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Original Research

Exploring DNA Polymorphisms of Leptin Gene within Indian Water Buffaloes

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Abstract

Leptin is a 16-kDa protein secreted predominantly from white adipose tissue and performs important roles in controlling the body weight, feed intake, immune function, production and reproduction. In present study, buffalo leptin gene was analyzed using DNA markers for polymorphism and its association with production traits. The PCR-RFLP analysis of leptin gene using BsaAI, Sau3AI and Kpn2I restriction enzymes revealed monomorphism. The PCR-SSCP followed by DNA sequencing of Murrah buffalo leptin gene spanning exon III revealed two SNPs viz., c.25 T>C and c.316A>G, which were used to genotype the Murrah, Surti and Bhadawari populations. The c.25 T>C allele frequencies was found 0.81 and 0.19 for C and T in Murrah; 0.65 and 0.35 for C and T in Surti and 0.65 and 0.35 for C and T in Bhadawari buffaloes respectively. The c.25T>C genotypes were not in Hardy-Weinberg proportions, however c.316A>G genotypes maintained equilibrium at this locus in the populations studied. The c.316A>G SNP allele frequencies were observed in Murrah 0.31 and 0.69 for A and G; Surti 0.3 and 0.7 for A and G and Bhadawari 0.3 and 0.7 for A and G respectively. The Murrah buffalo leptin allelic variant sequences revealed 99 % similarity with cattle sequence. The statistical analysis using general linear model procedure indicated that Murrah buffalo leptin c.25 T>C and c.316A>G SNP genotypes were not related significantly (P>0.01) with Murrah buffalo milk production traits viz; milk yield, fat percentage and SNF percentage.

Keywords: Leptin; DNA polymorphism; Buffaloes; PCR-RFLP; SSCP; DNA Sequencing

Introduction

In marker-assisted selection of dairy livestock, some genes are proposed as potential candidates associated with dairy performance traits. Recent developments in molecular biotechnologies have opened the possibility of identifying and using genomic markers and multiple genes for the genetic improvement of livestock (Margawati, 2012). It is being proposed by researchers that candidate gene analyses can be used to identify individual genes responsible for traits of economic importance (Komisarek and Dorynek, 2009). Many studies have reported the genetic polymorphisms of candidate genes viz; Leptin, STAT5A, DGAT and Butyrophilin which has been implicated for part of milk production trait variability in cattle (Winter *et al*., 2003; Brym *et al*., 2004; Liefers *et al*., 2005; Kale and Yadav, 2011).

Leptin is a 16 kDa protein synthesized by adipose tissue (Zhang *et al*., 1994) which is involved intricately in feed intake, energy metabolism, growth and reproduction of cattle. In the bovine species, Leptin has been mapped to chromosome 4 (Stone *et al*., 1996). The availability of the polymorphic markers within and adjacent to leptin gene can facilitate genetic studies to determine specific role of leptin. There are various reports regarding associations between leptin gene polymorphisms and economic traits (Liefers *et al*., 2002; Buchanan *et al*., 2003; Liefers *et al*., 2005). The molecular genetics techniques focused on genome analysis have opened new possibilities for evaluation of complex economically important traits in farm animals. The restriction fragment length polymorphisms (RFLP) are first DNA markers reported for linkage studies (Botstein *et al*., 1980). The PCR-SSCP analysis (Orita *et al*., 1989) is a technique based on the principle that single-stranded DNA molecules form specific sequence-based secondary

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structures under non-denaturing conditions.

The buffalo is the most important farm animal species in Asia, especially India, where it is extensively used for milk, meat, fuel and fertilizer production, as well as for draught power (Borghese, 2005). The Riverine buffalo contributes significantly to Indian agriculture economy. Nearly 55 % of milk production in India comes from buffaloes (FAO, 2007). Indian buffaloes are hardy, thrive well on poor quality nutrition and are good converter of inputs into milk as compared to cattle (Biswas *et al.*, 2003). However, their inherent potential for growth and production has not been exploited due to inadequate information about genetic basis and breeding strategies. River buffalo and domestic cattle are closely related because they belongs to same bovidae family, cytogenetically characterized, shares homology in chromosome banding (Gallagher and Womack, 1992) and gene mapping (Di Meo *et al*., 2005). The comparative genomics approach with various DNA marker techniques might prove feasible approach in analysing buffalo genome. Therefore the present work has been carried out to analyse Leptin gene polymorphism using PCR-RFLP and PCR-SSCP in riverine buffaloes (*Bubalus bubalis*). The association study was carried out to find possible relationship (if any) with milk production traits in Murrah buffaloes.

