Explor. Anim. Med. Res., Vol.2, Issue - 1, 2012, p. 7-17

ISSN 2277- 470X

IDENTIFICATION OF POLYMORPHISM IN LEPTIN GENE OF MURRAH BUFFALO BY PCR-RFLP

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ABSTRACT: Present study was undertaken with the objective to identify the genetic variation in exon 2 and exon 3 of leptin gene and to determine the association of variants with milk production and fat% in Murrah buffaloes. Genomic DNA was isolated from 150 lactating Murrah buffaloes. The targeted segment was amplified by PCR using oligonucleotide primers and respective amplicons of 289 bp and 405 bp size comprising of exon 2 and exon 3 were obtained. PCR-RFLP was carried out using a battery of restriction endonucleases *viz. AluI, AciI, MspI, PvuII, HindIII, HinfI, Eco32I, Eco147I* and *Kpn2I* to explore genetic variation. Data were recorded pertaining to parity-wise milk production and fat%. Results indicated monomorphic patterns of both the exons of leptin gene in Murrah buffalo. Data on milk yield and fat percentage were recorded. However, association study was not possible due to monomorphic exhibition of the gene in all the animals included in the study.

Key words: Leptin gene, PCR-RFLP, Genetic polymorphism, Murrah.

INTRODUCTION

Leptin, the name derived from the Greek word leptos, meaning 'thin' is a 16 kD 167-amino acid protein which is synthesized by adipose tissue. This is involved in regulation of feed intake, energy balance, fertility and immune functions (Fruhbeck *et al.* 1998). It is one of the most useful biomolecule to act 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 and Hallas 1998). The Leptin gene spans about 18.9 kb consisting of three exons and two introns of which the first exon is not transcribed into protein. Leptin gene

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has been mapped to chromosome 7 in human (Green *et al.* 1995) and Chromosome 4 in bovine (Stone *et al.* 1996). In buffaloes, the Leptin gene is located on chromosome 8 (Vallinato *et al.*, 2004). Leptin is one of the important candidate genes in dairy animals associated with traits related to milk production and milk composition. Exon 2 region of leptin gene has been reported to be highly polymorphic in cattle (Wilkins and Davey 1997, Yoon *et al.* 2005). Genetic polymorphism with significant association to milk yield, live weight, energy balance, feed intake and fertility were first reported in cattle (Liefers *et al.* 2002, Buchanan *et al.* 2002).

Buffaloes contribute about 54 per cent of the total milk produced in India. Although the economic importance of buffaloes has always been known, yet very little research work has been carried out to exploit the genetic potential of this important dairy animal. Though studies have been carried out on association studies between leptin gene polymorphisms but a little amount of information about polymorphism and association studies of leptin gene with phenotypic traits like milk production, fat percentage, protein yield etc. has been reported in buffaloes. Hence the present study was undertaken with the objective to identify polymorphisms within exons 2 and 3 of leptin gene and its association with milk and fat yield in Murrah buffalo.

MATERIALS AND METHODS

Animals

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

Sample and Data Recording

Blood samples were collected in vacutainers (Bacton-Dickinson vacutainer system) containing sodium EDTA as an anticoagulant from 150 lactating Murrah buffaloes maintained at National Dairy Research Institute, Karnal. Data on milk yield and fat percentage for 1st, 2nd, 3rd and 4th lactation were collected from the records maintained at cattle yard of the institute.

DNA Extraction

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 DNA samples devoid of smear were used for further study. The quality of DNA was checked on 0.8% agarose and quantity by UV spectrophotometer at A_{260}/A_{280} nm. The samples having OD ratio between 1.7-1.9 were considered good and used for polymerase chain.

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 (MJ Research) using the following program: For the amplification of 2nd exon,

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

25 µl of PCR reaction mix contained 3.000 µl genomic DNA (50 ng/ul), 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 MixTM (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. The primers designed, regions amplified, annealing temperatures and product sizes are given in Table 1.

