academicJournals

Vol. 12(42), pp. 6140-6146, 16 October, 2013
DOI: 10.5897/AJB2013.13061
ISSN 1684-5315 ©2013 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Genotypic profiling of coding region of leptin gene and their association studies on reproductive and milk production traits in Sahiwal and Frieswal cattle of India

Umesh Singh*, Sushil Kumar, Rajib Deb, Sandeep Mann and Arjava Sharma

Molecular Genetics Laboratory, Project Directorate on Cattle, (ICAR), Meerut Cantt., Meerut, Uttar Pradesh--250 001, India.

Accepted 20 September, 2013

Leptin gene has its role in appetite, metabolism, growth and milk production in cattle. Single nucleotide polymorphisms (SNPs) in leptin gene in different cattle breeds have been reported and subsequently associated with their production performance. The objective of this study was to evaluate the association of genetic differences in the bovine leptin gene with milk production, reproduction, milk constituents in Sahiwal and Frieswal cattle of India. In total, one hundred and seventy six cows were genotyped by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) to screen the presence of three SNPs in leptin gene. The testing of Hardy- Weinberg equilibrium for the three SNPs of within Frieswal and Sahiwal population indicated that the polymorphism site in the populations fitted with Hardy-Weinberg equilibrium (P > 0.05) except for C/*BspEI*/T and C/*NruI*/T position in Sahiwal. Polymorphism C/*NruI*/T have significant association with age at first service and age at first calving and heterozygotes have more prolonged age at fist service and age at first calving. For milk protein, C/*BspEI*/T and C/*HphI*/T was found to have significant effect. For lactose and SNF, C/*HphI*/T polymorphism has found to be significant. In case of combined genotyping, genotype CTCTCC (713.00±167.99 days) was found to have noticeable higher age at first service and age at first calving. But milk production higher first lactation yield was noted for CCCCCT (3987.00±337.86 kg).

Key words: Leptin gene, polymorphism, Frieswal, Sahiwal, polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP), combined genotype.

INTRODUCTION

Remarkable progress has been achieved in milk production since 1980, due to the intense selection of animals based on the production performance. But this resulted in the declining trend in various non-yield traits like reproductive performance which in turn resulted in the low economic output of the dairy farmers. Identification of single nucleotide polymorphisms (SNP) opened new vistas in animal breeding as these methods are quite cheaper and resulted in direct genotyping for candidate genes using polymerase chain reaction (PCR) (Karen et al., 1998; Mataves et al, 2003).

Leptin is a 167 amino acid or 16 kDa polypeptide,

*Corresponding author: usinghas@gamil.com.

Abbreviations: WBC, White blood cells; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **SNP,** single nucleotide polymorphisms.

Table 1. Details of the primers.

SNPs	Primer sequence	Product size (bp)	Annealing temperature (°C)	Reference
C/*BspEI*/T	F5ATGGGCTGTGGACCCCTGTATC 3' R 5' TGGTGTCATCCTGGACCTTCC 3'	94	60	Haegeman et al., 2000
C/*NruI*/T	F 5' CAAGATGGACCAGACATTCG 3' R 5'CTGGACTTTGGGAAGAGAGG 3'	317	58	Buchanan et al. 2002
C/*HphI*/T	F 5' GGGAAGGGCAGAAAGATAG 3' R 5' TGGCAGACTGTTGAGGATC 3'	331	54	Lagonigro et al., 2003

which is synthesized predominantly in the adipose tissue. It is involved in the growth and metabolism and plays a crucial role in the regulation of feed intake, energy balance, fertility, milk production and immune functions (Singh et al., 2012; Blache et al., 2000; Chilliard et al., 2005; Liefers et al., 2002; Nkrumah et al., 2005). The bovine leptin gene has been mapped to chromosome 4 (Stone et al., 1996; Pomp et al., 1997). It consists of three exons and out of which only two exons are translated into the proteins (He et al., 1995).

