

# 1 Exploring the relationship between polymorphisms of leptin and IGF-1 genes with milk 2 yield in indicine and taurine crossbred cows

Mohammad Rayees Dar<sup>1,\*</sup>, Mahendra Singh<sup>1</sup>, Sunita Thakur<sup>1</sup>, Archana Verma<sup>2</sup>

4 \*Corresponding Author: E-mail: rayeesr21@gmail.com, Tel: +91-700-65-72722

5 <sup>1</sup>Animal Physiology Division, <sup>2</sup>Animal Genetics and Breeding Division, ICAR-National Dairy Research Institute,  
6 Karnal - 132001, Haryana, India.

## 7 ABSTRACT

8 Leptin and IGF-1 plays a significant role in milk production and lactation in bovines. The present  
9 investigation was carried out to identify the novel polymorphisms in exon 3 region of leptin gene  
10 and exon 3 + partial intron 3 of IGF-1 gene and to analyze their association with the milk  
11 production performance in indicine and taurine crossbred (Karan Fries) cows. Blood samples  
12 were collected from 160 apparently healthy Karan Fries cows. Four SNPs at positions  
13 rs29004508 (C>T), rs29004509 (C>T), rs29004510 (T>C), rs29004511 (T>C) in Leptin gene  
14 and two SNPs at positions rs133251968 (C>A), rs137289661(C>T) in IGF-1 gene were found in  
15 Karan Fries cows, however rs29004509 (C>T) had positive correlation ( $p<0.05$ ) with milk yield.  
16 The genetic variants observed in exon 3 region of *leptin* gene and their association with milk  
17 yield traits revealed the importance of CT genotype, which had been useful for genetic  
18 improvement of Karan Fries cow for milk production traits and can also be utilized as a potential  
19 genetic marker to select appropriate animals.

20 **Key Words:** Polymorphisms; Leptin; IGF-1; Genotype; Genetic marker

21 1. INTRODUCTION

22 In the current era of genomic informatics, attempts are increasingly underway to find certain  
23 meaningful associations in the genome that can be exploited to select animals for favorable  
24 production traits to attain rapid genetic gain in short time periods (Kiyici *et al.*, 2019). Insulin-  
25 like growth factor-1 (IGF-1) gene is located on chromosome 5, consists of 4 exons spanning

26 66191602-66264083 bp length (Laron, 2001). IGF-1, a small peptide of 70 amino acids with a  
27 molecular weight of 7649 Dalton, is also considered to play an important physiological role in  
28 growth, development, metabolism and lactation in bovines (Mehmannavaz *et al.*, 2010). IGF-1 is  
29 believed to be one of the main mediators of the effects of energy balance on the reproductive  
30 performance of the dairy cows after calving. Its concentrations are highly associated to  
31 postpartum energy balance (Mehmannavaz *et al.*, 2010), follicular growth and resumption of  
32 ovarian cyclicity (Mullen *et al.*, 2011; Lynch *et al.*, 2010). SNPs, IGF1i3 A-G, IGF1i6 A-G,  
33 IGF1i7 A-G, rs29012855 A-G of IGF-1 have association ( $P < 0.05$ ) with functional survival milk  
34 protein, fat yield, somatic cell score, carcass conformation and BCS in cows (Mullen *et al.*,  
35 2011). SNP/SnaBI in IGF-1 with milk production traits showed no significant effects within first  
36 4 months of lactation but a significant association with interval to commencement of luteal  
37 activity in Holstein Friesian dairy cows have been reported (Nicolini *et al.*, 2013). SNPs, 89C/T,  
38 98G/T and 167T/C, of IGF-1 are associated with milk production and constituent traits in  
39 buffaloes (Fatima *et al.*, 2009). The leptin, an adipokine also plays a significant role in several  
40 physiological processes including regulation of energy balance, fertility, milk production and  
41 was indicated as a candidate gene for marker assisted selection for high yielding cows (Liefers *et*  
42 *al.*, 2005, Vohra *et al.*, 2011, Kononoff *et al.*, 2017). Leptin gene is located on chromosome 7,  
43 consists of 3 exons, spanning 1282412782-128257629 bp length. Only exons 2 and 3 are  
44 translated, expressing 167 amino acids from which 21 amino acids as signal peptide are removed  
45 to leave a 16.7 kilo Dalton protein of 146 amino acids, arranged as four anti-parallel helices (A,  
46 B, C and D), which are typical class 1 cytokines (Zhang *et al.*, 1997). Previously an association  
47 between A1457G and milk yield in British cows have been found (Banos *et al.*, 2008; Clempson  
48 *et al.*, 2011). However, there are contradictory reports regarding association of leptin

