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MOLECULAR CHARACTERIZATION OF THE LEPTIN GENE IN RIVERINE BUFFALOES

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ABSTRACT

The present study was undertaken with the objectives of sequence characterization and studying the genetic variation in leptin gene locus 2nd exon and 3rd exon in Murrah buffalo by PCR-RFLP. The leptin gene was amplified by PCR using oligonucleotide primers standardized for *Bos taurus* species. 289 bp fragment comprising of exon 2 and 405 bp fragment containing exon 3 of leptin gene were amplified and digested with *Alu I* , *AciI*, *MspI*, *Sau3AI*, *HphI* restriction enzyme using a cattle specific primer. The sizes of the amplification products were similar in cattle and buffalo. The leptin gene in buffalo reveals monomorphism since no variation was found. The result indicates strong conservation of DNA sequence between cattle and buffalo. Nucleotide sequence variations observed in the leptin gene between *Bubalus bubalis* and *Bos taurus* species revealed 97.0% nucleotide identity. Sequence comparison of buffalo with cattle reveals variation at eight nucleotide sequences at positions 983, 1083, 1147, 1152, 1221, 1371, 3318, 3333 when all the SNPs are synonymous resulting in no change in amino acids. The milk production traits namely milk yield and fat yield could not be associated with buffalo leptin genotypes due to their monomorphic haplotype.

Keywords: leptin gene, PCR-RFLP, genetic polymorphism, phylogenetic tree, haplotype

INTRODUCTION

Leptin is a 167-amino acid protein produced by the leptin gene (LEP), whose name is derived from the Greek word “leptos”, which means “thin”. 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). It is one of the most useful biomolecules as a marker for identifying high performing individuals leading to better adaptability and productivity. Leptin is also responsible for the regulation of body weight and energy homeostasis (Friedman *et al*., 1998).

Defects in leptin production cause severe hereditary obesity in animals. It has an important role in regulation of hematopoiesis, angiogenesis, wound healing, and the immune and inflammatory response. The LEP gene is the human homolog of the gene (ob) mutant in the mouse ‘obese’ phenotype (Zhang *et al*., 1997). Since the bovine leptin gene has been identified on chromosome 4, several SNPs have been previously identified in introns and exons of leptin among different breeds of cattle. The physiological role and biology of leptin is well reviewed (Hossner, 1998 and Houseknechcht *et al*., 1998). Polymorphic studies on bovine leptin gene

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have been reported (Pomp *et al*., 1997; Haegeman *et al*., 2000; Lien *et al*., 1997 and Wilkins and Davey, 1997, (Almeida *et al*., 2003). Lagonigro *et al*. (2003); Choudhury, 2004; Yoon *et al*. (2005).

The leptin gene consists of three exons and two introns which spanabout 18.9 kb, of which the first exon is not transcribed into protein. The leptin gene has been mapped to chromosome 7 in human (GreGreen *et al.,* 1995) and chromosome 4 in bovine (Stone *et al*., 1996). In buffaloes, the leptin gene is located on chromosome 8 (BBU 8q32) (Vallinato *et al*., 2004).

The milk production trait is a quantitative trait and polygenic in inheritance. Since the milk production trait is directly related to feed intake and energy balance, it is obvious that there is an effect of leptin on milk produntion. Pickavance *et al*. (1998) observed that the feed intake-induced leptin increase was eliminated during lactation and they speculated that the hypoleptinemia may be an important factor promoting the hyperphagia of lactation. This data demonstrated that the onset of the negative energy balance is largely responsible for the declining leptin concentrations towards parturition and the low leptin level during lactation probably induces the hyperphagia of lactation. Plasma leptin responds to isoenergetic glucose or lipid supplementation which is dependent on the stage of lactation. Holstein cows in late lactation exhibited a robust leptin response to parenteral glucose or lipid administration, whereas early-lactation cows did not (Chelikani *et al*., 2003). It was also reported that mammary adipocytes expressed leptin during early stages of development in sheep (Bonnet *et al*., 2002). 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).

The buffalo contributes about 54 percent 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. Though studies have been carried out on characterization in cattle, similar studies in buffaloes are scarce. Association studies between leptin gene polymorphisms and live weight, energy balance, feed intake and fertility were reported in cattle (Liefers *et al*., 2002), but little information about polymorphism and association studies of the leptin gene with phenotypic traits like milk production, fat percentage, protein yield etc. have been reported in buffaloes.

Hence the present study was undertaken with the objective of sequence characterization and identification of polymorphisms within exons 2 and 3 of the leptin gene and its association with milk and fat yield.

MATERIALS AND METHODS

Animals

The present study was conducted with 120 lactating Murrah buffaloes maintained at the cattle yard of the National Dairy Research Institute, Karnal Haryana, India.

Sample and data

About 10 ml venous blood was collected from the jugular vein of each animal into a sterile 50 ml polypropylene vial containing 0.5 M EDTA as anticoagulant.

Data on milk yield and fat percentage for the 1st, 2nd, 3rd and 4th lactations were collected from the records maintained at the cattle yard of the National Dairy Research Institute, Karnal Haryana, India.

DNA preparation

Genomic DNA was isolated from blood samples following the phenol-chloroform extraction method described by Sambrook and Russel (2001).

