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Association Analyses of Single Nucleotide Polymorphism in the Leptin Receptor Gene with Reproduction and Production Traits in High Yielding Indian Cow Breed

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ABSTRACT

The present study was designed with the aim to identify the polymorphism of bovine leptin receptor gene and their association with production and reproduction traits in population of Sahiwal cows. Blood samples were collected from 69 Sahiwal cows and genomic DNA was harvested for analyzing the genetic polymorphism in LEPR gene by PCR-RFLP (LEPR/*BseG*I) method. The results revealed three genotypes CT, CC, and TT in the population with 47.83, 36.23 and 15.94% gentotypic frequency, respectively and two alleles C and T with 0.60 and 0.40 allelic frequency, respectively. The LEPR/*BseG*I assay revealed significant association of genetic polymorphism on LP, TMY, MY300, and PY in third lactation in Sahiwal cows while PCR-RFLP assay did not reveal association of genetic polymorphism on reproductive traits. In conclusion, SNP identified in the *LEPR* gene and its association with production traits advocates that this gene might serve as a candidate genetic marker for selection of Sahiwal cattle with better milk yield. However, further studies are needed to validate this SNP of the *LEPR* gene in another breed and population of dairy cattle and its association with other production and reproduction traits further needed to be verified.

HIGHLIGHTS

- Genetic polymorphism in LEPR gene analyzed in Sahiwal cattle.
- Three genotypes CT, CC, and TT revealed 47.83, 36.23 and 15.94% gentotypic frequency, respectively
- LEPR/*BseG*I showed association with LP, TMY, MY300, and PY traits.

Keywords: LEPR gene, PCR-RFLP, Reproduction traits, Productions traits, Sahiwal cows

Sahiwal is one of the most common cattle breeds of India with average milk yield of 2325 kg per lactation (NBAGR, 2004). The milk yield of dairy cows primarily depends on energy metabolism which is principally affected by feeding behaviour, nutrition, physiological condition and genetic makeup of the animals. Nevertheless, the feed intake, energy homeostasis, lipid metabolism, growth and body condition of animals are primarily regulated by some of the hormones and leptin is one of that important ones synthesized largely in white adipose tissue (Moravcikova *et al.,* 2012a; 2012b; Pandey *et al.,* 2016; Pandey *et al.,* 2019). Additionally, leptin hormone also affects immune system functions and several aspects of reproduction (Houseknecht and Portocarrero, 1998) such as ovarian follicles, the placenta, and lactating mammary glands (Chillard *et al.,* 2005; Matteis *et al.,* 2012). This hormone performs its action through six receptors isoforms, the

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long form LEPR-b is fully functional and performs most of the physiological functions of hormone (Tartaglia, 1997). Most of its actions are mediated centrally, at the level of hypothalamus where it takes part in the energy homeostasis (feed intake) and in the regulation of the activity of the secretory organs. However widespread expression of LEPR-b receptor suggests its function in many peripheral tissues, including gonadal tissues (Silva *et al.,* 2002). The expression of LEPR in ruminants appears to be influenced by levels of nutrition (Chilliard *et al.,* 2005) and blood leptin concentrations which seem to impede secretion of luteinizing hormone and stimulate release of growth hormone (Nonaka *et al.,* 2006).

Furthermore several mutations reported in the bovine *LEP* gene were found to be associated with milk yield (Glantz *et al.,* 2012; Pandey *et al.,* 2017) and reproduction traits (Trakovicka *et al.*, 2013b; Rambachan *et al.*, 2017) in different breeds of cattle. Since leptin perform its physiological functions through receptors present in most bovine tissues (Silva *et al.,* 2002), the leptin receptor gene (LEPR) can also be considered as a candidate gene affecting productive and reproductive traits of dairy animals.

