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Sequencing, single nucleotide polymorphisms identification and development of genotyping tests for leptin gene in zebu cattle

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ABSTRACT

The present study was undertaken with the objectives of sequencing of *Bos indicus* leptin gene and identification of putative SNPs in entire gene. Nine novel SNPs and one reported SNP were identified in Sahiwal cattle population. Two polymerase chain reaction- restricted fragment length polymorphism (PCR-RFLP) tests were developed for SNP G to A in intron-1 and SNP C to T in exon-3 of leptin gene using DraI and MspI restriction enzymes, respectively. Allelic frequencies of G and A alleles were 71.29 and 28.71% and C and T alleles were 63.12 and 36.88% by DraI-RFLP and MspI RFLP, respectively, which reflects high variability, segregation of these putative DNA markers thus having ample scope of selection in this population. The MspI PCR-RFLP based DNA test for potential haplotype having 3 SNPs in exon-3 and the DraI DNA test with a single SNP in intron-1. Both newly developed DNA based tests and other identified SNPs could be of high relevance in association studies of Sahiwal zebu cattle of the region.

Key words: *Bos indicus,* Leptin gene, PCR-RFLP, SNPs

Interest in bovine leptin gene has increased during the last few years owing to its important role in productive and reproductive of cattle. The bovine leptin gene located at BTA 4q32 Leptin, the hormone product of the obese (leptin) gene located on BTA 4 (Stone *et al.* 1996, Pomp *et al.* 1997) is considered to play a role in the regulation of appetite, energy partition and body composition (Baile *et al.* 2000). This candidate gene is involved in growth and metabolism of animals and plays an important role in regulation of feed intake, immune function, growth, milk yield and reproduction of cattle. SNP concept has basically arisen from the need for very high densities of genetic marker for studies of complex traits. SNPs can provide valuable data on association between specific genes or other DNA structure and phenotypes, or on population and genome dynamics. Several studies in bovine *(Bos taurus*) leptin gene has described (Block *et al*. 2001, Liefers *et al.* 2002, 2003a, 2003b, Nkrumah *et al*. 2006, Sadeghi *et al*. 2008) involvement of the gene in complex traits. Choudhary *et al*. (2005), Dandapat *et al*. (2010) and Vohra *et al.* (2011) reported presence of *Sau*3AI, *Hph*I and *Aci*I RFLP, respectively, only in crossbred cattle absence of these polymorphisms in *Bos indicus*. Therefore, before any association study it is imperative to identify the putative SNPs and development of reliable test to screen the different genotypes in the population. The objectives of present study were to sequence, SNPs identification and to develop DNA based PCR- RFLP tests for leptin genotyping in *Bos indicus* cattle.

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MATERIALS AND METHODS

A panel of Sahiwal cattle (N, 202) was selected randomly from Uttar Pradesh (49), Uttarakhand (32), Punjab (39), Haryana (44) and Chhattisgarh (39) to include a diverse gene pool for polymorphism detection. Blood samples were collected from jugular vein into EDTA containing vacutainer tubes. DNA was extracted from whole blood following standard phenol–chloroform extraction method described by Sambrook and Russell (2001).

Five sets of primers were designed on the basis of available leptin gene sequence for *Bos taurus* under GenBank accession number BTU50365 (Table 1). These primers were utilized to sequence the complete cds (exon2, exon3) and partial 3'UTR region of the gene in *Bos indicus*. Another 5 sets of primers with SSCP polymorphic regions (previously published by the same groups, Dubey *et al*. 2008) of the gene, were used to identify the SNPs in *Bos indicus*. Polymerase chain reaction was carried out in a final reaction volume of 25µl, as described by the same group (Dubey *et al*. 2008).To