DNA was dissolved in TE buffer and was kept in water bath at 60°C for 2 h to dissolve the pellet properly in the buffer. The quality of the DNA was checked through spectrophotometry. DNA samples with O.D. ratios between 1.7 and 1.9 were considered as good and used for further study. The samples beyond this range were re-extracted by the phenol-chloroform extraction method. The DNA quality was also checked by running the sample in 0.8 percent agarose gel electrophoresis. The DNA samples devoid of smear were used for further study.

DNA amplification

Exon 2 and exon 3 of the leptin gene were amplified. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique was applied to explore the polymorphism in the leptin gene.

For the amplification of the 2nd exon, 25 µl of PCR reaction mix containing 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 minute, denaturation at 93°C for 1 minute, annealing at 59°C for 30 seconds, extension at 72°C for 1.3 minutes were carried out for 36 cycles The primers designed, regions amplified, annealing temperatures and product sizes are given in Table 1.

For the amplification of the 3rd exon, 25 µl of PCR reaction mix containing 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 minute, denaturation at 93°C for 1 minute, annealing at 56°C for 30 seconds, extension at 72°C for 1.3 minutes were carried out for 36 cycles. The primers designed, regions amplified, annealing temperatures and product sizes are given in

Table 1. Description of primers used and the amplified products of the different loci studied.

Locus	Primer sequence 5'-3'	T_m	Primer length bp	Primer source	Amplified product length
Primer I	F-5'- GGT GGT AAC GGA TCA CAT GG -3' R-5'- CCA CGG TTC TAC CTC GTC TC -3'	59 °C	20 20	**D**	289 bp fragment containing of exon II
Primer II	F-5'- GCA TAG CAG TCC GTC TCC TC -3' R-5'- TTC CCT GGA CTT TGG GAA G -3'	56 °C	20 19	**D**	405 bp fragment containing of exon III

All primers were obtained from IDT, United States of America, synthesized in 100 nM scales.

*D= Designed by using Primer 3 software.

Table 1.

RFLP and polyacrylamide gel electrophoresis

*Alu*I, *AciI*, *HphI* restriction enzymes (0.07 µl of 10 U/ µl) and *MspI*, *Sau3AI*, *PvuII*, *HindIII*, *HinfI*, *Eco32I*, *Eco147I* and *Kpn2I* (0.14 µl of 5 U/ µl) were applied on both the contigs (20 µl of PCR product each time). The reaction mixture for RE digestion was kept for incubation at 37^{o}C for 4 h. Horizontal electrophoresis on 2-3% agarose gel was used to resolve restriction fragments and was visualized by ethidium bromide staining. The ethidium bromide was added to the agarose gel of 1 µl/100 ml of gel. The agarose gel electrophoresis was performed in 1X buffer at 100 volts for 30, 60, and 90 minutes till complete separation of all fragments of RE digested gene fragments and PCR marker. The restriction digested gene fragments were visualized on UV transilluminator and photographed with a gel documentation system.

Nucleotide sequencing

The PCR products were concentrated to 50 ng/µl by pooling several tubes to precipitate by the isopropanol procedure. In order to obtain clean fragments 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 (bands were 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 markers (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). Representative samples from each of the variants obtained by RFLP analysis were also custom sequenced from M/s. Chromous biotech, India.

Sequence data analysis

Sequence data were analyzed mostly by DNASTAR software. Sequence data was 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 growth hormone gene was conducted using the BLAST algorithm available at the National Center for Biotechnology Information (NCBI, Bethesda, MD). 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.

Statistical analysis

The frequencies of gene and genotypes were estimated for the identified locus as per the method suggested by Falconer and Mackay (1998). Association study with the milk production parameters with the genotype of leptin gene could not be achieved due to monomorphism of the alleles of the leptin gene in buffalo.

RESULTS AND DISCUSSION

Identification of Genotypes

The PCR amplification generated a 289 bp for exon II (Figure 1) and a 405 bp for exon III segment (Figure 2) for the leptin gene of buffalo. The bubaline leptin gene is homologous to the cattle leptin gene of similar length (Ji *et al*., 1998), thus it indicates strong conservation of DNA sequences in both species. Nucleotide sequence organization is similar to other species, as goat, sheep, mouse, human, monkey, camel, cat, dog, donkey, elephant, horse, yak, pig and rat (www.ncbi.nlm. nic.in).

PCR-RFLP of Leptin gene

Size of various electrophoretic bands observed by PCR-RFLP analysis of the leptin gene with *various* restriction enzymes in Murrah buffaloes are given in Table 2.

In the present study, PCR-RFLP analysis using all the eleven enzymes did not reveal polymorphism in either of the exons of the leptin gene in Murrah buffaloes.

Alu-I digestion of theamplified product of the 2nd exon revealed two products of 189 and 100 bp (Figure 3). Aci-I digestion of the amplified product of the 2nd exon revealed two products of 89 and 200 bp (Figure 4). *MspI* digestion of the amplified product of the 2nd exon revealed two products of 79 and 210 bp (Figure 5). *Sau3AI* digestion of the amplified product of the 2nd exon revealed two products of 89 and 200 bp (Figure 6). *HphI* digestion of the amplified product of the 2nd exon revealed two products of 89 and 200 bp (Figure 7).