RFLP and Agarose Gel Electrophoresis

Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) technique was applied to explore the polymorphism in leptin gene. The restriction digestion of the PCR products were carried out with a battery of restriction enzymes (Fermentas) *viz. Alu*I, *Aci*I, restriction enzymes (0.07 µl of 10 U/ µl) and *MspI, 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 restriction digestion was kept for incubation at 37^0C temperature for 4 hours. The reaction was stopped by adding 0.5 M EDTA. Restriction fragments were resolved on 2-3% agarose gel electrophoresis and were visualized by ethidium bromide staining. The ethidium bromide 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 30, 60, and 90 minutes till complete separation of all fragments of restriction digested gene fragments and DNA marker. The restriction digested gene fragments were visualized on UV transilluminator and photographed with gel documentation system.

RESULTS AND DISCUSSION

DNA Extraction

The overall yield of DNA in Murrah buffalo ranged from 350-510 µg with a mean of 414.64± 4.87µg/ml and the overall purity of DNA ($OD_{260/280}$) ranged from 1.70-1.90 with a mean of 1.80±0.01.

Identification of Genotypes

The PCR amplification generated a 289 bp for exon II (Fig.1) and 405 bp for exon III segment (Fig. 2) for leptin gene of buffalo. Bubaline leptin gene is homologous to the cattle leptin gene of similar length (Ji *et al.* 1998), thus it indicates conservation of DNA sequences in both species.

PCR-RFLP of Leptin gene

Size of various electrophoretic bands observed by PCR-RFLP analysis of leptin gene with various restriction enzymes in Murrah buffaloes are given in table 1.

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

AluI digestion of amplified product of 2nd exon revealed two products of 189 & 100 bp (Fig. 3). *AciI* digestion of amplified product of 2nd exon revealed two products of 89 & 200 bp (Fig. 4). *MspI* digestion of amplified product of 2nd exon revealed two products of 79 & 210 bp (Fig. 5).

AluI digestion of amplified product of 3rd

Table 1: PCR-RFLP analysis of Leptin gene

REs used	Contig 1 (289 bp)		Contig 2 (405 bp)	
	Restriction Fragments	Pattern	Restriction Fragments	Pattern
AluI	189 and 100 bp	Monomorphic	55 and 350 bp	Monomorphic
AciI	89 and 200 bp	Monomorphic	135 and 270 bp	Monomorphic
MspI	79 and 210 bp	Monomorphic	255 and 150 bp	Monomorphic
HindIII	289 bp	-	405 bp	-
HinfI	289 bp	-	405 bp	-
Eco32I	289 bp	-	405 bp	-
Eco147I	289 bp	-	405 bp	-
Kpn2I	289 bp	-	405 bp	-
PvuII	289 bp	-	405 bp	-

exon revealed two products of 55 & 350 bp (Fig. 6). *AciI* digestion of amplified product of 3rd exon revealed two products of 135 & 270 bp (Fig. 7). *MspI* digestion of amplified product of 3rd exon revealed two products of 255 & 150 bp (Fig. 8).

Leptin gene in Murrah buffaloes included in present study is monomorphic as revealed by PCR-RFLP analysis using *AluI, AciI, MspI,* 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 above enzymes in leptin gene of cattle and other species. Adikari (2006) has also reported monomorphism in coding region of leptin gene using PCR-SSCP technique.

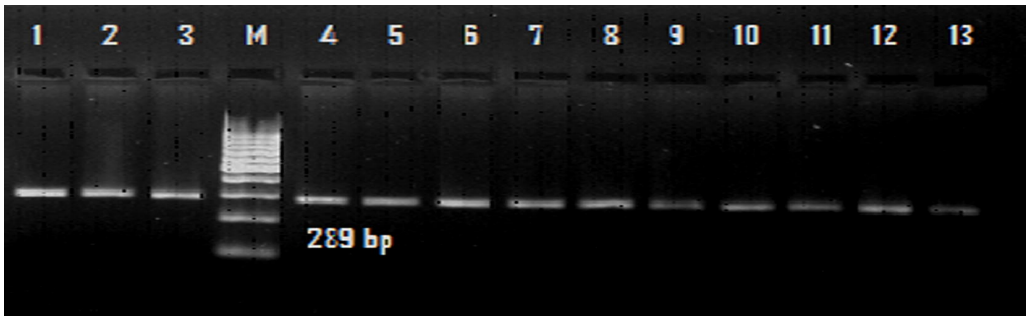
It might be because of the fact that the animals were in closed herd. Similar monomorphism of this gene in cattle was also observed by others in bubaline leptin gene. Kumar *et al.* (2003) reported the absence of polymorphism within 522 bp PCR product of leptin gene in buffalo digested with *HinfI* restriction enzyme. However, Javanmard *et al.* (2005) have reported three genotypes AA, AB and BB with genotypic frequencies as 0.100, 0.100 and 0.800 respectively in Iranian buffalo. All buffalo animals investigated in the present study are genotyped as AA where all the tested buffalo DNA amplified fragments were digested with *Sau3*AI endonuclease and gave one undigested fragment at 400 bp. Thus, the A gene and AA genotypic frequencies were found to be 1.00 respectively. Therefore, this monomorphism of the buffalo may be a species specific characteristic of buffalo.

