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Association of leptin gene polymorphism with economic traits in crossbred cattle

VIVEK CHOUDHARY[1], PUSHPENDRA KUMAR[2], CHINMOY MISHRA[3], TARUN KUMAR BHATTACHARYA[4], BHARAT BHUSHAN[5] and ARJAVA SHARMA[6]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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ABSTRACT

The present investigation was carried out to identify two polymorphisms in leptin gene by PCR-RFLP and to examine the possible association of the identified genotypes with growth, production and reproduction traits in 205 female crossbred cattle (½ Holstein Friesian × ½ Hariana). One fragment of 330 bp comprising partial intron 2 and exon 3, and another fragment of 94 bp comprising partial exon 2 of leptin gene were amplified, and digested with *Hph*I and *Kpn*2I restriction enzymes, respectively, for identification of genotypes. The animals with *Hph*I-RFLP-CT and *Kpn*2I-RFLP-AV genotypes had significantly higher birth weight than the *Hph*I-RFLP-CC and *Kpn*2I-RFLP-AA genotypes respectively. The *Hph*I-RFLP locus had significant effect on body weight at 12 months of age, age at first calving and average daily milk yield, while, *Kpn*2I-RFLP had significant effect on first lactation milk yield and average daily milk yield. Therefore, leptin gene could be used as a marker for genetic selection of economic traits in cattle.

Key words: Cattle, Crossbred, Economic traits, Gene, Leptin, PCR, Polymorphism

Leptin is a hormonal product of leptin (obese) gene secreted by the adipose tissue and plays a major role in the regulation of feed intake, energy metabolism and reproduction of animals (Trakovická *et al*. 2013, Dongre *et al*. 2014, Kononoff *et al*. 2017). Level of leptin circulating in blood stream is directly proportional to the total amount of body fat (Hossner 1998). The bovine leptin gene has been partially cloned and assigned to 4q32 chromosome (Stone *et al*. 1996). Various restriction fragment length polymorphisms have been detected in bovine leptin gene (Pomp *et al*. 1997, Lien *et al*. 1997, Haegeman *et al*. 2000, Sharifzadeh *et al*. 2010). Reports are also available on association of leptin genotypes with carcass traits in cattle (Fitzsimmons *et al*. 1998, Tessanne *et al*. 1999, Buchanan *et al*. 2002, de Carvalho *et al*. 2012, da Silva *et al*. 2012) along with very few reports on association studies with daily milk yield (Liefers *et al*. 2002, Buchanan *et al*. 2003, Dandapat *et al*. 2009, Clempson *et al*. 2011) and calving interval (Almeida *et al*. 2003). However, study on association of leptin genotypes with some other important economic traits such as birth weight, body weight at six months intervals up to 2 years of age and age at first calving have not yet been reported. The present investigation was planned to study the association of polymorphisms in leptin gene with various growth, production and reproduction traits in crossbred cattle.

Present address: [1]Assistant Research Scientist (vchoudhary @gru.edu), Augusta University, Augusta, GA, USA. [2]Principal Scientist and Head (pushpendra64@gmail.com), [5]Principal Scientist (bhushan.drbharat@gmail.com), Animal Genetics Division. [3]Assistant Professor (drchinmoymishra @gmail.com), Department of Animal Breeding and Genetics, College of Veterinary Science and Animal Husbandry, OUAT, Bhubaneswar. [4]National Fellow (bhattacharyatk@gmail.com), Directorate of Poultry Research, Hyderabad, Andhra Pradesh. [6]Ex-Director (arjava@yahoo.com), National Bureau of Animal Genetic Resources, Karnal, Haryana.

MATERIALS AND METHODS

The present study was undertaken on 205 female crossbred (½ Holstein Friesian × ½ Hariana) cattle from Cattle and Buffalo Farm of the institute. All the animals were recorded for growth traits i.e. birth weight (Bwt), body weight at 6 months of age (BW6M), body weight at one year of age (BW12M), body weight at 18 months of age (BW18M), body weight at two years of age (BW24M), daily body weight gain at 6 to 12 months of age (WG12), daily body weight gain at 12 to 18 months of age (WG18), daily body weight gain at 18 to 24 months of age (WG24); production traits i.e. first lactation milk yield (FLMY), average daily milk yield during first lactation (DMY) and reproduction traits i.e. age at first calving (AFC). The data was grouped under three seasons, i.e. summer (March-June), monsoon (July-October) and winter (November-February). Blood samples were randomly collected from animals during winter season in a 15 ml polypropylene tube containing ethylene diamine tetraacetic acid (EDTA) as an anticoagulant and kept in frozen state till the isolation of DNA.