Alu-I digestion of the amplified product of the 3rd exon revealed two products of 55 and 350 bp (Figure 8). Aci-I digestion of the amplified product of the 3rd exon revealed two products of 135 and 270 bp (Figure 9). *MspI* digestion of the amplified product of the 3rd exon revealed two products of 255 and 150 bp (Figure 10). *Sau3AI* digestion of the amplified product of the 3rd exon revealed two products of 105 and 300 (Figure 11). *HphI* digestion of the amplified product of the 3rd exon revealed two products of 105 and 300 bp (Figure 12).

Since the Murrah buffaloes included in the present study were found to be monomorphic, it was not feasible to analyze the data with respect to milk and fat yield (Table 3). It might be because of the fact that the animals were in a closed herd. Similar monomorphism of this gene in cattle was also observed by others in the bubaline leptin gene. Kumar *et al*. (2003) reported the absence of polymorphism within 522bp PCR product of leptin gene in buffalo digested with HinfI restriction enzyme. Vallinoto *et al*. (2004) amplified promoter and exon 1 with primers designed from the bovine leptin gene. Three SNPs and one microsatellite were identified. No polymorphisms were detected in exon 2. Similar monomorphism of buffalo were also reported by PCR-RFLP in growth hormone gene (Pal and Chatterjee, 2010). Therefore, this monomorphism of the buffalo may be a species specific characteristic of buffalo. Thus, the gene and genotypic frequencies were found to be 1.00.

However in cattle (*Bos taurus*), polymorphism was detected for the leptin gene by a number of researchers. Kulig *et al.* (2009) investigated how leptin gene polymorphisms affected milk production traits such as milk yield, fat and protein yield, and fat and protein content in Jersey cows. Two single-nucleotide polymorphisms (SNPs) were genotyped, using *Sau3AI* RE. RFLP polymorphisms within the bovine leptin gene were detected by using *HinfI* restriction enzyme and it was found that allele A positively affected milk production traits in Friesian cattle and they

Table 2. PCR-RFLP analysis of the leptin gene with different Res.

REs used	**Contig 1 (1173-1344)**			**Contig 2 (3103-3462)**		
	PCR product	**Cutting sites**	**Pattern**	**PCR product**	**Cutting sites**	**Pattern**
AluI	289 bp	189 & 100 bp	Monomorphic	405 bp	55 & 350 bp	Monomorphic
AciI	289 bp	89 & 200 bp	Monomorphic	405 bp	135 & 270 bp	Monomorphic
MspI	289 bp	79 & 210 bp	Monomorphic	405 bp	255 & 150 bp	Monomorphic
Sau3AI	289 bp	89 & 200 bp	Monomorphic	405 bp	105 & 300 bp	Monomorphic
HphI	289 bp	89 & 200 bp	Monomorphic	405 bp	105 & 300 bp	Monomorphic
HindIII	289 bp	No	-	405 bp	No	-
HinfI	289 bp	No	-	405 bp	No	-
Eco32I	289 bp	No	-	405 bp	No	-
Eco147I	289 bp	No	-	405 bp	No	-
Kpn2I	289 bp	No	-	405 bp	No	-
PvuII	289 bp	No	-	405 bp	No	-

Table 3. Lactation wise maximum and minimum milk and fat % in animals.

Lactation	**Milk production**			**Fat %**		
	Minimum (Kg)	**Maximum (Kg)**	**Average (Kg)**	**Minimum**	**Maximum**	**Average**
First	1994	3339	2089.93	6.00	10.00	7.31
Second	1922.5	3620	2294.60	5.9	10.2	7.45
Third	1915	3414.5	2107.65	5	8.2	7.48
Fourth	1713	3748	2033.00	6	8.5	7.79

indicated significant superiority of allele A over allele B for milk and milk protein yields and body conformation traits (Khaleel *et al.,* 2009).

Pannier *et al.* (2009) reported four SNP loci which were found to be in linkage disequilibrium and thus, the frequencies of each of the 16 possible haplotypes were inferred by maximum likelihood. No significant association between any individual SNP and haplotype was found with intramuscular fat values in *Bos taurus.* Fortes *et al.* (2009) have reported three genotypes in *Bos taurus* x *Bos indicus* crossbred cattle with 7.7 % higher frequency of the T allele.

Buchanan *et al*. (2003) genotyped 416 Holstein cows by using restriction enzyme Kpn21 and compared lactation performance data using a mixed model. Animals homozygous for the T allele produced more milk and had higher somatic cell count linear scores, without significantly affecting milk fat or protein percent over the entire lactation.

Dandapat *et al.* (2010) observed polymorphism using *HphI* -PCR-RFLP in *Bos taurus* x *Bos indicus* crossbred cattle exhibited AA, AV and VV genotypes with their respective frequency of 0.57, 0.36 and 0.07 and gene frequency as 0.75 and 0.25 for the A and V alleles, respectively. However, they have reported monomorphic pattern in Sahiwal cattle. Since no mutation was found in Sahiwal cattle and only the A allele was present throughout the population studied, the frequency of the A allele was 1.