49 polymorphisms with composition and quality of milk (Ferreira *et al.*, 2019). Despite the studies  
50 on polymorphisms of leptin with respect to milk yield, there is no available literature on the  
51 relationship of these two genes on the same aspect. The present investigation was undertaken  
52 with a hypothesis that polymorphisms at the leptin and IGF-1 gene locus might play a role in  
53 milk production. The associations between leptin and IGF-1 gene polymorphisms and milk yield  
54 will provide insight into the underlying mechanisms of leptin and IGF-1 gene with milk  
55 production, and results may be used in future breeding programs. This study was undertaken to  
56 evaluate the association of single nucleotide polymorphism in bovine leptin and IGF-1 gene with  
57 the milk production performance in crossbred cows.

58 **2. MATERIALS AND METHODS**

59 **2.1. Experimental animals and DNA extraction**

60 The present experiment was carried out in indicine and taurine crossbred (Karan Fries)  
61 lactating cows of II and III parity at NDRI, Karnal, situated at an altitude of 250 meter above  
62 mean sea level, latitude and longitude position being 29° 42" N and 79° 54" E respectively.  
63 Experiment was approved by the Institutional Animal Ethics Committee (IAEC) constituted as  
64 per the article no.13 of the CPCSEA rules, laid down by Govt. of India. Crossbred Karan Fries  
65 cows (n=160) were selected from the institute livestock research center of ICAR-NDRI, Karnal  
66 during the month of June. The cows were maintained in loose housing system and were fed as  
67 per the ICAR (2013) feeding standard, received green fodder and concentrate mixture as per the  
68 requirement in the ratio of 60:40. The cows were machine milked three times a day in the  
69 morning, noon and evening and the milk yields were recorded. Ten ml of blood was collected in  
70 EDTA coated vaccutainer tubes in morning and was stored at -20°C until DNA isolation.  
71 Genomic DNA was extracted from the blood samples using phenol - chloroform extraction

72 method with minor modifications (Sambrook and Russell, 2001). The quality of DNA was  
73 checked by 1.5% agarose gel electrophoresis. Quality and quantity of DNA was also estimated  
74 by Biospec-nano spectrophotometer (Shimadzu co-operation, Japan). The ratio between OD<sub>260</sub>  
75 and OD<sub>280</sub> was observed for each sample. DNA sample with a ratio of 1.8 was further diluted to a  
76 final concentration of 30 ng/μl and was stored at -20°C for further analysis.

77 **2.2. Polymerase chain reaction (PCR) primers and amplifications**

78 The primer was designed based on the bovine leptin and IGF-1 gene sequence (NCBI  
79 GenBank AC\_000162.1 and AC\_000161.1) using Primer3 software (Table 1). The PCR  
80 reactions were carried with 25 μl total volume containing template DNA of 3 μl (30 ng/μl), 1.0 μl  
81 of forward and reverse primer, PCR Master Mix (2x) (Fermentas) of 12.5 μl, and 8.5 μl of water.  
82 Amplification was performed in a Thermal cycler (MJ research and BioRad, T100). The thermal  
83 cycling conditions involved an initial denaturation at 95°C for 3 min, followed by 35 cycles with  
84 initial denaturation at 95°C for 30s, annealing temperature of 57.9°C and 57.3°C for 30 sec  
85 respectively, extension at 72°C for 1 minute. PCR program for both the primers was similar and  
86 the PCR products were detected by electrophoresis on 2% agarose gel stained with ethidium  
87 bromide.