Table 1. Primers designed for sequencing of *Bos indicus* leptin gene

Primers	Primer sequence 5' to 3'	Annealing temperature	Nucleotide covered (BTU50365)	Product length (bp)
SQ1F	CTCTGGCCTTCAGGTCTTTG	62°C	75–1018	944
SQ1R	GTTACCACCCGCATCCAC			
SQ2 F	CACTGCTGCGTGGTCTACAG	62°C	836 –1737	902
SQ2 R	TCTATGGTTGCAGGTCTGAGC			
SQ 3F	TCAGACCTGCAACCATAGATGA	63°C	1719–2698	980
SQ3 R	TTTCTTCCCCAGGCATTGA			
SQ4 F	CTGCTTGGGGTGGAAGAGTT	65°C	2635–3497	863
SQ4 R	CAGCTGAGAGGAGCGAGAGA			
SQ5 F	CCCTGGAGAGCTTGGAGAG	62°C	3232–3963	732
SQ5 R	CCAGTCCCTGGAGAACAAACT			

Table 2. Primers used for SNP identification

Primers	Primer sequence 5' to 3'	Annealing temperature	Nucleotide covered (BTU50365)	Product length (bp)
LEPII F	ACATCCGTTGTTCACTGTGG	65°C	422–761	340
LEPII R	TGCAGGCATATCCCATAACC			
LEPIV F	GTGCCACGTGTGGTTTCTTC	65°C	1051–1260	210
LEPIV R	CCTCCCTACCGTGTGTGAGA			
LEPV F	ACACACGGTAGGGAGGGACT	65°C	1245–1589	345
LEPV R	GCTCAGTTACCAGGCAGGAA			
LEPXI F	GCTCTTGCTCTCCCCTTCCT	64°C	2977–3406	430
LEPXI R	GGTTTCTTCCCTGGACTTTGG			
LEPXIII F	CTGGGATTTTCACAGC GTCT	64°C	3601– 4031	431
LEPXIII R	TCGAGATCCATTCAGAGCAA			

Table 3. Single nucleotide polymorphisms (SNPs) identified in *Bos indicus* leptin gene

Primer/fragment Number	*Position (EU313203)*	*SNPs*	*Accession number*	Specific region
LEPII	520	G/A	EU366460–61	Intron-1
LEPIV	1047	C/T	EU366462-63	Exon-2, non synonymous mutation Arginine to Cystin (25)
LEPV	1385	G/C	EU366464-65	Intron-2
LEPXI	3126	T/C	EU366466-67	Exon-3
LEPXI	3138	T/C	EU366466-67	Exon-3
LEPXI	3222	C/T	EU366466-67	Exon-3
LEPXIII	3734	C/T	EU366468-69	3' UTR
LEPXIII	3739	G/A	EU366468-69	3' UTR
LEPXIII	3770	G/A	EU366468-69	3' UTR
LEPXIII	3772	C/T	EU366468-69	3' UTR

sequence the complete *Bos indicus* leptin gene, all 5 PCR products were cloned into the plasmid vector T4 and sequencing was carried out using a kit. Further to identify the putative SNPs, PCR product from each SSCP patterns were purified and sequenced.

To identify the putative SNPs, nucleotide sequences were aligned and compared with already reported leptin gene sequences using MEGALIGN programme of the Lasergene software package. To search the restriction enzyme site, online available NEB cutter software (http://tools.neb.com/NEBcutter2/index.php) was utilized. PCR products of intron-1 (LEPII, 340bp) and exon-3 (LEPIX, 430bp) fragments were digested as per manufacturer's protocols with *DraI* and *MspI* restriction enzymes respectively as follows: PCR reaction mixture 10µl, 10 × buffer Tango 2µl, *DraI* enzyme 1µl/*MspI* 1µl, nuclease free water 17µl (total reaction mixture 30µl /

Table 4. Allelic and genotypic frequency of leptin gene for *DraI*-RFLP and *MspI*-RFLP in Sahiwal cattle population

PCR-RFLP test	Genotypes	Frequency %	No. of animals	Alleles	Frequency %
DraI-RFLP	GG	52.97	107	GA	71.2928.71
	GA	36.63	74		
	AA	10.40	21		
Total	3	100%	202	2	100
MspI-RFLP	CC	40.59	82	CT	63.1236.88
	CT	45.05	91		
	TT	14.36	29		
Total	3	100%	202	2	100

sample). All the ingredients were mixed gently and spun down for a few seconds before incubation. The digested PCR products (5µl) with the loading dye (1µl) were run on 10% PAGE at 200 V for 4 h. The gels were silver stained as per Bassam *et al*. (1991). Both PCR-RFLP tests in intron-1 and exon-3 using *DraI* and *MspI* digestion, respectively, were utilized to screen all the genotypes for Sahiwal cattle population (N=202).The allelic and genotypic frequencies were calculated according to Falconer and Mackay (1996).