Several polymorphisms in this gene have been found which have significant role in production, reproduction, milk constituents, and growth traits and carcass characteristics (Lien et al., 1997; Haegeman et al., 2000; Buchanan et al., 2002; Lagonigro et al., 2003; Kulig and Kmei, 2009). So the objective of the present work was formulated to distinguish the allelic variation of leptin gene coding exons (exon 2, 3) among Frieswal (Holstein Friesan X Sahiwal) and an indigenous breed viz. Sahiwal of Indian origin. It is also aimed to associate the effect of individual SNP effect on the production and reproduction traits as well as milk constituents. Since genotypic effect of one SNP may be influenced by other SNPs and the genotype combination effect is a reflection of interactions of multiple SNPs, the study was designed to identify the association of haplotypes of leptin gene coding exons (exon 2, 3) with the milk production and reproductive traits.

MATERIALS AND METHODS

DNA isolation and genotyping of animals

Blood samples were collected randomly from 126 Frieswal and 50 Sahiwal cows of Indian origin maintained at Military Farm, Meerut, Uttar Pradesh, India under the same management regimen. Genomic DNA was isolated from white blood cells (WBC) pellet using standard phenol chloroform extraction method (Sambrook and Russel, 2001). The PCR-restriction fragment length polymorphism (RFLP) technique was used to screen the DNA polymorphisms of the leptin gene. Two regions in exon 3 (317 and 331 bp) and one region in exon 2 (94 bp) of leptin gene were amplified. Amplification of the desired leptin gene fragments was performed with published primer pairs (Table 1) (Haegeman et al., 2000; Buchanan et al., 2002; Lagonigro et al., 2003). PCR were performed using genomic DNA template (approximately 100 ng) in a final reaction volume of 25 µl containing 1X PCR buffer (Sigma Aldrich, India), 1.5 mM $Mgcl_2$ (Sigma Aldrich), 200 µM dNTPs (Sigma Aldrich, India), 0.5 µM of each primer and 1 U Taq DNA polymerase (NEB, India). Initial denaturation for 5 min at 94°C followed by 35 cycles of 94°C (30 s), variable annealing temperature (30 s), 72°C (30 s) and a final extension at 72°C for 10 min. The PCR products were isolated and verified by agarose gel electrophoresis methods using 1.5% agarose gel with ethidium bromide for 20 min and visualized under UV trans-illuminator and Gel Documentation System (AlphaImager EP, USA). The restriction digestion was carried out in a final volume of 15 µl reaction. The PCR products for each sample was digested for 4 h at 37°C with 8 U of restriction enzyme BspEI (Haegeman et al., 2000) for exon 2 SNP and 10 U of restriction enzymes that is, NruI (Buchanan et al., 2002) and HphI (Lagonigro et al., 2003) for two SNPs at exon 3 regions. Digested products were separated in horizontal gel electrophoresis using 2.5% agarose gel. Digested fragments' size was estimated by comparing them against DNA ladder (low molecular weight ladder for exon 2 and 2-log DNA ladder for both regions of exon 3).

Statistical analysis

For each breed, calculation of allele and genotypes frequencies was based on direct counting. The Chi-square ($\chi 2$) analysis was performed to test whether the genotype distributions obtained were in accordance with the Hardy-Weinberg equilibrium. Allele frequencies between breeds were compared by Fisher's exact test. Analysis of associations between the genotypes of SNPs reproduction and production, were carried out with the GLM procedure, using SPSS software by the following model:

$$Y_{ijklm}=\mu+G_i+B_j+Y_k+S_l+e_{ijklm}$$

Where, Y_{ijklm} is the observed value; μ is the overall mean; G_i is the effect of genotype ; B_j is the effect of season; Y_k is the year of calving; S_l is the effect of season of calving, and e_{ijklm} is the random error. Values of $P < 0.05$ were considered to be significant.

In case of reproduction traits like age at first service and age at first calving, year and season of birth are considered in place of year and season of calving. But for milk constituents, the effect of breed is excluded as only one breed is under consideration and the model is:

$$Y_{ijkl}=\mu+G_i+Y_j+S_k+e_{ijkl}$$

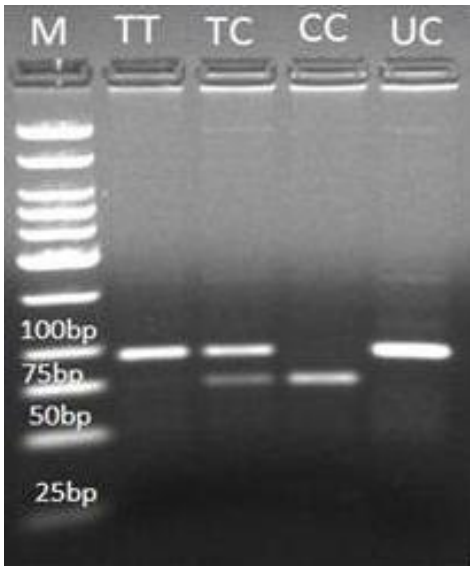

Figure 1. A 2.5% agarose gel displaying *BspEI* digestion of an amplified portion (94 bp) of leptin gene exon 2 (C/*BspEI*/T) of animals with genotypes TT, TC and CC. M, Low molecular weight DNA ladder; UC- uncut.

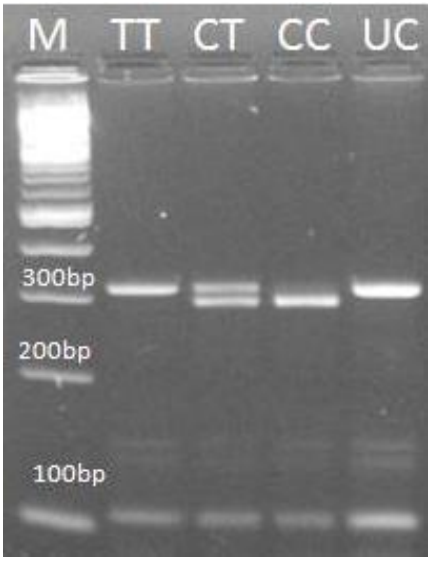

Figure 2. A 2.5% agarose gel displaying *Nru*Idigestion of an amplified portion (317 bp) of leptin gene exon 3 (C/*Nru*I/T) of animals with genotypes TT, CT and CC. M, 2-log DNA ladder; UC, uncut.

RESULTS AND DISCUSSION

Identification and genotyping of SNPs

Three SNP were identified in the bovine leptin gene using PCR-RFLP method. The PCR product of fragment 1 of exon 2 was digested with the BspEI enzyme. The three possible genotypes were defined by three distinct banding patterns: CC (75 and 19 fragments), CT (94, 75 and 19 fragments) and TT (94 fragment) as shown in Figure 1. This is in confirmation with the study of Konfortov et al. (1999) and Buchanan et al. (2002) who described a cytosine (C) to thymine (T) substitution (C—T substitution) in exon 2 of the leptin gene of *Bos taurus and its cross*breds. For the polymorphism of C/*Nru*I/T at exon 3, three digestion patterns were found in the leptin gene among Frieswal. Three genotypes were found as shown in Figure 2; an intact 317 bp fragment as TT genotype, 297 and 20 bp as CC and 317, 297 and 20 bp as TC genotype after digesting with *Nru*I (Buchanan et al., 2002). Similar pattern was observed in in Golpayegani, Najdi, Sarabi and Sistani by Ali Asghar et al. (2010). However, Nassiry et al. (2008) in Golpayegani and Choudhary et al. (2005) in Hariana, Sahiwal, Gir and Nimari cattle couldnot detect TT genotypes. The PCR product of Fragment 3 was digested with HphI enzyme (Lagonigro et al., 2003), and identified three genotypes which were CC (331 bp fragments), CT (331, 311 and 20 bp fragments) and TT (311 and 20 bp fragments) as shown in Figure 3. DNA sequencing analysis confirmed a C/T transition which resulted in the Alanine to Valine (A59V) change in the secreted protein. The genotypic and allelic frequencies of the leptin gene in 176 cattle are presented in Table 2. The testing of Hardy- Weinberg equilibrium for the three SNPs within Frieswal population indicated that the polymorphism site in the populations fitted with Hardy-Weinberg equilibrium ($P > 0.05$). But it has been found that within Sahiwal breed, polymorphisms at C/*BspEI*/T and C/*Nru*I/T position showed significant departures from Hardy Weinberg equilibrium as shown in Table 2. Fisher's exact test revealed that two breeds differ significantly. From the results of Fisher's exact test, it is clear that frequency of genotpes between two breeds differ significantly as shown in Table 2. The frequency of T allele was found to be comparatively lower in Frieswal crossbred. This is in confirmation with the reports of Konfortov et al. (1999) and Choudhary et al. (2005).