The LEPR gene is located on bovine chromosome 3q33 (Pfister-Genskow *et al.,* 1997) consisting of 20 exons divided over 1.75 Mb and several polymorphisms have been mapped in this chromosome in earlier studies (Kappes *et al.,* 1997). Liefers *et al.* (2004) described a single nucleotide polymorphism (T945M) in the leptin receptor gene in which there was substitution of cytosine to thymine base at position 115 in exon 20, which results in a substitution of the amino acid threonine by methionine at residue 945 (T945M) which may have introduced an alteration in the structure of intracellular domain of the LEPR (Liefers *et al.,* 2004). Furthermore, LEPR gene polymorphisms were found significantly associated with milk performance (Komisarek and Dorynek, 2006), reproduction (Clempson *et al.,* 2011) and growth traits (Silva *et al.,* 2012). Thus, this study was designed with the aim to identify the polymorphism of bovine leptin receptor gene and their association with production and reproduction traits in population of Sahiwal cows.

MATERIALS AND METHODS

Animals and DNA extraction

Blood samples were collected from 69 Sahiwal cows maintained at Instructional Livestock Farm Complex (ILFC), College of Veterinary Science and Animal Husbandry, Mathura. The genomic DNA was extracted from WBCs of collected blood samples by phenol-chloroform method (Sambrook and Russell, 2001) and further optical density of extracted DNA was determined at wavelength 260 and 280 nm using UV-Visual spectrophotometer for estimation of quality and quantity of DNA.

PCR amplification

A fragment of LEPR gene was amplified using 5'-ACTACAGATGCTCTACTTTGG-3' as forward and 5'-TGCTCCTCCTCAGTTT-3' (Almeida *et al.,* 2008) as reverse primers which resulted in amplification of 197 bp amplicon. The amplification was done in a total volume of 25 µl PCR mix in thermal cycler (Bio-Rad, USA). The PCR mix was prepared containing 2.5 µl PCR dream buffer 10X, 2.5 µl of 2 mM dNTPs, 10 pM (0.5 µl) from each primer, 1 U *Taq* DNA polymerase and 100-150 ng of template DNA. The PCR program was 94°C for 3 min, followed by 35 cycles of 94 °C for 30s, 51.6 °C for 50s and 72 °C for 50 sec. The final step prolonged for 7 min at 72°C.

Restriction reaction

LEPR/*BseG*I polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was executed for determining the genetic polymorphism in LEPR gene. Each digestion reaction carried out at 50°C for 4.5 hours in 10 µl reaction mixture containing 5 µl PCR products, 2 µl buffer 10X, 5 U (0.5 µl) *BseG*I restriction enzyme (Fermentas) and 7.5 µl deionized water. The restriction fragments were visualized on 2% agarose gels in 1X TBE stained with ethidium bromide (0.5 µg/ml) (Fermentas).

Statistical analysis

The allelic and genotypic frequencies of LEPR/*BseG*I

polymorphism were determined for estimating the deviation from Hardy-Weinberg equilibrium using χ^2 test. The statistical significance was determined by ANOVA followed by Tukey's post-hoc multiple comparison test using SPSS software (version 16.0). The data are presented as mean ± SE and a p value < 0.05 was considered to be statistically significant.

RESULTS

LEPR gene polymorphism and restriction pattern

The amplified fragments of the *LEPR* gene revealed amplicon of 197 bp (Fig. 1) which on digestion with *BseG*I restriction enzyme revealed three types of band patterns (genotypes); CC, CT and TT genotypes. The amplicon of CC genotypes were having single restriction site thus revealed two fragments of 130 and 67 bp. The amplicon of CT genotype was containing 3 restriction sites thus revealed 4 fragments of 130, 93, 67 and 37 bp. The third TT genotype containing 2 restriction sites revealed 3 fragments of 93, 67 and 37 bp (Fig. 2). This result revealed that the studied population of Sahiwal cattle were polymorphic in nature with two types of alleles C and T with three types of genotypes CC, TT and CT genotypes in the population.