Nucleotide sequencing and SNP detection

The nucleotide sequences for exon 2 and exon 3 for the bubaline leptin gene have been depicted in Figure 13 and Figure 14 respectively. The sequence obtained for the Murrah is compared and aligned with sequence of *Bubalus bubalis* using the MegAlign program of DNASTAR software. Amplified regions of both the contigs were custom sequenced by using forward and reverse primers. The leptin gene sequences for exon 2 and exon 3 have been submitted to the gene bank. The PCR amplification procedure for two contig regions of Leptin gene has been standardized which yielded consistent and specific amplification. The leptin gene in Murrah buffaloes included in present study is monomorphic as revealed by PCR-RFLP analysis using *AluI, AciI, MspI, Sau3AI, HphI* restriction enzymes. *HindIII, HinfI, Eco32I, Eco147I, Kpn2I* and *PvuII* restriction enzymes did not reveal any cutting site in both the contigs. However, reports were available regarding the cutting site of the above enzymes in the leptin genes of cattle and other species. Sequence data were analysed using chromas (Ver.1.45, http://www.technelsium.com.au/chromas.html).

Clustal W multiple alignments with *Bubalus bubalis* sequence revealed nucleotide changes at eight positions in the gene. These variations are found in nucleotide sequences at positions 983 in intron 1; at 1083, 1147, 1152 and 1221 in exon 2; at 1371 in intron 2; at 3318 and 3333 at exon 3 (Figures 13 and 14). Eight SNPs were discovered by studying variation at eight nucleotide sequences at positions 983, 1083, 1147, 1152, 1221, 1371, 3318, 3333 while comparing leptin gene sequences with other species. However, all these nucleotide changes are synonymous, i.e. there is no change in amino acids.

Phylogenetic analysis of buffalo with other species

The nucleotide sequences deduced for the respective exons of the leptin gene in the Murrah buffaloes were arranged to represent the coding region and were compared with other leptin

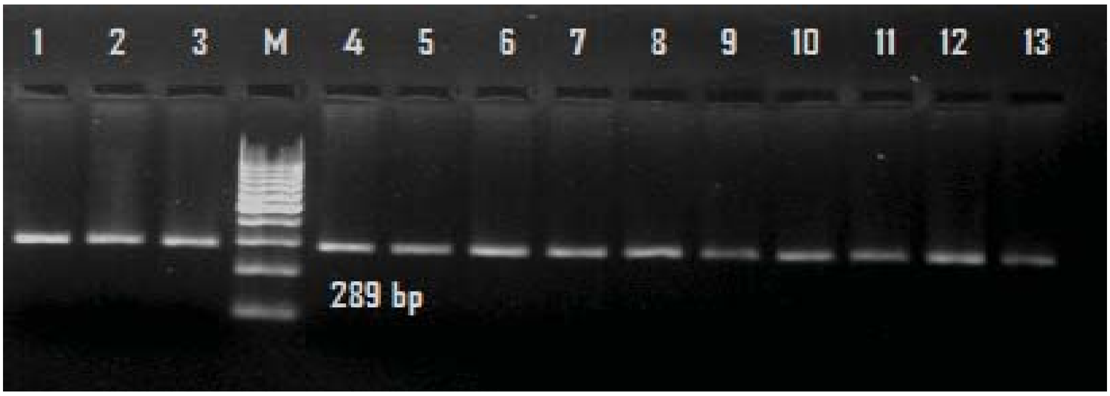

Figure 1. Resolution of PCR amplified product of Primer I on 1.5% agarose gel.

Lane 1-9 and 10-19 : PCR product (289 bp)
Lane M : 100 bp Molecular Marker

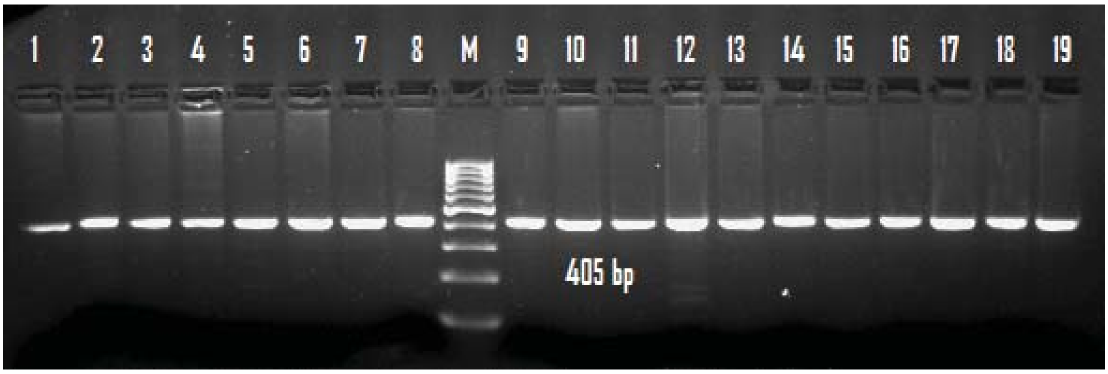

Figure 2. Resolution of PCR amplified product of Primer II on 1.5% agarose gel.