Association of the leptin gene polymorphisms with production and reproduction traits

The breed, year and season of calving are found to be significantly associated with production traits like first lactation milk yield and peak yield. Least squares means of these traits with respect to three SNPs are given in Table 3. The three SNP providing genotypes were not significant predictors of the traits used as first lactation milk yield and peak yield. In the present study, none of the polymorphism was found to have a significant effect

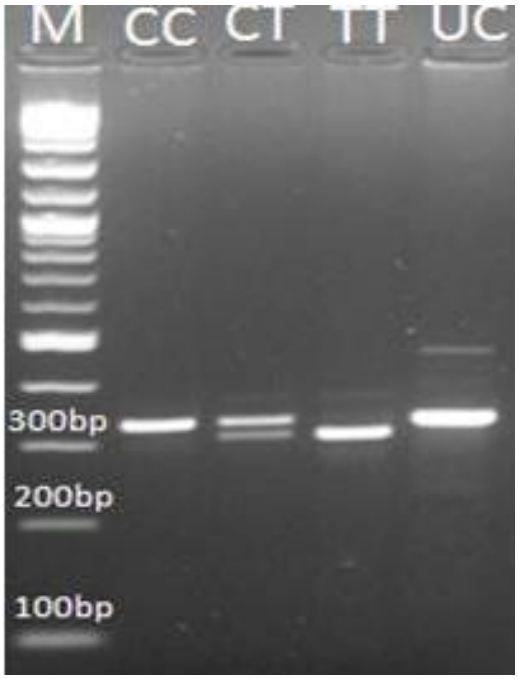

Figure 3. A 2.5% agarose gel displaying *HphI* digestion of an amplified portion (331bp) of leptin gene exon 3 (C/*HphI*/T) of Animals with genotypes CC, CT and TT. M, 2-log DNA ladder; UC, uncut.

Table 2. Gene and Genotype frequencies of three regions of leptin gene after PCR-RFLP in Frieswal and Sahiwal cattle.

Polymorphism	Breed	CC	CT	TT	C	T	Hardy Weinberg equilibrium χ2 test	Fishers exact test
C/*BspEI*/T	Frieswal	0.38 (49)	0.51 (63)	0.11 (14)	0.64	0.36	0.88^{ns}	p<0 .001
	Sahiwal	0.08 (4)	0.88 (42)	0.04 (2)	0.52	0.48	27.22**	
	Total	0.30 (53)	0.60 (105)	0.09 (16)	0.61	0.39	12.13**	
C/*NruI*/T	Frieswal	0.27 (34)	0.51 (64)	0.22 (28)	0.52	0.48	0.04^{ns}	p<0 .001
	Sahiwal	0.41 (21)	0.55 (28)	0.04 (2)	0.69	0.31	3.86*	
	Total	0.31 (55)	0.52 (92)	0.17 (30)	0.57	0.43	0.65^{ns}	
C/*HphI*/T	Frieswal	0.58 (73)	0.38 (48)	0.04 (5)	0.77	0.23	0.71^{ns}	p<0 .001
	Sahiwal	0.96 (48)	0.04 (2)	0 (0)	0.98	0.02	0.02^{ns}	
	Total	0.69 (121)	0.28 (50)	0.03 (5)	0.83	0.17	0.0^{ns}	

** P < 0.001; * P < 0.05; ns-non-significant.

on production. This is in confirmation with the study of Madeja et al. (2004) and Leifers et al. (2002). On the contrary, many of the researchers could establish a significant association between leptin gene polymorphism and milk production traits (Veerkamp et al., 2000; Buchanan et al., 2002; Sadeghi et al., 2008; Dandapat et al., 2009).

In case of reproductive traits like age at first service and age at first calving, year of birth of animals is found to have a significant effect. Least square means of age at first service and age at first calving are presented in Table 3 with respect to SNPs. Heterozygotes have more prolonged age at fist service and age at first calving when compared with both homozygotes in cases of three polymorphisms studied and for which C/*NruI*/T is found to be significant. Contrary to the results, Dandapat et al. (2009) and Moussavi et al. (2006) observed non-

Table 3. Least squares mean and standard errors for production and reproduction traits of different Leptin genotypes.