However in cattle (*Bos taurus*), polymorphism was detected for leptin gene by a number of researchers. 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. Choudhary *et al.* (2005) reported polymorphism in crossbred cattle using *Bsa*AI and *Kpn*2I-RFLP. Kulig *et al.* (2009) investigated how leptin gene polymorphisms affect 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 *Sau3*AI RE. RFLP polymorphisms within the bovine leptin gene were detected by using *Hinf*I restriction enzyme and it was found that A allele positively affected milk production traits in Friesian cattle and they indicate significant superiority of allele A over allele B for milk and milk protein yields and body conformation traits (Khaleel *et al.* 2009).Whereas, Vohra *et al.* (2011) reported that Karan Fries cattle with TT genotype showed significantly higher 305 days milk yield as compared to cattle with CC genotype

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 T allele.

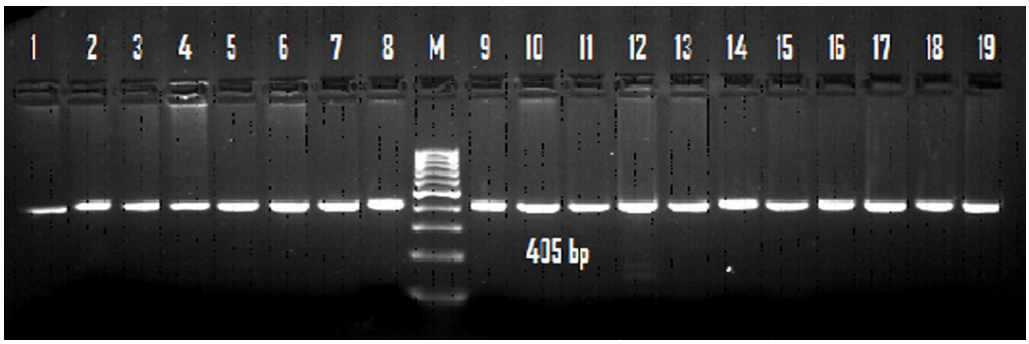
Buchanan *et al.* (2003) genotyped 416 Holstein cows by using restriction enzyme *Kpn*21 and compared lactation performance data using a mixed model. Animals homozygous for the T allele produced more

Fig.1: Resolution of PCR amplified product of Exon II on 1.5% agarose gel



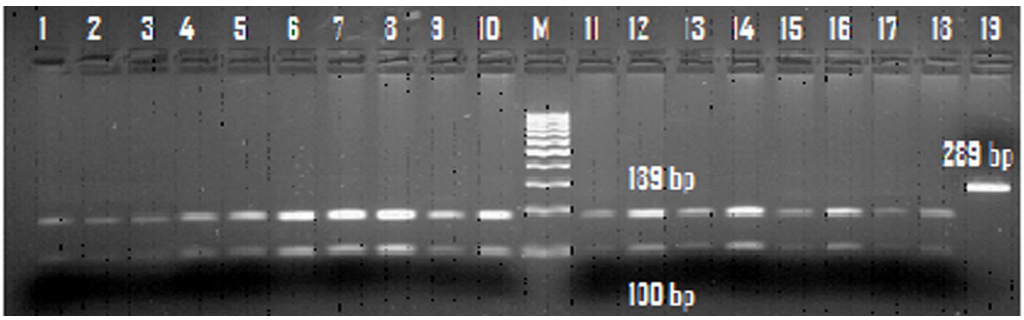
Lane 1-9 and 10-19 : PCR product (289 bp)
Lane M : 100bp Molecular Marker