88 The gene and genotypic frequencies of different genotypes were estimated by standard  
89 procedure POPGENE version 1.32 (University of Alberta, Canada) (Yeh *et al.*, 1999). The  
90 association of SNP genotypes with milk yield was analyzed using General Linear model (SAS  
91 Version 9.2) as follows:

92 
$$Y_{ij} = \mu + G_i + e_{ij}$$
  
93 where,

$Y_{ij}$  = Adjusted value of milk yield of  $j^{\text{th}}$  animal of  $i^{\text{th}}$  genotype

$\mu$  = Overall mean

$G_i$  = Effect of  $i^{\text{th}}$  Genotypes

$e_{ij}$  = Residual error  $\text{NID}(0, \sigma^2_e)$

94 **3. RESULTS**

95 **3.1. Analysis of sequence data association of milk yield traits with SNPs**

96 The PCR product with the amplicon size of 496 and 226 bp was successfully amplified,  
97 covering exon 3 region of *leptin* gene and exon 3 + partial intron 3 of *IGF-1* gene in Karan Fries  
98 cows, respectively (Figure 1, 2). The final sequence of the contig for Karan Fries cows were  
99 deduced from the raw sequences by using BioEdit software. Clustal Omega software, with a  
100 reference sequence of *Bos taurus* (NCBI GenBank AC\_000162.1 and AC\_000161.1) was used  
101 for determining the polymorphism in exon 3 of Karan Fries cow. The comparison of nucleotide  
102 sequences of exon 3 of leptin and exon 3 + partial intron 3 of IGF-1 gene with that of reference  
103 sequence of *Bos taurus* by Clustal Omega multiple alignments revealed 6 mutations including 5  
104 transitions and 1 transversion. Four SNPs were found at positions rs29004508 (C>T),  
105 rs29004509 (C>T), rs29004510 (T>C), rs29004511 (T>C) in Leptin gene and two SNPs at  
106 positions rs133251968 (C>A), rs137289661(C>T) in IGF-1 gene as compared to *Bos taurus*  
107 (Ref. Seq. AC\_000161.1 and AC\_000162.1; Table 2 and Figure 3). The association of leptin and  
108 IGF-1 gene with milk yield elucidated SNP rs29004509 amplified by the primer 1 of leptin gene  
109 correspond to two genotypes CT and CC (Table 3). However, CT genotype of SNP rs29004509  
110 had positive effect ( $P < 0.05$ ) on milk production with mean of  $3827.06 \pm 145.394$  (Table 4). Rest  
111 of the identified SNPs like rs29004508 (C>T), rs29004510 (T>C), rs29004511 (T>C),  
112 rs133251968 (C>A), rs137289661(C>T) were not correlated with milk yield. The genotype and  
113 allelic frequencies of leptin and IGF-1 gene are represented in table 3, respectively.

114

115 **4. DISCUSSION**

116 The present study revealed presence of four SNPs at positions rs29004508 (C>T),  
117 rs29004509 (C>T), rs29004510 (T>C), rs29004511 (T>C) in Leptin gene and two SNPs at  
118 positions rs133251968 (C>A), rs137289661(C>T) in IGF-1 gene in crossbred cows in  
119 comparison to *Bos taurus*. Only rs29004509 (C>T) showed positive correlation ( $p<0.05$ ) with  
120 the milk production in Karan Fries cows. Similar to our results, Nobari *et al.* (2011) reported that  
121 AB genotype had a significant effect on milk production, days open and milking days compared  
122 to other genotypes in Brown Swiss cows and Liefers *et al.* (2002) reported that AB genotype had  
123 higher milk production in Holstein heifers. Association between polymorphism of leptin gene  
124 and milk production have been reported in several studies, leptin SNP A1457G association with  
125 milk production was found significant in cattle (Banos *et al.*, 2008) and high producing cattle  
126 (Clempson *et al.*, 2011). The HphI-RFLP locus had significant effect on average daily milk  
127 yield, while, Kpn2I-RFLP had significant effect on first lactation milk yield and average daily  
128 milk yield (Choudhary *et al.*, 2019). An association of single nucleotide polymorphisms (SNP) in  
129 the leptin gene with production efficiency as well as milk protein and milk yield has been  
130 reported (Barendse *et al.*, 2004). The association of leptin gene polymorphism with growth traits  
131 and reproduction traits has been reported in crossbred and indigenous cattle (Choudhary, 2004).  
132 GH-TaqI, LEP-Sau3AI and MYF5-TaqI polymorphisms of the genes investigated had significant  
133 effects, especially on the 305-day milk yield of Holstein cows (Sahin and Akyuz, 2017, Kiyici *et*  
134 *al.*, 2019). Trakovicka, (2013) reported that the SNP LEP/Sau3AI significantly influence milk,  
135 protein and fat yield ( $P<0.05$ ) in cows. Similarly polymorphism with respect to leptin gene was  
136 highly significant with milk yield and milk composition traits in Polish Black and White cows  
137 (Polish Friesian) (Flisikowsk *et al.*, 2004). Lende *et al.*, (2005) assessed that the Leptin SNP