SNP	Genotypes	Age at first service	Age at first calving	First lactation milk yield	Peak yield
	CC	638.14±22.91	925.67±21.79	2585.06±219.62	11.76±0.83
C/*BspEI*/T	CT	678.56±14.62	955.44±13.90	2687.06±178.88	13.00±0.67
	TT	651.87±43.60	940.49±41.46	2113.34±456.72	9.75±1.72
	CC	631.40±19.43^{b}	916.57±18.44^{b}	2756.86±196.34	12.74±0.76
C/*NruI*/T	CT	692.47±16.24^{a}	970.87±15.42^{a}	2538.27±183.37	12.35±0.71
	TT	670.73±27.85^{ab}	940.42±26.44^{ab}	2922.35±302.81	13.50±1.17
	CC	669.04±14.57	948.43±13.79	2631.79±170.11	12.68±0.65
C/*HphI*/T	CT	671.44±22.26	952.24±21.07	2748.67±240.65	12.30±0.92
	TT	572.99±64.90	854.39±61.43	2115.38±522.71	9.55±2.00

Mean values with the different superscript lower case letters in the same mutational site and column denote significant difference, P < 0.05.

Table 4. Least squares mean and standard errors for milk constituents of different Leptin genotypes in Sahiwal cattle.

SNP	Genotypes	Fat (Year)	Protein	Lactose	SNF
	CC	4.14±0.06	3.05±0.02^{a}	4.62±0.03	8.53±0.04
C/*BspEI*/T	CT	4.03±0.05	2.99±0.02^{b}	4.58±0.02	8.50±0.03
	TT	3.99±0.09	3.07±0.04^{a}	4.56±0.05	8.48±0.06
	CC	4.09±0.05	3.02±0.02	4.57±0.03	8.49±0.03
C/*NruI*/T	CT	4.02±0.06	3.01±0.02	4.60±0.03	8.52±0.03
	TT	4.06±0.08	3.00±0.03	4.60±0.04	8.48±0.05
	CC	4.02±0.05	2.99±0.02^{b}	4.55±0.02^{b}	8.46±0.03^{b}
C/*HphI*/T	CT	4.12±0.05	3.04±0.02^{a}	4.64±0.02^{a}	8.56±0.03^{a}
	TT	4.05±0.14	3.03±0.05^{ab}	4.56±0.06^{ab}	8.50±0.08^{ab}

Mean values with the different superscript lower case letters in the same mutational site and column denote significant difference at P < 0.05.

significant association in reproduction traits.

Association of the leptin gene polymorphisms with milk constituents in Frieswal cattle

Least square means of various milk constituents like fat, protein, lactose and SNF with respect to three SNPs are presented in Table 4. Year and season of calving is found to have a significant effect on the milk constituents. Among the polymorphisms, none of the SNPs was found to have significant association between fat content in milk. But for protein, C/*BspEI*/T and C/*HphI*/T was found to have significant effect. In C/*BspEI*/T polymorphism, heterozygotes are found to have significantly lower protein in milk when compared to both homozygotes. On the contrary C/*HphI*/T polymorphism, CT and TT genotypes have found to be higher protein in comparison to CC genotypes. For lactose and SNF, C/*HphI*/T polymorphism was found to be significant. As in the case of protein, homozygote dominant genotypes (CC) are found to have lower lactose and SNF content in milk in comparison to both genotypes. This is in confirmation with the findings of Leifers et al. (2002) who could establish a significant association between per cent of lactose in milk.

Association studies with combined genotypes

The genotype effect of one SNP may be influenced by other SNPs and the genotype combination effect is a reflection of interactions of multiple SNPs. Therefore, the analysis of genotype combination is superior to the analysis of one single SNP. So we have made an attempt to study the association between combined genotypes and the traits. In this work, 16 combined genotypes consisting

Table 5. Mean±SE of age at first service, age at first calving, first lactation milk yield and peak yield with respect to combined genotypes.