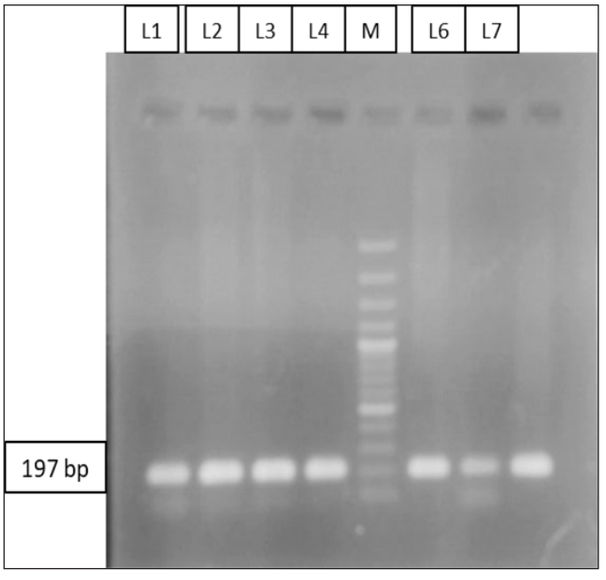

Fig. 1: PCR amplified products of the *LEPR* gene revealed amplicon of 197 bp**.**

Population analysis

Table 1: Genotypic and allelic frequencies of LEPR/*BseG*I gene in Sahiwal cattle

Breed	Genotypic frequency (%)			Allelic Frequency	
	CC	CT	TT	C	T
Sahiwal (N = 69)	36.23 (n = 25)	47.83 (n = 33)	15.94 (n = 11)	0.60	0.40

Where; N = Sample size, n = Number of animals in particular genotype.

The results disclosed that in studied population of Sahiwal cattle, the most frequent genotype was CT (47.83%) followed by the homozygote CC (36.23%), and TT genotype (15.94%) and the allele frequency of C and T alleles were 0.60 and 0.40, respectively. The allelic and genotypic frequencies of LEPR/*BseG*I in Sahiwal cattle were calculated and presented in Table 1.

The χ^2 calculated value for LEPR/*BseG*I genes in Sahiwal cattle were 10.78, while χ^2 table values were 9.210 at 5% and 1% level of significance, respectively for degree of freedom 2. These results revealed that $\chi^2_{(cal)} > \chi^2_{(tab)}$ at 1% level of significance *i.e.* selected population of Sahiwal cattle was not found in Hardy-Weinberg equilibrium.

Association of *LEPR* polymorphism with reproduction and milk production traits

The association studies of LEPR/*BseG*I assay on reproduction traits of Sahiwal cows has been depicted in Table 2. The LEPR/*BseG*I PCR-RFLP assay did not reveal significant influence of CC, CT and TT genotypes on reproduction traits in Sahiwal cattle. The association of LEPR/*BseG*I on milk production traits of Sahiwal cows have been presented in Table 3. The LEPR/*BseG*I assay revealed significant association of genetic polymorphism of LEPR/*BseG*I on LP, TMY, MY300, and PY in third lactation in which TT genotype showed longest LP and highest TMY, MY300 and PY compared to CT and CC genotypes in Sahiwal cows.

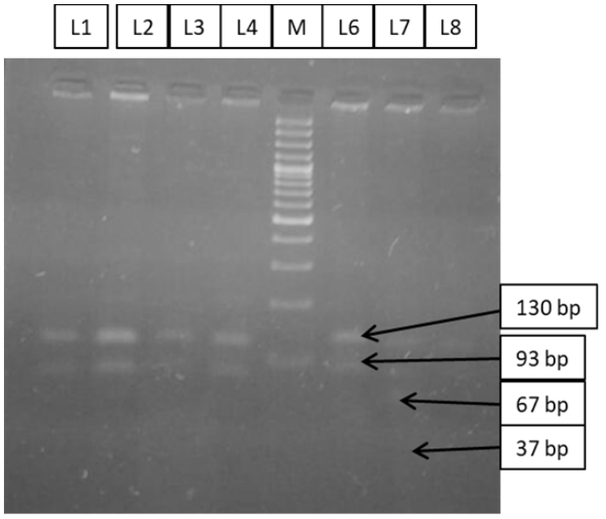

Fig. 2: LEPR/*BseG*I PRC-RFLP pattern showing genotype pattern in 2% agarsoe gel; Lane 7: CT genotype (130, 93, 67 & 37 bp); Lane 1-4: CC genotype (130 & 67 bp); Lane 8: TT genotype (93, 67 & 37 bp); Lane 5 (M): Marker (100 bp Ladder)