Materials and methods

Experimental Animals and DNA Extraction

The leptin gene analysis was conducted using primer LEPE3 on 65 Murrah, 10 Surti and 10 Bhadawari breeds of buffaloes. Similarly the analysis of the other region using LEP3C, the blood samples were collected from 42 Murrah,10 Surti and 10 Bhadawari buffaloes from Haryana and Punjab, Gujarat and Utter Pradesh and Madhya Pradesh states of India respectively. Blood samples (10 ml) were collected by jugular veinipuncture using vacuum tubes containing acid citrate dextrose solution (ACD) as an anticoagulant. Besides, 50 ml of milk was collected from each animal to estimated 305 days lactation milk yield, milk fat percentage and SNF percentage and total protein percentage. Genomic DNA isolation was performed from blood using the phenol chloroform extraction protocol (Clamp *et al*.,1993) with some modifications. All stock DNA samples were kept at -80°C for longer storage, and the working aliquots were maintained at -20°C.

PCR Amplification

The Leptin gene primers reported in cattle were used to screen the PCR-RFLPs in buffaloes (Table1). The other PCR primers (Table 1) for PCR-SSCP analysis were designed on the basis of cattle GenBank sequence encompassing polymorphic restriction site using PRIMER3 software (Rozen and Skaletsky, 1998). The polymerase chain reaction (PCR) was carried out on about 100 ng/µL of genomic DNA in a 25 µL reaction volume. The reaction mixture consisted of 2.5 µL of 10x PCR assay buffer containing 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 0.75 U Taq DNA polymerase and 10 pM of each primer (Integrated DNA Technologies, Inc). Amplification was carried out using PCR cycling conditions as (95°C for 5 min) and 34 cycles of 45 seconds at 95°C, annealing temperature (TA°C) and 72°C consecutively, followed by a five minute final extension at 72°C. In a process of PCR amplification annealing temperature (TA) was optimised for each primer sequence (Table 1). The amplified products (5 µL) were detected on 2% agarose gel using 1 µL of loading dye as a stop dye, electrophoresed and visualized using UV light after ethidium bromide staining.

PCR-RFLP Analysis

The PCR-RFLP polymorphism analysis of Leptin gene fragments was performed by using restriction enzymes to identify PCR-RFLP in buffaloes. In this PCR amplified DNA products (15 µL) with distilled water (4.75 µ L) were subjected to RE digestion with restriction buffer (2.5 µL) and respective restriction enzymes (0.5 µL). The restriction enzymes used in respect to the primers are given in the Table 1. The mixtures were digested at 37°C for 3 hours visualised bands under UV transilluminator.

PCR-SSCP Analysis and DNA Sequencing

The Leptin gene PCR products were resolved by SSCP in which various factors were optimized as amount of PCR product (5µL), acrylamide-bisacrylamide concentration (12%), glycerol (10%), voltage (200 volts), electrophoresis run time (12 hours)

Table 1. Details of primer sequences used for buffalo leptin gene polymorphism analysis

Name of Primer	Primer	Sequence	T^A / RE
LI2C	FP	5'-GTCTGGAGGCAAAGGGCAGAGT-3'	65°C/ BsaAI
	RP	5'-CCACCACCTCTGTGGAGTAG-3'	
LE2A	FP	5'-ATGCGCTGTGGACCCCTGTATC-3'	63°C/ Kpn2I
	RP	5'-TGGTGTCATCCTGGACCTTCC -3'	
LI2A	FP	5'- GTCACCAGGATCAATGACAT- 3'	58°C/ Sau3AI
	RP	5'- AGCCCAGGAATGAAGTCCAA- 3'	
LEPE3	FP	5'-CAATGACCTGGAGAACCTC-3'	62°C/ exon3
	RP	5'-ACATAGGCTCTCTTCTCCTGT-3'	
LEP3C	FP	5'-CCCTCTCTCCCACTGAGCTC-3'	62°C/ exon3
	RP	5'-TAAAGGATGCCCACATAGGC-3'	

