Fig.2 .

Orru *et al.* (2007) while investigating River Buffalo and two Egyptian River Buffalo reported 12 new single nucleotide polymorphism (SNP); two polymorphic sites G3333A and G3441A previously identified in exon 3 by Vallinoto *et al.* (2004) and originally referred to as 276-SNP, 384-SNP and one of the new SNP, C1221T occur in exon 2 and three G3195A, G3318A and G3434A in exon 3 while SNPs T1015C, C1071T, G1072A, T1081C, T1131G, T1143C, T1145G and A1151G are all in intronic region. They also reported a mutation at G3434A in the exon 3, resulting in Arg to Gln substitution in the codon 159 but it has been observed only one Italian Buffalo while all other animals were homozygote GG genotype. In exon 3, the SNP G3318A was found polymorphic only in the Egyptian Buffaloes, while it resulted monomorphic in Italian Buffaloes. Position G3441A was found monomorphic in both Egyptian and Italian Buffalo.

Fitzsimmones *et al.* (1998) reported 4 SNPs in exon 2 in *Bos taurus*. Whereas, Haegman *et al.* (2002) found 2 SNPs in exon 2 in *Bos taurus*. Adikari (2006) has also reported nucleotide changes at position 11, 365, 369 and 371 in intron 2 region. Six SNPs in exon 2 of

Table 2: SNPs Identified in Leptin Gene (Murrah buffaloes)

INTRON/EXON	Position in aligned data	Nucleotide change	SNP type	Amino acid change
Intron 1	983	G > (G/A)	Heterozygous	None
Exon 2	1083	C > T	Homozygous	None
Exon 2	1147	T > (T/G)	Heterozygous	None
Exon 2	1152	A > G	Homozygous	None
Exon 2	1221	C > (C/T)	Heterozygous	None

Fig.1: Clustal W Alignment with Reference sequence of Leptin Gene (Exon-2) in Murrah Buffalo

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GGATTAGTGCCTTTCATTACTGTCATTTCTAGACAATGAATTGTCTTTGAGGAGATGATAGCCATGGCAGACAGC
AAATCTC(A/G)TTGTTATCTGCATCCGAAGACGTGGATGCGGGTGGTAACGGATCACATGGGTGTTCTCTGAGA
TCGGCGACG(G/T)GCCACGTGTGGTTTCTTCTGTTTTCAGGCCCCAGAAGCCCATCCCGGGAAGGAAAATGCGC
TGTGGACCCCTGTA(C/T)CAATTCCTGTGGCTTTGGCCCTATCTGTCCTACGTGGAGGCTGTGCCCATCCGCAA
GGTCCAGGATGACACCAAAACCCTCATCAAGACGATTGTCACCAGGATCAATGACATCTCACACACGGTAGGGAG
GGACTGGGAGATGAGGTAGAACTGTGGCCATCCCTGGGGGAACCCCCAGAGGGC
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Fig. 2: NCBI BLAST data (Query: Reference Exon 2 sequence data, Subject: Aligned sequence data of the sample)

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Query  2    AGTGCCTTTCATTACTGTCATTTCTAGACAATGAATTGTCTTTGAGGAGATGATAGCCAT  61
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  412  AGTGCCTTTCATTACTGTCATTTCTAGACAATGAATTGTCTTTGAGGAGATGATAGCCAT  353

Query  62   GGCAGACAGCAAATCTCGTTGTTATCCGCATCCGAAGACGTGGATGCGGGTGGTAACGGA  121
            |||||||||||||||| |||||||| ||||||||||||||||||||||||||||||||||
Sbjct  352  GGCAGACAGCAAATCTCATTGTTATCTGCATCCGAAGACGTGGATGCGGGTGGTAACGGA  293

Query  122  TCACATGGGTGTTCTCTGAGATCGGCGACGTGCCACATGTGGTTTCTTCTGTTTTCAGGC  181
            |||||||||||||||||||||||||||||| ||||| ||||||||||||||||||||||||
Sbjct  292  TCACATGGGTGTTCTCTGAGATCGGCGACGGGCCACGTGTGGTTTCTTCTGTTTTCAGGC  233