DISCUSSION

LEPR gene polymorphism and restriction pattern

The present study revealed genetic polymorphism in LEPR/*BseGI* with two types of alleles C and T and showed three types of genotypes; CC, CT and TT genotypes. Similarly, Komisarek and Dorynek (2006), Komisarek (2010), Silva *et al.* (2012) and Ghanbari Baghenoey *et al.* (2014) also reported all the three genotypes in cattle population, corroborated the findings of present study. The frequency pattern of C and T alleles in LEPR/*BseG*I assay was 0.60 and 0.40, respectively. This finding was in agreement with the observations of various authors in different breeds of cattle who reported higher allelic frequency of C than T (Komisarek and Dorynek, 2006; Komisarek, 2010; Silva *et al.,* 2012; Trakovicka *et al.,* 2011, 2013a, 2013b, 2015; Ghanbari-Baghenoey *et al.,* 2014). This difference in allele frequency may be due to different history of the breeds, long-term geographical isolation, and selection towards high milk yield.

However, Trakovicka *et al.* (2011, 2013a, 2013b, 2015) did not observed TT genotype in the studied population. In present investigation the genotypic frequency of CT genotype was highest which was similar to the findings of other scientists (Komisarek, 2010; Silva *et al.,* 2012; Ghanbari-Baghenoey *et al.,* 2014). The high frequency of heterozygous animals indicated effective selection for polymorphism in the population of animals. However, Komisarek and Dorynek (2006) and Trakovicka *et al.* (2011, 2013, 2013a, 2015) reported highest frequency of CC genotype compared to CT and TT genotypes in cattle population. This variation in frequency of genotypes might be due to population size and geographical distribution.

Table 2: Association of LEPR/*BseG*I genotypes reproduction traits

Lactation	Genotype	AFC	GP	DP	CI
First	CC	1664.70 ± 59.28	280.24 ± 3.13	188.15 ± 33.33	540.05 ± 27.04
	CT	1683.60 ± 56.87	279.52 ± 1.77	246.59 ± 24.78	566.88 ± 38.48
	TT	1646.40 ± 91.92	283.00 ± 3.24	176.89 ± 38.51	519.44 ± 74.09
	Total	1670.90 ± 37.08	280.14 ± 1.55	211.37 ± 18.38	548.54 ± 23.18
Second	CC		283.71 ± 2.64	189.67 ± 31.79	480.00 ± 23.40
	CT		281.37 ± 2.52	162.59 ± 30.58	513.53 ± 36.18
	TT		287.25 ± 4.28	136.25 ± 27.83	468.75 ± 39.96
	Total		283.34 ± 1.67	167.48 ± 18.36	492.00 ± 19.19
Third	CC		302.75 ± 27.04	267.12 ± 59.17	505.00 ± 42.78
	CT		283.62 ± 1.69	178.09 ± 24.71	463.90 ± 31.21
	TT		286.75 ± 4.39	158.86 ± 40.18	527.57 ± 33.96
	Total		291.33 ± 9.76	200.31 ± 24.31	494.46 ± 20.45

[abc]Means bearing different superscript in a column for one lactation differ significantly ($P < 0.05$).