RESULTS AND DISCUSSION

In the present study, 3.897kb *Bos indicus* (Sahiwal cattle) leptin gene having full length open reading frame was sequenced (GenBank Accession number EU313203). BLAST analysis revealed 99, 96, 98, 99, 92, 95% and 84% homology with taurine cattle (*Bos taurus*), buffalo (*Bubalus bubalis*), yak (*Bos grunniens*), mithun (*Bos frontalis*), goat (*Capra hircus*), sheep (*Ovis aries*) and pig (*Sus scrofa*) respectively. The multiple sequence alignment revealed 4 nucleotide substitutions, one insertion and on deletion in *Bos indicus* as compared to *Bos taurus.* Sequencing of all SSCP variants resulted in 10 SNPs in entire gene and submitted to the GenBank EU366460 -EU366469.

PCR products of different DNA samples of Sahiwal for intron-1 and exon-3 in leptin gene were digested with *DraI* and *MspI* restriction enzymes, respectively, as per standard protocol. As a result 3 genotypes were found. For *DraI* namely GG homozygote of 340bp, AA homozygote of 232 bp and 108bp and heterozygote of 340bp, 232bp and 108bp (Fig.2a) and for *MspI* enzyme, 3 genotypes namely CC homozygote of 197bp, 132bp, 51bp and 50bp, TT homozygote of 197bp, 132bp and 101bp and CT heterozygote of 197bp, 132bp, 101bp, 51bp and 50bp were detected. All the animals (N=202) were screened using both newly developed PCR-RFLP tests. Allelic and genotypic frequency estimated with high range of genetic variability is presented in Table 3. Genetic polymorphism in the bovine leptin gene was described (Pomp *et al*. 1997, Haegeman *et al*. 2000, Buchanan *et al.* 2003). In the present study we are reporting leptin gene sequence with complete coding DNA sequence (CDs) in *Bos indicus* which was further used to identify the putative SNPs in entire gene (Fig. 2b).

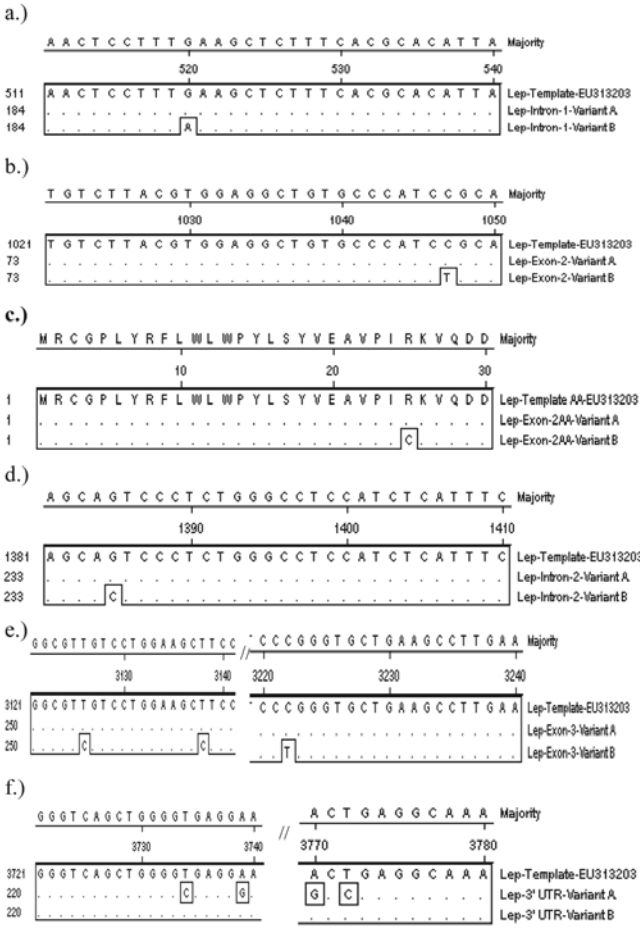

Fig. 1. Alignment of polymorphic regions, showing SNPs in leptin gene including amino acid sequence (Exon 2) with template EU313203; a. intron1; b. exon 2; c. amino acid sequence for exon2; d. intron2; e. exon3; f. 3' UTR region.