Fig. 2: Resolution of PCR amplified product of Exon III on 1.5% agarose gel



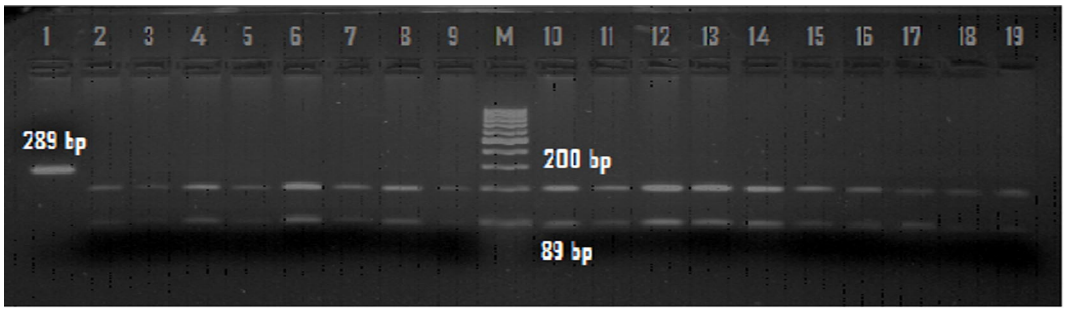
Lane 1-8 and 9-19 : PCR product (405 bp)
Lane M : 100bp Molecular Marker

Fig. 3: PCR-RFLP of primer 2 of Leptin gene on 2.5% agarose gel using AluI RE in Murrah buffaloes



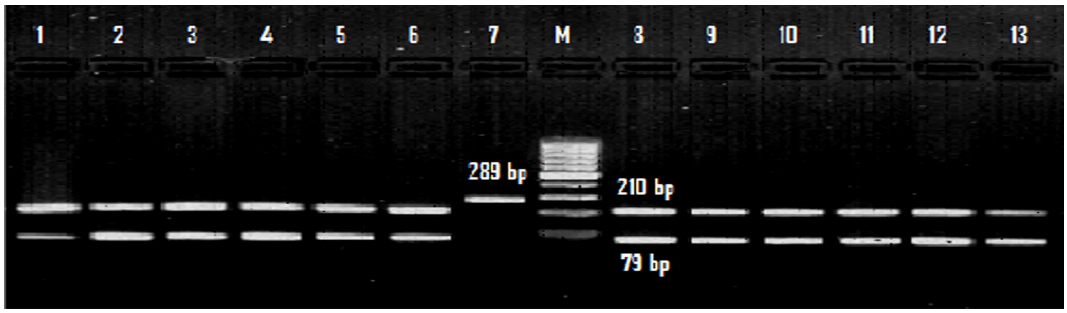
Lane 1-10 and 11-18 : 2 Bands (100 bp and 189 bp)
Lane 19 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

Fig. 4: PCR-RFLP of primer 2 of Leptin gene on 2.5% agarose gel using AciI RE in Murrah buffaloes



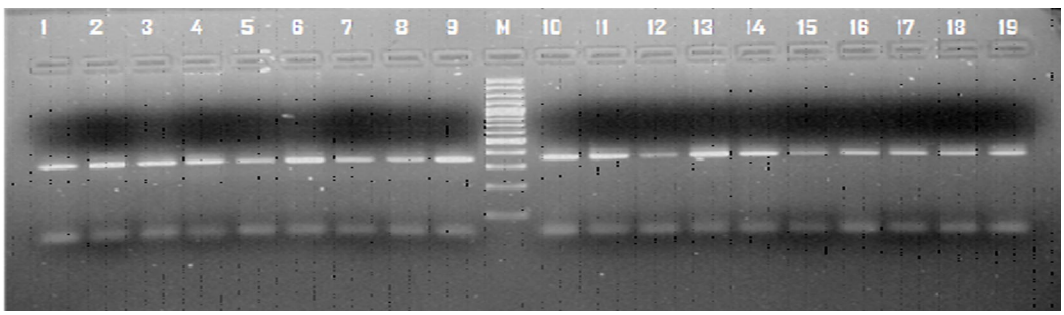
Lane 2-9 and 10-19 : 2 Bands (200 bp and 89 bp)
Lane 1 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