Lane 1-8 and 9-19 : PCR product (405 bp)
Lane M : 100bp Molecular Marker

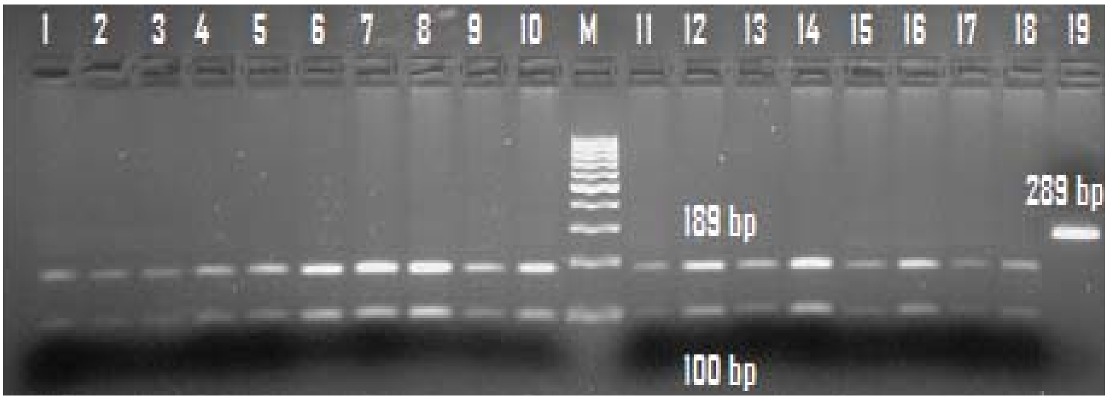

Figure 3. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *AluI* RE in Murrah buffaloes**.**

Lane 1-10 and 11-18 : 2 Bands (100 bp and 189 bp)
Lane 19 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

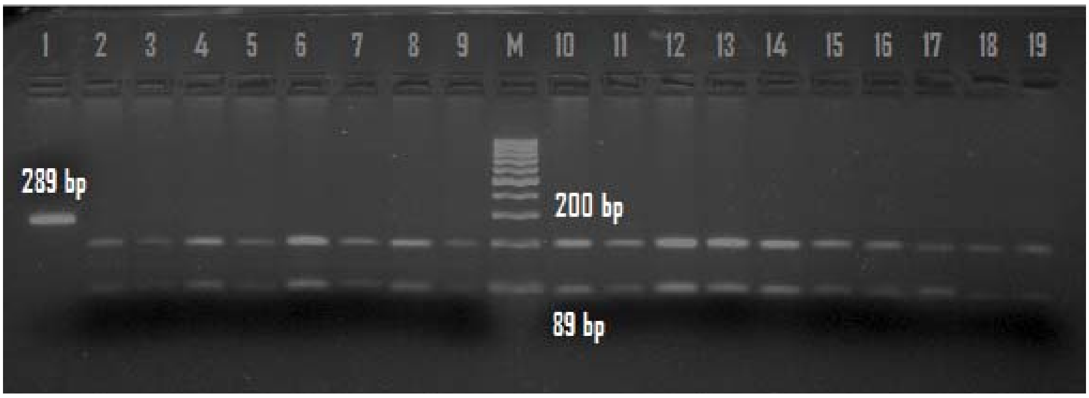

Figure 4. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *AciI* RE in Murrah buffaloes.

Lane 2-9 and 10-19 : 2 Bands (200 bp and 89 bp)
Lane 1 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

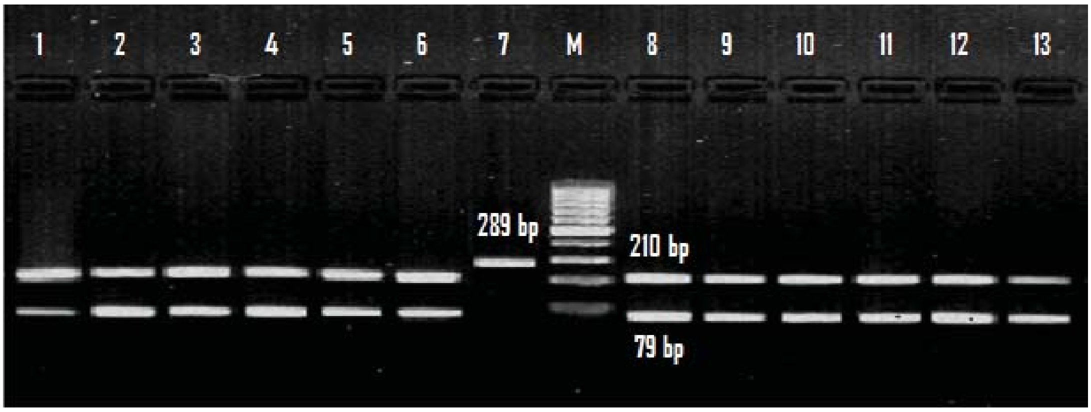

Figure 5. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *MspI* RE in Murrah buffaloes.