Combined genotype	n	Age at first service	Age at first calving	First lactation milk yield	Peak yield
CCCCCC	5	643.57±157.71	928.14±156.38	2245.00± 659.63	11.20±3.19
CCCCCT	3	577.20± 42.71	863.20± 44.57	3987.00± 337.86	15.67±1.53
CCCTCC	6	661.64±115.00	945.73±113.16	2421.67±1501.82	12.67±5.75
CCCTCT	4	613.82±127.14	896.18±130.15	2036.00±1151.46	12.25±4.19
CCCTTT	2	559.67± 72.42	844.00± 84.66	2031.00± 79.20	10.50±0.71
CCTTCC	6	589.38± 93.32	876.00± 95.22	2727.33±747.48	12.17±5.00
CCTTTT	1	506.00±0.00	791.00±0.00	2446.00±0.00	11.00±0.00
CTCCCC	24	640.14±131.19	922.69±132.69	2265.29± 811.25	11.13±3.71
CTCCCT	1	515.25± 28.25	822.00± 66.30	2130.00±0.00	10.00±0.00
CTCTCC	25	713.00±167.98	983.44±171.49	2087.20±1076.81	10.76±3.99
CTCTCT	7	685.44±151.94	966.50±152.27	2551.00± 356.91	12.00±1.15
CTTTCC	4	669.30± 89.04	950.70± 94.82	2928.75±1603.71	16.00±7.12
CTTTCT	3	695.80±205.96	902.80±143.08	2592.67± 195.37	14.33±1.53
TTCCCC	1	531.00±0.00	822.00±0.00	1715.00±0.00	10.00±0.00
TTCTCC	3	671.43±122.04	955.71±120.65	2068.00± 128.85	9.33±1.15
TTTTCC	1	496.00±0.00	778.00±0.00	3471.00± 0.00	17.00±0.00

of three SNPs were identified in Frieswal cattle, whereas 8 combined genotypes in Sahiwal. The frequencies of some of the combined genotypes were very low. So no statistical analysis was taken up in the case of combined genotypes. The Mean ± SE of age at first service, age at first calving, first lactation milk yield and peak of all the combined haplotypes are presented in Table 5. The combined genotype CTCTCC (713.00 ± 167.99 days) were found to have noticeable higher age at first service, followed by CTTTCT (695.80±205.95 days) and CTCTCT (685.44±151.94 days) in age at first service. But for age at first calving, CTCTCC (983.44±171.49) is followed by CTCTCT (966.50 ± 152.27) and TTCTCC (955.71 ± 120.653 day). But milk production was higher first before lactation yield was noted for CCCCCT (3987.00±337.86 kg).

In conclusion, the present study suggests that single nucleotide polymorphisms in leptin gene can be ideal markers for reproductive traits and milk constituents like fat, protein, lactose and SNF. Findings of this study in relation with combined genotypes need to be carried out in a large population before suggesting the haplotype pairs to be convincing molecular markers. So leptin gene is an ideal candidate gene that may assist in marker assisted selection for production as well as reproduction which is the need of the time.

ACKNOWLEDGEMENTS

Authors are thankful to the Director, Project Directorate on Cattle, ICAR, Meerut, Uttar Pradesh, India for providing necessary facilities to carry out the present work. Authors also express their gratitude to the Military Farm, Meerut, UP, India for providing blood and milk samples for initiation of the present study.

REFERENCES

Ali Asghar A, Nassiry MR, Farajollahi H, Mahdavi M, Abbasi H, Javadmanesh A (2010). Polymorphism in Exon 3 of Leptin Gene in Iranian Native Cattle Breeds. J. Appl. Anim. Res. 37: 159-162.

Blache D, Tellam RL, Chagas LM, Blackberry MA, Vercoe PE, Martin GB (2000). Level of nutrition affects leptin concentrations in plasma and cerebrospinal fluid in sheep. J. Endocrinol. 165: 625-637.

Buchanan FC, Fitzsimmons CJ, Van Kessel AG, Thue TD, Sim DCW, Schmutz SM (2002). Association of a missense mutation in the bovine leptin gene with carcass fat content and leptin mRNA levels.Genet. Sel. Evol. 34: 105-116.

Chilliard Y, Delavaud C, Bonnet M (2005). Leptin expression in ruminants: Nutritional and physiological regulations in relation with energy metabolism. Domestic. Anim. Endocrinol. 29:3-22.