and temperature (20°C). The PCR products were diluted in denaturing solution and heat denatured at 95°C for ten minutes. The PCR products were resolved on a non-denaturing acry1amide:bis-acrylamide (49:l) gel. The vertical gel electrophoresis was carried out in a Bio-Rad Protean® II Xi Cell electrophoreses unit using 1X TBE buffer at 10-12.5 volts/cm for 12 hr at room temperature. Gels were silver-stained (Sambrook and Russell, 2000) and photographed using digital camera. The PCR products representing different SSCP patterns in Murrah buffalo were directly got sequenced and nucleotide sequence alignments were carried out using alignment tools viz; Geneious software, ClustalW and BLAST. The DNA sequence polymorphism observed were used to genotype riverine buffalo populations.

Statistical Analysis

The frequency of polymorphic alleles, genotypes and their accordance with Hardy-Weinberg law was assessed by POPGENE 1.31 software (http://www.ualberta.ca/~fyeh). The association between polymorphic allelic variants of Leptin genes and milk production traits were analysed using GLM procedure (SYSTAT). The following model was used: $Y_{ijkl} = \mu + g_i + s_i + p_j + h_k + e_{ijkl}$
Y_{ij}: observation on j^{th} animal i^{th} genotype, μ: population mean, g_i: effect of i^{th} genotype (i=1, 2), s_i: effect of i season, p_j: effect of j parity, h_k: effect of k year, e_{ijkl}: random error

Results

PCR-RFLP Analysis

The digestion of 522 bp fragment of Murrah buffalo intron 2 of leptin gene with 10 U of BsaAI enzyme yielded uncut fragment indicating the frequency of AA genotype as 1. The polymorphic restriction site was absent in Murrah buffaloes studied exhibiting monomorphic pattern in the studied population.

The restriction digestion of 1820 bp intron 2 fragment using 3 U of Sau3AI enzyme exhibited AB genotype (Fragments of 730, 690, 400, 310 and 90) in all the animals indicating the absence of polymorphic restriction site for the Sau3AI enzyme in this gene fragment.

The 94 bp region of exon 2 of Murrah, Surti and Bhadawari leptin gene was amplified and digested using 10U of Kpn2I enzyme, which yielded uncut fragment indicating uncut T allele with 1.0 gene frequency. The polymorphic restriction site was absent in Murrah buffaloes exhibiting monomorphic pattern in the studied population.

PCR-SSCP Analysis

The PCR-SSCP analysis of exon 3 of leptin gene using primers LEPE3 and LE3C (Table 1) resulted in two different SSCP patterns each i.e. A and B. Since no homo or heterozygotes can be distinguished SSCP patterns were simply indicated by A and B alphabets. The SSCP patterns for LEPE3 and LE3C primers were best resolved in 12% and 20% acrylamide-bisacrylamide respectively with other conditions kept same viz. voltage (200 volts), temperature (25°C), time (8hrs) with 10% glycerol. The SSCP analysis of amplified exon 3 fragment of the leptin gene using primer LEPE3 resulted in two different patterns viz; A and B (Figure 1) with the following frequencies in 65 Murrah (A = 0.38, B = 0.62). The SSCP pattern frequency in Surti was A = 0.7 and B = 0.3 and in Bhadawari (A = 0.7

and B = 0.3). The LEP3C amplified fragments also exhibited two SSCP patterns in riverine buffaloes studied (Fig. 1). The SSCP pattern frequency in 42 Murrah was, A = 0.31 and B = 0.69 and in Surti and Bhadawari it was, A = 0.3 and B = 0.7. This study revealed polymorphic nature of exon 3 region of leptin gene in riverine buffaloes.