Lane 1-6 and 8-13 : 2 Bands (210 bp and 79 bp)
Lane 7 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

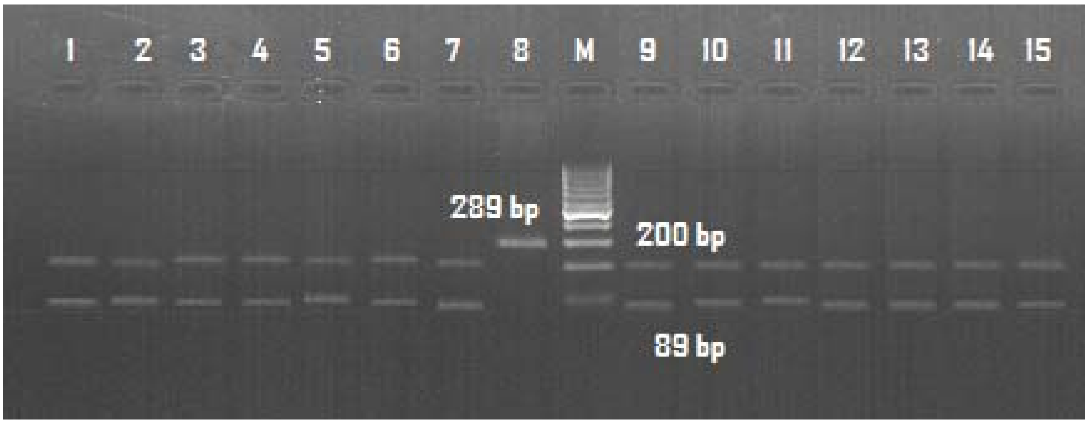

Figure 6. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *Sau3AI* RE in Murrah buffaloes.

Lane 1-7 and 9-15 : 2 Bands (200 bp and 89 bp)
Lane 8 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

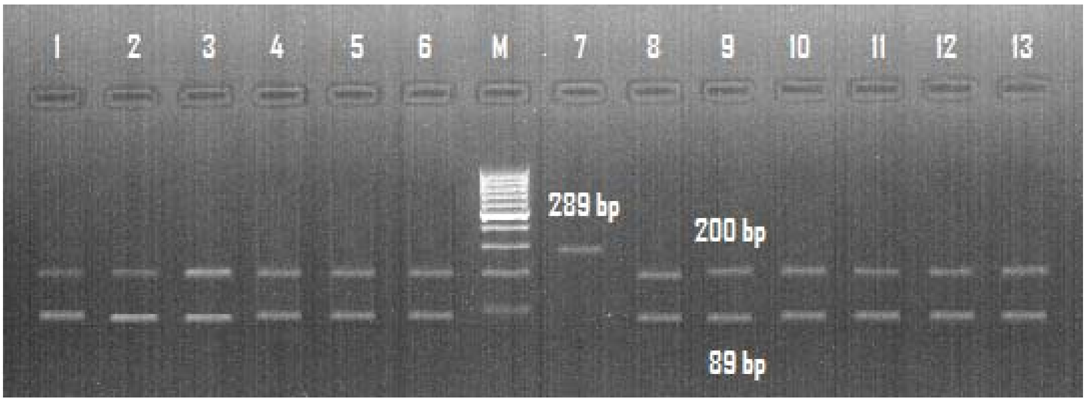

Figure 7. PCR-RFLP of primer I of the leptin gene on 2.5% agarose gel using *HphI* RE in Murrah buffaloes.

Lane 1-6 and 8-13 : 2 Bands (200 bp and 89 bp)
Lane 7 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

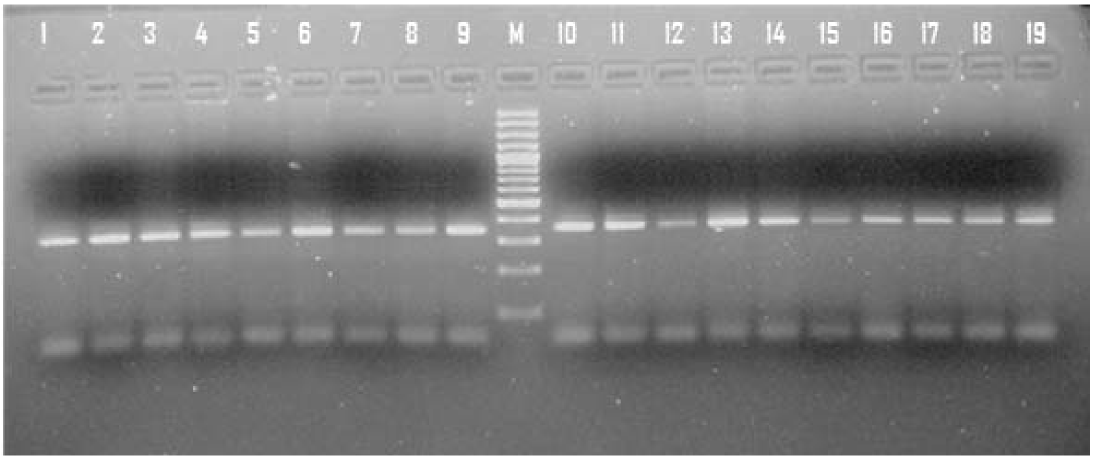

Figure 8. PCR-RFLP of primer II of the leptin gene on 2.5% agarose gel using *AluI* RE in Murrah buffaloes.
Lane 1-9 and 10-19 : 2 Bands (55 bp and 350 bp)
Lane M : 100 bp Molecular Marker