138 have association with milk yield, milk composition and DMI in dairy cows. Many SNPs were  
139 detected on the promoter region as well as exon regions of the leptin gene, and were found to be  
140 highly associated with different milk traits. Karan Fries cattle with TT genotype showed  
141 significantly higher 305 days milk yield as compared to cattle with CC genotype (Vohra *et al.*,  
142 2011). Other reports showed that C/BspEI/T and C/HphI/T poly-morphisms of leptin gene is  
143 associated with milk protein percentage, whereas the C/HphI/T locus of leptin is significantly  
144 associated with Solid non fat (SNF) percentage (Singh *et al.*, 2014). Contrary to our findings,  
145 Ferreira *et al.* (2019) reported that polymorphisms leptin do not influence the composition and  
146 quality of milk from 1/2, 3/4 and 7/8 Holstein x Guzera cows kept in a hot climate.

147 Kulig and Kmiec (2009) evaluated that the selection for the A59V CC and CT animals might  
148 contribute to enhance in milk yield as well as fat and protein yields in Jersey cattle. Contrary to  
149 our findings, Mullen *et al.* (2011) reported that SNPs, IGF1i3 A-G, IGF1i6 A-G, IGF1i7 A-G,  
150 rs29012855 A-G, of IGF-1 are associated ( $P < 0.05$ ) with functional survival and chest width,  
151 milk protein and fat yield, milk fat concentration, somatic cell score, carcass conformation and  
152 fat. In another study, (Lynch *et al.*, 2010) SNPs, IGF1iA-T, IGF1i2C-T, IGF1i3G-A, in IGF-1 were  
153 associated with milk production traits and BCS; and are in the agreement with our findings.  
154 Other studies reported that the polymorphism of bovine IGF-1 gene in exon 4 were associated  
155 with production traits in Bali cattle (Maskur *et al.*, 2012) but polymorphism in the intron 4 of  
156 IGF-1 gene was not associated with production traits in mixed population of Charolais and Beef  
157 master cattle (Reyna *et al.*, 2010).

158 It has been found that the SNP IGF-1/SnabI with milk production traits have no significant  
159 effects on either milk yield, fat corrected milk or Total solids yield within first 4 months of  
160 lactation (Nicolini *et al.*, 2013). In dairy cattle, some studies have analyzed the association of this

161 SNP (IGF-1/SnaBI) with milk production traits (Hines *et al.*, 1998; Siadkowska *et al.*, 2006;  
162 Bonakdar *et al.*, 2010; Mehmannavaz *et al.*, 2010; Mullen *et al.*, 2011; Ruprechter *et al.*, 2011).  
163 Mehmannavaz *et al.* (2010) proved a significant effect of the SNP *IGF-1/SnaBI* on estimated  
164 breeding values (EBV) for milk production traits in Iranian Holstein bulls, as animals with AB  
165 genotype had higher EBV for milk and fat yields than homozygous genotypes. The interval from  
166 calving to commencement of luteal activity postpartum (CLA) has been suggested as a suitable  
167 selection criteria for fertility, as early CLA is an important factor for a new pregnancy after  
168 calving, it presents higher heritability values (16 to 25%) than traditional fertility traits, and it is  
169 genetically favorably correlated with traditional fertility traits (Darwash *et al.*, 1997; Petersson *et*  
170 *al.*, 2007; Nicolini *et al.*, 2013). SNPs, 89C/T, 98G/T and 167T/C, of IGF-1 are reported to be  
171 significantly associated with milk production and constituent traits in buffaloes (Fatima *et al.*,  
172 2009).