Fig. 5: PCR-RFLP of primer 2 of Leptin gene on 2.5% agarose gel using MspI RE in Murrah buffaloes



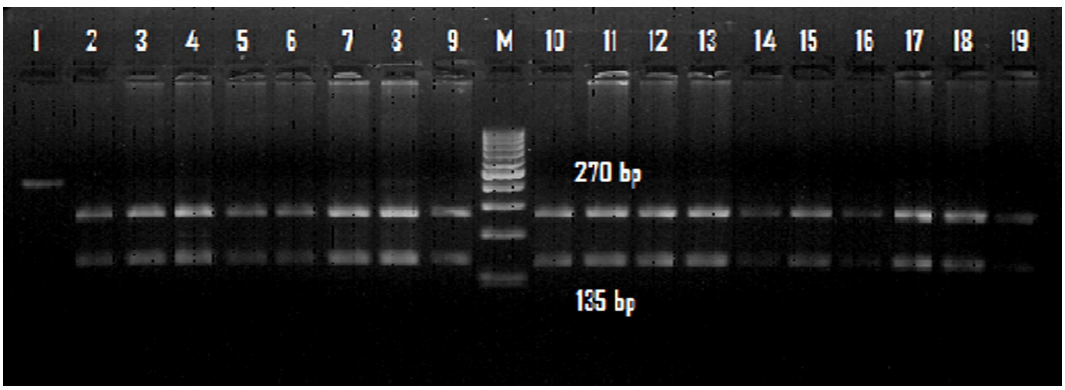
Lane 1-6 and 8-13 : 2 Bands (210 bp and 79 bp)
Lane 19 : PCR Product (289 bp)
Lane M : 100 bp Molecular Marker

Fig. 6: PCR-RFLP of primer 3 of Leptin gene on 2.5% agarose gel using AluI RE in Murrah buffaloes



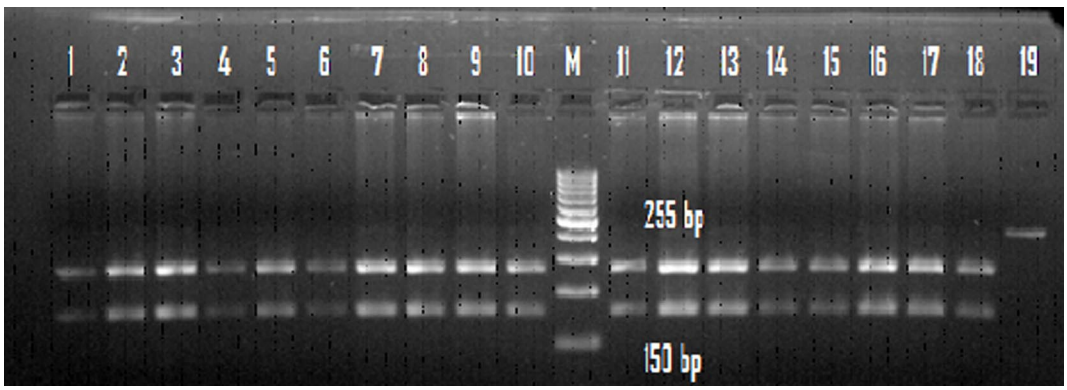
Lane 1-9 and 10-19 : 2 Bands (55 bp and 350 bp)
Lane M : 100 bp Molecular Marker

Fig. 7: PCR-RFLP of primer 3 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

Fig. 8: PCR-RFLP of primer 3 of 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

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 A and V alleles, respectively. However, they have reported monomorphic pattern in Sahiwal cattle. Since no mutation was found in Sahiwal cattle and only A allele was present throughout the population studied, the frequency of A allele was1.

Association with milk production and fat % data pertaining to milk production and fat% were recorded (Table 2) since 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: 2. Lactation wise maximum and minimum milk production and fat % in Murrah buffaloes included in present study.

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

CONCLUSION

Bovine leptin gene specific primers amplified the buffalo leptin gene and PCR amplification yielded amplicon of exon 2 and exon 3 of bubaline leptin gene. All Murrah buffaloes included in present study are monomorphic as revealed by PCR-RFLP analysis using *AluI, AciI, MspI,* restriction enzymes. Thus the monomorphic pattern of leptin gene in buffaloes may be a species specific characteristic of buffalo.

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