Choudhary V, Kumar P, Bhattacharya TK, Bhushan B, Sharma A (2005). DNA polymorphism of leptin gene in Bosindicus and Bostaurus cattle. J. Genet. Mol. Biol. 28:740-742.

Dandapat A, Kumar D, Ghosh AK, Banerjee D (2009). Association of leptin polymorphism with growth, milk production and reproduction traits in Sahiwal and crossbred cattle. Ind. J. Anim. Sci. 79(9): 892-896.

Haegeman A, van Zeveren A, Peelman LJ (2000). New mutation in exon 2 of the bovine leptin gene. Anim. Genet. 31(1):79.

He Y, Chen H, Quon MJ, Reitman M (1995). The mouse obese gene: Genome organization, promoter activity, and activation by CCAAT/ enhancer-binding protein alpha. J. Biol. Chem. 270: 28887-28891.

Konfortov BA, Licence VE, Miller JR (1999). Re-sequencing DNA from a diverse panel of cattle reveals a high level of polymorphism in both intron and exon, Mamm. Genome 10 1142-1145.

Kulig H, Kmie M (2009). Association between Leptin Gene Polymorphisms and Growth Traits in Limousin Cattle. Russ. J. Genet. 45(6): 738-741.

Lagonigro, Wiener RP, Pilla F, Woolliams JA, Williams JL (2003). A new mutation in the coding region of the bovine leptin gene associated with feed intake. Anim. Genet. 34: 371-374.

Liefers SC, Te Pas MFW, Veerkamp RF, Van Der Lende T (2002). Associations between Leptin Gene Polymorphisms and Production, Live Weight, Energy Balance, Feed Intake, and Fertility in Holstein Heifers. J. Dairy. Sci. 85:1633.

Lien S, Sundvold H, Klungland H, Vage DI (1997).Two novel polymorphisms in the bovine obesity gene (OBS). J. Anim. Genet. 28: 245-245.

Madeja Z, Adamowicz T, Chmurzynska A, Jankowski T, Melonek J, Switonski M, Strabel T (2004). Effect of leptin gene polymorphisms on breeding value for milk production traits. J. Dairy Sci. 87: 3925-3927.

Moussavi AH, Ahouei M, Nassiry MR, Javadmanesh A (2006). Association of Leptin Polymorphism with Production, Reproduction and Plasma Glucose Level in Iranian Holstein Cows. Asian-Aust. J. Anim. Sci. 19: 627-631

Nassiry MR, Shahroudi FE, Mousavi AH, Sadeghi B, Javadmanesh A (2008).The diversity of leptin gene in Iranian native, Holstein and Brown Swiss cattle. Afr. J. Biotechnol. 7 (15): 2685-2687.

Nkrumah JD, Li C, Yu J, Hansen C, Keislar DH, Moore SS (2005). Polymorphisms in the bovine leptin promoter associated with serum leptin concentration, growth, feed intake, feeding behavior and measures of carcass merit. J. Anim. Sci. 83(1): 20-28.

Pomp D, Zou T, Clutter AC, Barendse W (1997). Mapping of leptin to bovine chromosome 4 by linkage analysis of a PCR-based polymorphism. J. Anim. Sci. 75(5):1427.

Sadeghi M, Babak MMS, Rahimi G, Javaremi AN (2008). Effect of leptin gene polymorphism on the breeding value of milk production traits in Iranian Holstein. Animal 2(7):999-1002

Sambrook J, Russel DW (2001). Molecular Cloning: A Laboratory Manual. 3rd edn, Vol. 1, Cold Spring Harbor Laboratory Press, New York, USA.

Singh Umesh, Kumar S, Deb R (2012). Monograph on bovine leptin gene: A Biomarker Associated with Dairy Milk Production. ISBN: 978-3-659-13582-8, Lambert Academic Publishing, Germany (In press).

Stone RT, Kappes SM, Beattie CW (1996). The bovine homologue of the obese gene maps to chromosome 4, Mammal. Genome 7: 399-400.

Veerkamp RF, Oldenbroek JK, Van der Gaast HJ, Van der Werf JH (2000). Genetic correlation between days until start of luteal activity and milk yield, energy balance, and live weights. J. Dairy Sci. 83:577–583