DNA Sequencing and Genotyping

The gene fragments amplified using LEPE3 and LEP3C primer revealed two SSCP patterns in Murrah, Surti and Bhadawari breeds of buffalo. The PCR product representing polymorphic SSCP patterns in Murrah were got directly sequenced using automated DNA sequencing services (Bangalore Genei). The nucleotide sequence analysis using alignment tools (Geneious software) revealed one SNP (T-C substitution) in exon 3 at 25th nucleotide position (c.25 T>C) within Murrah leptin gene (Fig. 1). The nature of mutation was T-C transversion between SSCP pattern A and B confirming them as variants (A and B). The nucleotide sequences arising out of polymorphism were submitted in GenBank (EU078405, EU030441). The DNA sequence analysis of PCR products amplified using primer LEP3C revealed one SNP (A>G substitution) in exon 3 at 316th nucleotide position (c.316A>G) within Murrah buffalo leptin gene. The nucleotide sequences arising out of this polymorphism were submitted in GenBank (EU194869, EU199796). The identified experimental SNPs viz. c.25T>C and c.316A>G in exon 3 of Murrah leptin gene were used to genotype the Murrah, Surti and Bhadawari populations (Table 2). The c.25T>C genotypes were not in Hardy-Weinberg proportions however c.316A>G genotypes maintained equilibrium at this locus in the studied population.

Comparison of sequences of polymorphic leptin allelic variant sequence of buffaloes with cattle

The polymorphic Murrah leptin allelic variants sequences (EU078405, EU030441) identified using LEPE3 primer were compared with Bos taurus reference sequence using alignment tool (Geneious Software). It revealed five computational SNPs viz; C-T at 25th, 67th, 181st and T-C at 85th and 97th nucleotide position. The Murrah leptin allelic variant sequence (EU078405, EU030441) was found to have 99 % pairwise similarity with cattle sequence (AJ132764). The polymorphic Murrah leptin allelic variants sequences (EU194869, EU199796) identified using LEP3C primer were compared with *Bos taurus* reference sequence using alignment tool (Geneious Software). It revealed six computational SNPs viz; C-T at 134th, 221st, 335th; T-C at 239th, 251st and G-A at 316th position of nucleotide sequence. The Murrah leptin allelic variant sequence (EU194869, EU199796) was found to have 99 % similarity with cattle sequence (AJ132764).

Association of leptin allelic variants with milk production traits in Murrah buffaloes

In the marker assisted selection of dairy animals some genes are proposed as potential candidates associated with dairy performance traits. It is believed that SNPs occurring within such genes may influence the milk production trait or at least be an effective DNA marker of a subregion of the dairy animal genome. In current study, the results of variance analysis indicated that the effect of leptin allelic variants (c.25T>C and c.316A>G) on milk yield, fat percentage and SNF percentage was found non-significant (P>0.01). The least square mean values of milk production traits not differing in their leptin c.25T>C and c.316A>G genotypes are given in Table 3. The effect of non-genetic factors viz. season, parity and year of calving on milk yield and SNF percentage were found to be non-significant (P>0.01) while their effect was significant (P<0.01) on fat percentage.

Discussion

In the present study PCR-RFLP analysis using BsaAI, Sau3AI and Kpn2I restriction indicated single restriction pattern in Murrah, Surti and Bhadawari breeds of riverine buffaloes. The results of this study did not agree with those of Lien *et al*. (1997) who for the first time described a guanine (G) to adenine (A) substitution in position 1620 in intron 2 of the leptin gene of Norwegian cattle identified by BsaAI digestion. Chaudhary *et al*. (2005) reported digestion of 522 bp PCR products with the BsaAI restriction enzyme revealed three genotypes in all the breeds of Bos indicus, Bos taurus and Jersey cattle. The bovine leptin gene polymorphism (Pomp *et al.*, 1997) using the enzyme Sau3AI revealed two alleles. The effect of Sau3AI

Table 2. The frequency of alleles in buffalo leptin locus, SNP c.25T>C and c.316A>G

Buffalo Breed	N	c.25T>C Allele Frequency		N	c.316A>G Allele Frequency	
		C	T		A	G
Murrah	65	0.81	0.19	42	0.31	0.69
Surti	10	0.65	0.35	10	0.3	0.7
Bhadawari	10	0.65	0.35	10	0.3	0.7

Table 3. Mean and standard error (SE) of milk production Traits In reference to Murrah Buffalo Leptin SNP, c.25T>C and c.316A>G

SNP Genotype	N	Milk Yield ± SE	FAT*±SE	SNF*±SE
c.25T>C CC	40	2052.87±290.38	0.297±0.004	0.315±0.001
c.25T>C CT	25	2421.37±280.95	0.294±0.004	0.315±0.001
c.316A>G AA	13	2143.51±276.02	0.292±0.004	0.315±0.001
c.316A>G GG	29	1989.04±268.07	0.293±0.004	0.314±0.001