Query  182  CCCAGAAGCCCATCCCGGGAAGGAAAATGCGCTGTGGACCCCTGTACCAATTCCTGTGGC  241
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  232  CCCAGAAGCCCATCCCGGGAAGGAAAATGCGCTGTGGACCCCTGTACCAATTCCTGTGGC  173

Query  242  TTTGGCCCTATCTGTCCTACGTGGAGGCTGTGCCCATCCGCAAGGTCCAGGATGACACCA  301
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  172  TTTGGCCCTATCTGTCCTACGTGGAGGCTGTGCCCATCCGCAAGGTCCAGGATGACACCA  113

Query  302  AAACCCTCATCAAGACGATTGTCACCAGGATCAATGACATCTCACACACGGTAGGGAGGG  361
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  112  AAACCCTCATCAAGACGATTGTCACCAGGATCAATGACATCTCACACACGGTAGGGAGGG  53

Query  362  ACTGGGAGACGAGGTAGAACCGTGGCCATTCTGTGGGGGA--CCCCAGAGG  410
            |||||||| |||||||||| ||||||| ||  |||||||  |||||||||
Sbjct  52   ACTGGGAGATGAGGTAGAACTGTGGCCA-TCCCTGGGGGAACCCCCAGAGG  3
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Yellow: Exon 2 region
Green: SNP
Red: Leptin gene coding region

Jersey cattle were reported by Konfortov *et al.* (2006). Recent research in India with *Bos grunniens* revealed 4 SNPs in both exon 2 and 3 as reported by Dongre *et al.* (2008). Among all these results, there was change of amino acids in protein product in some cases (*i.e.* non-synonymous mutation). Whereas, in few cases, no changes in amino acids were observed (*i.e.* synonymous mutation)

Jhala *et al.* (2009) have reported three point mutations at 42, 44 and 250 nucleotide positions in Mehsana buffalo. Orru *et al.* (2011) reported a total of 10 SNP: 2 in intronic region, 2 in exon 2 and 6 in exon 3 out of which three SNP (C978T; C3100T; A3157G) have been reported to affect muscle fat in Simmental bulls. Scatà *et al.* (2012) reported eight SNPs in 5' flanking and exon 1 of Bubaline leptin gene :A83G, A90G, A121G, G256T, A283G, G959T, A1010C and G1254A. Tanpure *et al.* (2012) reported five single nucleotide polymorphic sites 98, 111, 172, 209 and 266 in intron 1, which correspond to three haplotypes in Mehsana buffalo.

Sequence Homology of buffalo leptin gene with other species

The nucleotide sequences deduced for the respective exons of leptin gene in Murrah buffaloes were arranged to represent the coding region and were compared with other leptin sequences of the related species available in NCBI Gene Bank viz Bovine, caprine using NCBI web site Basic Local Alignment Search Tool (BLAST). There exists 97%, 97%, 99%, 98% and 80% sequence homology with *Bos taurus, Bos indicus, Ovis aries, Capra hircus* and *Homo sapiens* respectively. The higher sequence similarity of buffaloes with sheep and goat than cattle might be due to lower query coverage (47%) in these two species.

CONCLUSION

Bovine leptin gene specific primers amplified the buffalo leptin gene and PCR amplification yielded an amplified product of exon 2 and exon 3 of bubaline leptin gene. Eight SNPs were identified while comparing bubaline leptin gene with that of other species, none of which resulted in change in amino acid sequence i.e. synonymous mutations. There exists 97%, 97%, 99%, 98% and 80% sequence homology with *Bos taurus, Bos indicus, Ovis aries, Capra hircus* and *Homo sapiens* respectively. Because the leptin gene plays an important role in influencing economic traits in cattle, the novel detected single nucleotide polymorphisms might be used in association studies to assess their potential of being genetic markers for selection.

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