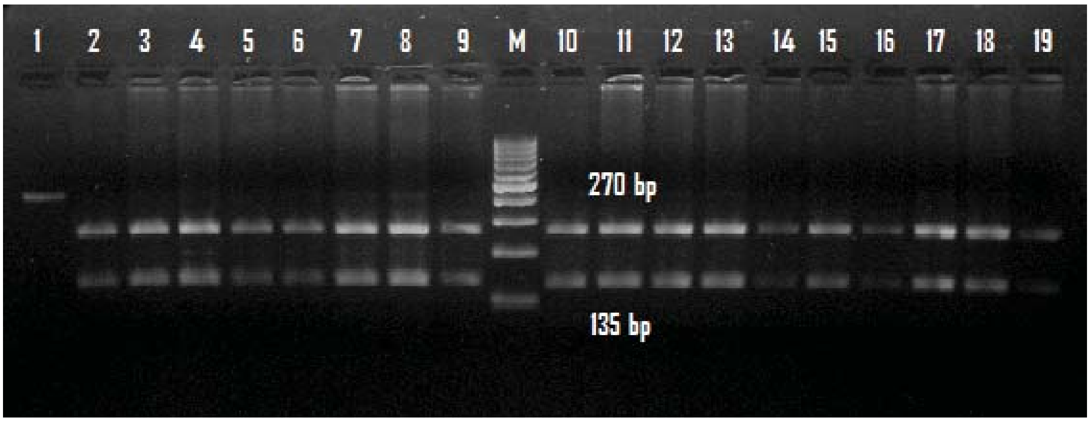

Figure 9. PCR-RFLP of primer II of Leptin gene on 2.5% agarose gel using *AciI* RE in Murrah buffaloes.
Lane 2-9 and 10-19 : 2 Bands (135 bp and 270 bp)
Lane 1 : PCR Product (405 bp)
Lane M : 100 bp Molecular Marker

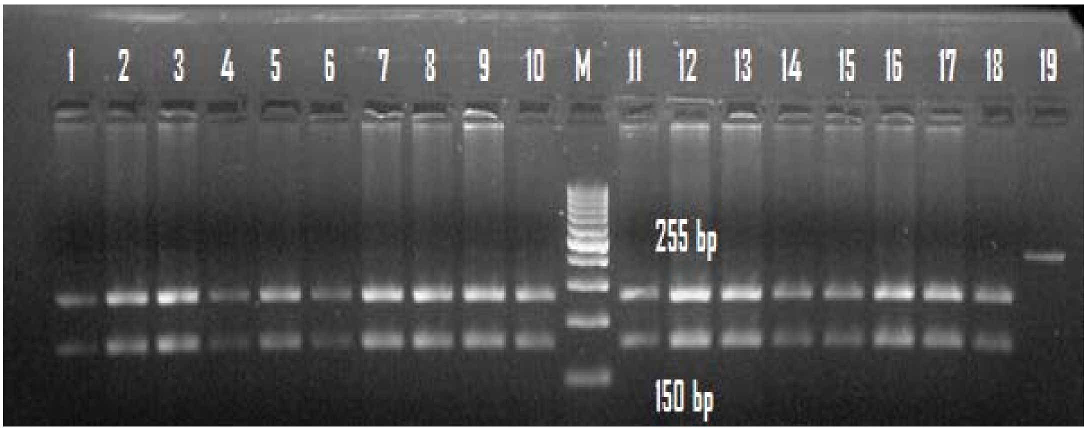

Figure 10. PCR-RFLP of primer II of the leptin gene on 2.5% agarose gel using *MspI* RE in Murrah buffaloes.

Lane 1-10 and 11-18 : 2 Bands (150 bp and 255 bp)
Lane 19 : PCR Product (405 bp)
Lane M : 100 bp Molecular Marker

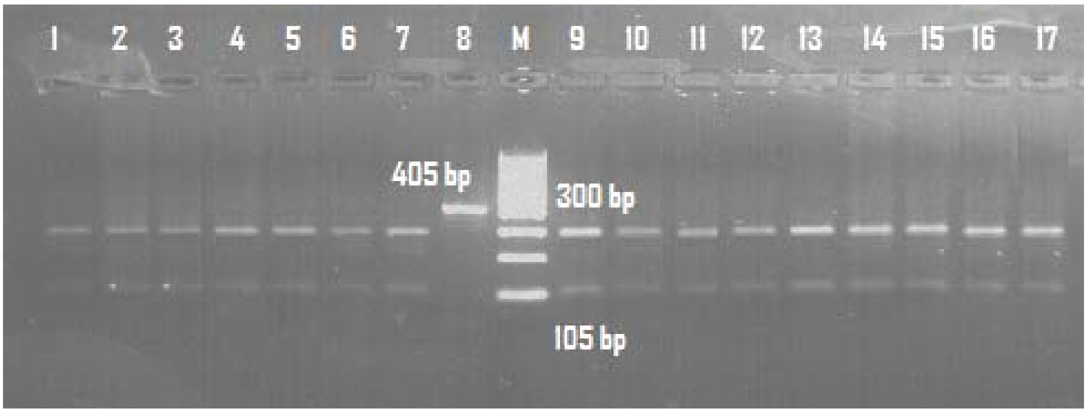

Figure 11. PCR-RFLP of primer II of Leptin gene on 2.5% agarose gel using *Sau3AI* RE in Murrah buffaloes.