The genomic DNA was isolated by phenol-chloroform extraction method (Sambrook and Russell 2001). A 330 bp fragment of leptin gene spanning over a part of intron 2 and a part of exon 3 was amplified using a set of forward (5'-GGG AAG GGC AGA AAG ATA G-3') and reverse (5'-AGG CAG ACT GTT GAG GAT C-3') primers. Another fragment of 94 bp of leptin gene comprising partial exon 2 was amplified using a set of forward (5'-ATG CGC TGT GGA CCC CTG TAT C-3') and reverse (5'-TGG TGT CAT CCT GGA CCT TCC-3') primers (Buchanan *et al*. 2002). The PCR programme for amplification of 330 bp fragment of leptin gene consisted of an initial denaturation for 5 min at 94°C followed by 35 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, extension at 72°C for 45 sec and the final extension for 5 min at 72°C. The programme for amplification of 94 bp fragment of leptin gene consisted of an initial denaturation at 94°C for 5 min, followed by a 35 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 45 sec, extension at 72°C for 30 sec and the final extension of 72°C was given for 5 min.

An aliquot of each 15 µL PCR product of 330 bp and 94 bp was digested overnight at 37°C with 5 units of *Hph*I and *Kpn*2I restriction enzyme respectively. The enzymatic reaction was stopped by adding 6× loading dye (0.25% bromophenol blue, 40% sucrose). The digested PCR products were electrophoresed in 4% agarose gel at 5–6 volts/cm for 2 h. Ethidium bromide was added to agarose gel (0.5 µg/mL of agarose gel solution) prior to its casting. After completion of electrophoresis, the gel was visualized under gel documentation system.

The genotypes were identified according to the restriction fragment patterns observed for both the RFLPs. The gene and genotype frequencies were estimated (Falconer and Mackay 1996). For effect of RFLP genotypes on various traits of economic importance, least squares analysis of variance (Harvey 1990) technique was used (Table 1). Mutant homozygotes were very few in number in both the RFLPs studied. Hence, they were ignored during the analysis as interpretation from such a scanty number of observations will not be statistically relevant. The number of animals analyzed under each trait varied with the availability of records on that particular trait. The following models were used for the analysis:

For growth traits:

$$Y_{ijklmn} = \mu + Sire_i + HphI\text{-}RFLP_j + Kpn2I\text{-}RFLP_k + Sbirth_l + b_1(Bwt)_m + e_{ijklmn}$$

where Y_{ijklmn} is the observation on n^{th} animal sired by i^{th} sire having j^{th} *Hph*I-RFLP genotype and k^{th} *Kpn*2I-RFLP genotype born in l^{th} season and having m^{th} regression of birth weight on the trait; µ is the overall mean of the population for the trait; $Sire_i$ is the random effect of i^{th} sire; $HphI\text{-}RFLP_j$ is the effect of j^{th} *Hph*I-RFLP genotype on the trait; $Kpn2I\text{-}RFLP_k$ is the effect of k^{th} *Kpn*2I-RFLP genotype on the trait; $Sbirth_l$ is the effect of l^{th} season of birth; $b_1(Bwt)_m$ is the regression of birth weight on the trait; e_{ijklmn} is the random residual effect. Model for birth weight was same as above except the $b_1(Bwt)$ factor, i.e. effect of regression of birth weight was not considered in the model.

RESULTS AND DISCUSSION

Digestion of the 330 bp PCR product with *Hph*I restriction enzyme revealed three different band patterns forming three genotypes. The AA genotype was having no *Hph*I restriction site with 330 bp size and AV genotype with 330 bp, 310 bp and 20 bp; VV genotype with 310 bp and 20 bp. The smaller fragment of 20 bp could not be resolved on the agarose gel. The exact size of the restricted fragments were estimated after online analysis of nucleotide sequence with leptin gene of HF cattle (NCBI accession no. AY534919). The digestion of 94 bp PCR product with *Kpn*2I restriction enzyme, showed three different band patterns indicating three genotypes, viz. CC genotype (75 bp and 19 bp), CT genotype (94 bp, 75 bp and 19 bp) and TT genotype (94 bp) (Fig. 2). The 19 bp fragment could not be resolved on agarose gel.