RFLP in the leptin gene was studied on bull breeding values for milk production trait in bovines (Madeja *et al*., 2004). Kulig (2005) reported statistically significant (P=0.01) relation between the leptin combined genotypes and milk, protein, and fat yield were observed. Chaudhary *et al*. (2005) reported that digestion of the 94 bp fragment with the Kpn2I restriction enzyme; three genotypes were observed in HF, Jersey and crossbred cattle. Similarly, Buchanan *et al.* (2002) reported that C allele cleaved in to two fragments 75 and 19 bp, while T allele remained uncut at 94 bp in Bos Taurus.

In current study the identified SSCP polymorphisms after direct DNA sequencing revealed two SNPs at positions 25 and 316 (EU078405; EU194869) in exon 3 of leptin gene indicating polymorphic nature of exon 3 of Murrah leptin gene. The large-scale comparative DNA sequencing could be the only method of accurately accessing the extent of sequence diversity (Haegeman *et al*., 2000). There are reports that Vallinoto *et al*. (2004) sequenced approximately 6 kb of the bubaline leptin gene including the 5' region, which revealed three SNPs and one microsatellite. The association between milk production traits and genetic markers are being investigated on wide scale. In present study, the identified SNP genotypes at leptin gene locus were not associated significantly with Murrah milk production traits. Similarly Liefers *et al*. (2002) reported that heifers with the RFLP1-AB genotype produce 1.32 kg/d more milk and consume 0.73 kg/d more food compared with the RFLP1-AA genotype. Buchanan *et al*. (2003) reported that animals homozygous for the T allele produced more milk (1.5 kg/d vs. CC animals) without significantly affecting milk fat or protein percent over the entire lactation. Liefers *et al*. (2005) reported that leptin promoter mutations were found to be associated with fertility, energy balance and protein yields. These studies suggest that leptin is established candidate genetic marker, which could be used to improve genetic potential of livestock.

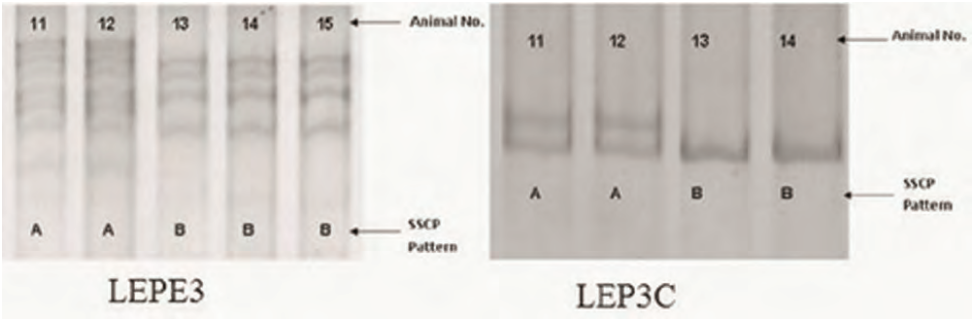

Fig. 1. PCR-SSCP using primer LEPE3 and LEP3C resolved on 12 and 20% non-denaturating PAGE respectively in Riverine buffaloes. where A and B: SSCP patterns

Conclusion

The present study revealed that PCR-SSCP followed by DNA sequencing is one of the effective molecular biological techniques to detect DNA sequence variation at candidate gene loci in buffaloes. The identified SSCP within exon 3 of Murrah leptin gene followed by DNA sequencing revealed two SNPs (c.25T>C and c.316A>G). The association of these identified allelic variants with Murrah milk production traits revealed non-significant effect on Murrah milk production traits. The possible reasons for non-significant effect might be due to the fact that sample size was small, absence of some genotypes, high standard error and uneven distribution of data. However, studies concerning analysis of leptin gene polymorphism and their relation with production traits in riverine buffaloes are fairly scarce. Nowadays leptin is emerging as established candidate genetic marker in bovines for production traits, it is necessary to screen all the regions of leptin genes and to continue association studies using large sample size to identify DNA marker for production traits in buffaloes.

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