A total of 10 SNPs were identified which constitute 9 novel and 1 reported R4C SNP (Figs 1 c and d) in entire leptin gene. This R4C polymorphism is due to C→T change leads to amino acid change from arginine to cystin in exon-2 of leptin gene in *Bos taurus* (Buchanan *et al*. 2003). Dandapat *et al*. (2010) studied exon-2 polymophism for leptin gene in *Bos indicus* using *Hph*I RFLP and reported monomorphic result. Interestingly, we identified R4C SNP

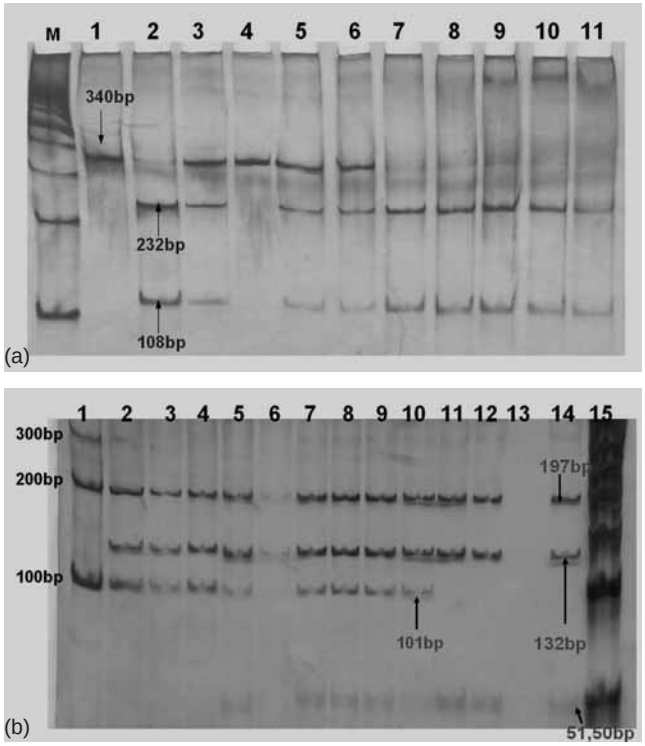

Fig. 2. PCR-RFLP tests developed for leptin genotypes in Sahiwal cattle *(Bos indicus).* a. for SNP G'!A (LEP II, intron-1) using DraI restriction digestion. M= 100bp ladder; Lane 1 and 4, GG genotype; lane 2 and 7–11, AA genotype; lane 3, 5 and 6, GA genotype. b. For SNP C'!T (LEP XI, third SNP of exon-3) using MspI restriction digestion. lane1,100bp ladder; Lane 2–4, 6,TT genotype; lane 5, 7–10,TC genotype; lane 11, 12 and 14,CC genotype; 13,negative and 15,50bp ladder.

in same region of the gene and also searched restriction site for enzyme *Aci*I to genotype the R4C polymorphism in cattle population by *in silico* analysis. Presence of this polymorphism and found to have association with 305 days milk yield in Karan Fries crossbred cattle has been reported (Vohra *et al*. 2011) and reflecting ample scope for further association study in Zebu cattle.

The frequency of allele A in *DraI*-RFLP was low (0.29), suggesting that there may be a selection force acting against the A allele or favoring the G allele in the population. In case of *MspI*–RFLP the frequency of allele T in Sahiwal cattle population was also low (0.37), suggesting that there may be a selection force acting against the T allele or favoring the C allele in the population. Chaudhary *et al.* (2005) also studied the polymorphism in exon-3 of the gene for *Bos indicus* and reported monomorphic result. Thus, we are reporting 2 new PCR-RFLP tests in *Bos indicus* cattle for leptin gene, which could be utilized for future breeding programme and conservation strategy for Sahiwal zebu cattle.

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