The allelic frequencies and genotype frequencies for both RFLPs were calculated (Table 2 and 3). In both the RFLPs, the frequency of mutant homozygotes (*Hph*I-RFLP-VV and *Kpn*2I-RFLP-TT) was very low (Table 2). Only CC and CT genotypes in exon 2 were reported earlier. This may be due to lack of polymorphism and presence of only wild

Table 1. Genotype-wise least squares mean (kg) and their standard error for various economic traits in crossbred cattle

Trait	*Hph*I-RFLP genotype		*Kpn*2I-RFLP genotype	
	AA	AV	CC	CT
Bwt (kg)	22.19±0.36 (89)	23.50±0.41* (84)	22.19±0.32 (119)	23.55±0.46* (54)
BW6M (kg)	81.94±2.66 (85)	81.42±2.73 (75)	79.92±2.58 (115)	83.44±2.89 (45)
BW12M (kg)	151.36±4.69 (84)	159.77±4.96* (76)	152.02±4.61 (111)	159.11±5.19 (49)
BW18M (kg)	237.70±6.46 (87)	234.96±6.85 (64)	236.38±6.42 (103)	236.28±7.09 (48)
BW24M (kg)	303.62±9.55 (77)	308.75±9.86 (73)	302.35±9.45 (103)	310.03±10.23 (47)
WG12 (kg)	0.383±0.015 (82)	0.409±0.017 (61)	0.381±0.015 (105)	0.411±0.018 (38)
WG18 (kg)	0.415±0.012 (83)	0.406±0.015 (59)	0.412±0.012 (100)	0.408±0.016 (42)
WG24 (kg)	0.380±0.016 (77)	0.387±0.018 (62)	0.371±0.016 (96)	0.397±0.020 (43)
FLMY (kg)	2146.86±121.53 (59)	2345.40±133.61 (49)	2060.43±117.42 (70)	2431.84±138.53* (38)
DMY (kg)	6.32±0.39 (55)	7.36±0.46* (44)	6.01±0.39 (69)	7.82±0.49** (30)
AFC (days)	951.09±15.81 (55)	888.05±19.91* (44)	916.87±14.47 (69)	922.26±22.42 (30)

Table 2. Allelic frequencies of *Hph*I-RFLP and *Kpn*2I-RFLP in crossbred cattle

Allele	*Hph*I-RFLP		*Kpn*2I-RFLP	
	A	V	C	T
Allelic frequency	0.74	0.26	0.82	0.18

Table 3. Genotypic frequencies of *Hph*I-RFLP and *Kpn*2I-RFLP in crossbred cattle

Genotypic	*Hph*I-RFLP			*Kpn*2I-RFLP		
	AA	AV	VV	CC	CT	TT
Genotypic frequency	0.53	0.42	0.05	0.68	0.27	0.05

allele (A) in the Hariana breed (Choudhary *et al*. 2003). Thus, Hariana animals contribute only wild allele to the crossbred population. The lower frequency of the mutant allele (V) in Belgium Blue crossbreds (0.13) and Indian crossbred (0.07) than the present finding was reported (Haegeman *et al*. 2000, Dandapat *et al*. 2009). The frequencies of T allele (*Kpn*2I-RFLP) was reported to be 0.58 in Angus breed, 0.34 in Charolais breed, 0.55 in Hereford breed and 0.32 in Simmental breed (Buchanan *et al*. 2002) in purebred taurine cattle and 0.17 in crossbred cattle (Kaplanova *et al*. 2009).

*Hph*I-RFLP and *Kpn*2I-RFLP loci of leptin gene had significant (P<0.05) association with the birth weight of animals. The *Hph*I-RFLP-AV heterozygotes showed higher birth weight than *Hph*I-RFLP-AA homozygotes (Table 1). Animals having *Kpn*2I-RFLP-CT genotype were heavier at birth than the animals possessing *Kpn*2I-RFLP-CC genotype (Table 1). Heterozygous animals that have an extra alanine in their leptin gene tend to have more weight at birth which is similar to earlier finding (Dandapat *et al*. 2009). The mutation in exon 2 of leptin gene (94 bp *Kpn*2I-RFLP, i.e. R25C/E2FB/AY138588.1:c 305C>T) causes change in amino acid from cysteine to arginine (Konfortov *et al*. 1999) and is more likely to alter the function of the leptin (Buchanan *et al*. 2002).