Lane 1-7 and 9-17 : 2 Bands (300 bp and 105 bp)
Lane 8 : PCR Product (405 bp)
Lane M : 100 bp Molecular Marker

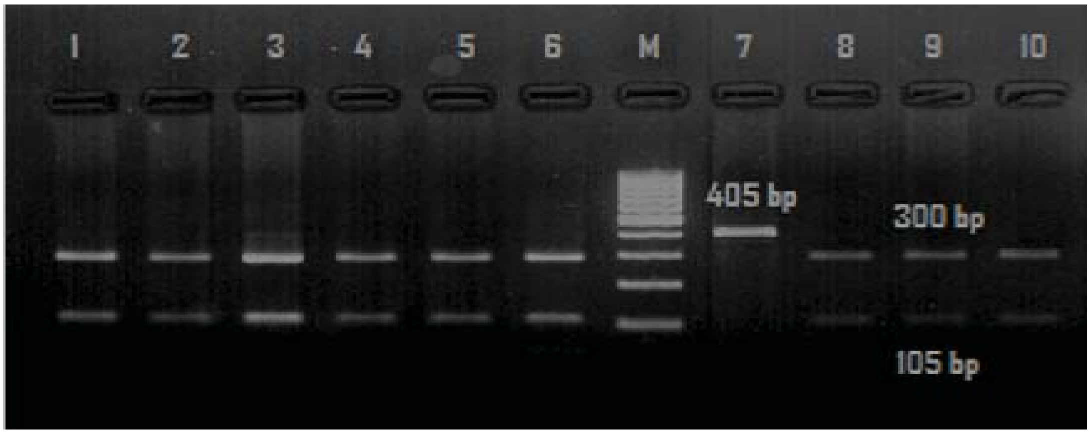

Figure 12. PCR-RFLP of primer II of the leptin gene on 2.5% agarose gel using *HphI* RE in Murrah buffaloes.

Lane 1-7 and 9-17 : 2 Bands (300 bp and 105 bp)
Lane 8 : PCR Product (405 bp)
Lane M : 100 bp Molecular Marker

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GGATTAGTGCCTTTCATTACTGTCATTTCTAGACAATGAATTGTCTTTGAGGAGATGATAGCCATGGCAGACAGC
AAATCTC(A/G)TTGTTATCTGCATCCGAAGACGTGGATGCGGGTGGTAACGGATCACATGGGTGTTCTCTGAGA
TCGGCGACG(G/T)GCCACGTGTGGTTTCTTCTGTTTTCAGGCCCCAGAAGCCCATCCCGGGAAGGAAAATGCGC
TGTGGACCCCTGTA(C/T)CAATTCCTGTGGCTTTGGCCCTATCTGTCCTACGTGGAGGCTGTGCCCATCCGCAA
GGTCCAGGATGACACCAAAACCCTCATCAAGACGATTGTCACCAGGATCAATGACATCTCACACACGGTAGGGAG
GGACTGGGAGATGAGGTAGAACTGTGGCCATCCCTGGGGGAACCCCCAGAGGGC
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Figure 13. Clustal W alignment with reference sequence of the leptin gene (Exon-2) in Murrah buffalo depicting SNPs in green.

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TGTCACATGGGCACAAGAAGTAAGGGCCCAGGGAGGATGGTGTGGAAGTGGGGAGGAAGAACCTCTATGCTCTAG
GGAAAGGCAGAGTCAGGGGAGCTCTGAGGAGCTGCCCTCTCTCCCACTGAGCTCTTGCCCTCCCCTTCCTCCTGC
ATAGCAGTCCGTCTCCTCCAAACAGAGGGTCACTGGTTTGGACTTCATCCCTGGGCTCCACCCTCTCCTGAGTTT
GTCCAAGATGGACCAGACATTGGCGATCTACCAACAGATCCTCACCAGTCTGCCTTCCAGAAATGTGGTCCAAAT
ATCTAATGACCTGGAGAACCTCCGGGACCTTCTCCACCTGCTGGCCGCCTCCAAGAGCTGCCCCTTGCCGCAGGT
CAGGGCCCTAGAGAGTTTGGAGAGCTTGGGCGTCGTCCTGGAAGCCTCCCTCTACTCCACCGAGGTGGTGGCCCT
GAGCCGGCTGCAGGGGTCACTACAGGACATGTTGCGGCAGCTGGACCTCAGTCCTGGGTGCTGAAGCCTTGAAGG
CCTCTCTTCCCAAAGTCCAGGGAAGAAACCTGAGCTTCTGGCTGTCCACAGGAGA
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Figure 14. Clustal W alignment with reference sequence of the leptin gene (Exon-3) in Murrah buffalo depicting SNPs in green.

sequences of the related species available in NCBI Gene Bank viz Bovine, caprine using the 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 the bubaline leptin gene. The leptin gene in the Murrah buffaloes included in present study is monomorphic as revealed by PCR-RFLP analysis using *AluI, AciI, MspI, Sau3AI, HphI* restriction enzymes. Thus the monomorphic pattern of leptin gene in buffaloes may be a species specific characteristics of buffalo. Eight SNPs were identified while comparing the bubaline leptin gene with the leptin genes of other species. There exists 97%, 97%, 99%, 98% and 80% sequence homology with *Bos taurus, Bos indicus, Ovis aries, Capra hircus* and *Homo sapiens*, respectively.

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