Table 3: Association of LEPR/*BseG*I genotypes on milk production traits

Lactation	Genotype	LP	TMY	MY300	PY	DRPY
First	CC	369.09 ± 21.96	1827.20 ± 122.72	1479.80 ± 51.80	7.63 ± 0.35	42.13 ± 3.68
	CT	365.62 ± 24.18	1830.00 ± 156.65	1463.50 ± 61.61	7.59 ± 0.31	42.67 ± 3.48
	TT	385.00 ± 51.90	2086.60 ± 475.95	1466.80 ± 172.31	7.89 ± 0.81	45.56 ± 4.97
	Total	370.00 ± 15.75	1868.70 ± 110.53	1470.40 ± 42.48	7.65 ± 0.23	42.90 ± 2.24
Second	CC	329.43 ± 32.53	1886.70 ± 227.78	1678.40 ± 75.64	8.16 ± 0.52	42.19 ± 3.91
	CT	369.53 ± 31.01	2055.50 ± 190.92	1657.50 ± 79.38	9.05 ± 0.56	41.26 ± 3.20
	TT	375.43 ± 28.56	2303.30 ± 237.44	1894.90 ± 249.09	8.69 ± 0.98	37.25 ± 5.54
	Total	356.52 ± 19.14	2039.80 ± 126.83	1706.30 ± 62.57	8.65 ± 0.36	40.86 ± 2.24
Third	CC	211.45^{a} ± 33.60	977.91^{a} ± 191.34	1317.70^{a} ± 86.53	7.29^{a} ± 0.60	37.25 ± 5.30
	CT	276.50^{a} ± 35.65	1703.20^{ab} ± 274.09	1736.00^{ab} ± 137.41	9.35^{b} ± 0.67	34.77 ± 3.33
	TT	403.38^{b} ± 27.43	2397.20^{b} ± 221.73	1793.40^{b} ± 141.06	9.63^{b} ± 0.73	46.00 ± 3.91
	Total	286.16 ± 23.34	1624.90 ± 167.68	1602.40 ± 79.16	8.67 ± 0.42	38.39 ± 2.56

^{abc}Means bearing different superscript in a column for one lactation differ significantly (P < 0.05).

Association of *LEPR* polymorphism with reproduction and milk production traits

The association studies of LEPR gene by *BseGI*/PCR-RFLP assay revealed significant influence of genetic polymorphism on LP, TMY, MY300 and PY in studied population of Sahiwal cows. The association study showed that T may be selected as a desirable allele because TT genotype exhibited longer LP, higher TMY, MY300 and PY compared to CT and CC genotypes. However, the LEPR/*BseG*I genetic polymorphism did not show significant association with AFC, DP, CI, and DRPY in Sahiwal cattle breeds. Almeida *et al.* (2008), and Giblin *et al.* (2010) also did not observe significant effect of T945M on milk production and reproduction performance in Holstein cattle, but significant associations was observed between other polymorphisms in LEPR gene and energetically expensive process of lactogenesis, energy storage and fertility performance, therefore they considered LEPR gene as a potential candidate gene for selection of improved fertility in dairy cows. Additionally, Komisarek (2010) and Trakovicka *et al.* (2013a) did not observed significant influence of T945M of LEPR gene on production or reproduction traits except for calving interval and age at first insemination, respectively.

However, in contrast to the findings of present study, Trakovicka *et al.* (2015) reported significant higher milk, protein and fat yield in CC genotype of LEPR gene in Pinzgau cows and Komisarek and Dorynek (2006) reported negative significant association between fat and protein content in milk and T allele of T945M polymorphism in Jersey cows. Besides, Chen *et al.* (2004), Mackowski *et al.* (2005) and Schenkel *et al.* (2006) also found genetic variations of the bovine LEPR gene which were associated with milk production traits and fatness traits.

Carvajal *et al.* (2010) reported significant associations of T945M for milk production, fat and protein percentages but not with milk yield in Chilean dairy cattle. Clempson *et al.* (2011) reported only a weak association of this SNP with milk yield and days to first service in Holstein cows. De Matteis *et al.* (2012) reported four novel SNPs in LEPR gene (LEPR01, LEPR03, LEPR09 and LEPR16) which significantly influenced milk production traits (ME fat%, ME protein%, test day fat% and test day protein %) in Holstein cows. For SNP *LEPR/T945M* effect on milk production (Glantz *et al.,* 2012), reproduction (Clempson *et al.,* 2011; Glantz *et al.,* 2011; Komisarek, 2010) and growth traits (Almeida *et al.,* 2008, Silva *et al.,* 2012) were confirmed.

CONCLUSION

In conclusion, SNP identified in the *LEPR* gene and its association with production traits advocates that this gene might serve as a candidate genetic marker for selection of Sahiwal cattle with better milk yield. However, further studies are needed to validate this SNP of the *LEPR* gene in another breed and population of dairy cattle and its

association with other production and reproduction traits further needed to be verified.

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