*Hph*I-RFLP showed significant (P<0.05) association with body weight at 12 months of age. Animals with AV genotype showed higher body weight than the animals with AA genotype (Table 1). The study of body weight at various age groups provides a measure of growth and size. This reflects the stability of a breed or a strain to a particular production system, and is important for the early maturity and general adaptability to environment. As the birth weight is higher in *Hph*I-RFLP-AV genotype, the body weight at 12 months of age was also higher in animals with this genotype as compared to *Hph*I-RFLP-AA genotype which is similar to earlier findings (Liefers *et al*. 2002, Dandapat *et al*. 2009). But, the *Kpn*2I-RFLP locus though had effect on birth weight, was not found to be associated with body weight at 12 months of age. Allelic variation at the same locus has been reported to be associated with increased fat deposition and mRNA levels in beef cattle (Buchanan *et al*. 2002, Liefers *et al*. 2002). A 10.8 kg difference was observed between reported leptin genotypes (Pomp *et al*. 1997, Liefers *et al*. 2002). Similarly, it was observed that the heterozygotes had higher weight at first calving than the homozygotes having wild alleles (Clempson *et al*. 2011).

The leptin genotypes seem to have some effect on body weight gain at 6 to 12 months of age (Liefers *et al*. 2002, Kulig and Kmie 2009). Both the loci did not show any significant effect on body weight at 18 and 24 months, and body weight gain during 12 to 18 and 18 to 24 months of age. Usually the serum leptin level increases gradually from birth to 22 months of age of heifers and does not change thereafter (Tokuda and Yano 2001). So it may be suggested that the mutant alleles of leptin may be causing some minor functional loss in the leptin protein and so tends to increase body weight around 12 months of age (Liefers *et al*. 2002). The effect of leptin on weight of carcass (Buchanan *et al*. 2003) is due to excessive accumulation of fat in the body during late fattening period (Silva *et al*. 2014).

Significant (P<0.05) association of *Kpn*2I-RFLP was found with first lactation milk yield. Animals having CT genotype produced 371.41 kg more milk in their first lactation as compared to animals with CC genotype (Table 1). The *Hph*I-RFLP genotypes did not show any significant effect on first lactation milk yield. It was reported that animals homozygous for mutant allele (*Kpn*2I-RFLP, T allele) produce more milk as compared to homozygous wild type animals (CC), and had higher somatic cell count and linear scores, without significantly affecting milk fat or protein percent (Buchanan *et al*. 2002). However, CC genotype was associated with lowest fat-to-protein ratio with statistically significant value in Holstein cows (Kadlecová *et al*. 2014). Taking linkage disequilibrium into account, significant differences was found between other leptin genotypes AA and AB (Liefers *et al*. 2002) for milk yield, protein yield, feed intake and lactose yield. Similar to the present findings, no significant association of *Hph*I-RFLP genotypes with the lactation traits was observed (Singh *et al*. 2013).

The *Kpn*2I-RFLP genotypes had highly significant (P<0.01) association with the average daily milk yield of first lactation. The animals with CT genotype had higher daily milk yield than the animal with CC genotype by 1.81 kg (Table 1). The *Hph*I-RFLP genotypes were also having a significant (P<0.05) effect on this trait with AV genotype producing 1.04 kg more daily milk yield than the AA genotype. The *Kpn*2I-RFLP genotypes having extra cysteine in the leptin protein were significantly affecting the daily milk yield of first lactation in our study similar to taurine cattle (Pomp *et al*. 1997, Liefers *et al*. 2002) a direct effect of the obese locus with BM1501 microsatellite, no association was found with milk production traits. However, leptin genotypes in cattle were reported to be associated with milk and milk constituent traits (Liefers *et al*. 2002) suggesting the positive effect of the mutation in the exon 2 of the leptin gene on higher milk production in cattle. As

the first lactation milk yield is a moderately heritable trait (Choudhary *et al*. 2003), there are high prospects of improvement in this trait through marker-assisted selection.

Significant (P<0.05) association was found between *Hph*I-RFLP genotypes and age at first calving. The animals with AV genotype calved 63 days earlier than those of AA genotype (Table 1). *Kpn*2I-RFLP genotypes did not show any significant effect on the age of first calving. The *Hph*I-RFLP 'V' allele in heterozygous animals seems to be of great value if utilized in selection program. Earlier studies suggest that leptin modulates reproductive function and provides a direct link between reproduction and the nutritional status of an organism (Kennedy and Mitra 1963, Chehab *et al*. 1997, Friedman 2002) which supports the present observation that *Hph*I-RFLP-AV genotype causes significant increase in body weight and simultaneously has lower age at first calving.

Leptin gene had significant effect on growth, production and reproduction traits. The heterozygous animals were superior to the wild homozygous animals from economic point of view. Although further analysis from other populations is required to confirm these results, the association of genetic markers with better productive and reproductive performance is a very interesting observation and could be used in marker-assisted selection to improve growth, production and reproduction in cattle populations which will ultimately lead to higher milk production.

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