## 173 CONCLUSIONS

174 In this study novel SNPs were detected in indicine and taurine crossbred cows. SNP at  
175 position rs29004509 (C>T) in Leptin gene had positive correlation ( $p<0.05$ ) with milk yield. The  
176 genetic variants observed in exon 3 region of *leptin* gene and their association with milk yield  
177 traits revealed the importance of CT genotype, which can be utilized as a potential genetic  
178 marker to select elite cows for genetic improvement in future.

## 179 ACKNOWLEDGMENTS

180 The authors are thankful to the Director, National Dairy Research Institute, Karnal Haryana  
181 for providing the necessary facilities to carry out this experiment. This work was supported by  
182 Board of Research in Nuclear Science (BRNS) Mumbai, India under project no.  
183 2013/35/48/2013-BRNS/RTAC.

184 **AUTHOR'S CONTRIBUTIONS**

185 MR, MS and AV designed the study. MR and ST provided the data and performed the  
186 analysis. MR, MS and AV drafted the manuscript. MR, MS, ST and AV contributed to the  
187 interpretation of results, the discussion and commented on the manuscript. All authors read and  
188 approved the manuscript.

189 **ORCID**

190 **rayeesr21@gmail.com** <http://orcid.org/0000-0002-6683-2008>

191 **chhokar.ms@gmail.com** <http://orcid.org/0000-0003-3541-7239>

192 **REFERENCES**

193 Banos, G., Woolliams, J. A., Woodward, B. W., Forbes, A. B., and Coffey, M. P. (2008). Impact  
194 of single nucleotide polymorphisms in leptin, leptin receptor, growth hormone receptor and  
195 diacylglycerol acyltransferase (DGAT1) gene loci on milk production, feed and body energy  
196 traits of UK dairy cows. *Journal of Dairy Science*, 91, 3190-3200.

197 Barendse, W., Bunch, R. J., and Harrison, B. E. (2004). The leptin C73T missense mutation is  
198 not associated with marbling and fatness traits in a large gene mapping experiment in  
199 Australian cattle. *Animal Genetics*, 36, 86-98.

200 Bonakdar, E., Rahmani, H. R., Edriss, M. A., and Sayed Tabatabaei, B. E. (2010). IGF-I gene  
201 polymorphism, but not its blood concentration, is associated with milk fat and protein in  
202 Holstein dairy cows. *Genetics and Molecular Research*, 9, 1726–1734.

203 Choudhary V., Pushpendra K., Chinmoy M., Tarun K. B., Bharat B. and Arjava S., (2019).  
204 Association of leptin gene polymorphism with economic traits in crossbred cattle Indian  
205 *Journal of Animal Sciences*, 89(9), 974-978.

206 Choudhary, V. (2004). Molecular studies on leptin and insulin-like growth factor binding  
207 protein-3 (IGFBP-3) genes in cattle, Ph.D. thesis submitted to Indian Veterinary Research  
208 Institute, Bareily, India.

209 Clempson, A. M., Pollott, G. E., Brickell, J. S., Bourne, N. E., Munce, N., and Wathes, D. C.  
210 (2011). Evidence that leptin genotype is associated with fertility, growth, and milk  
211 production in Holstein cows. *Journal of Dairy Science*, 94, 3618-3628.

212 Darwash, A. O., Lamming, G. E., and Woolliams, J. A. (1997). Estimation of genetic variation  
213 in the interval from calving to postpartum ovulation of dairy cows. *Journal of Dairy*  
214 *Science*, 80, 1227–1234.

215 Fatima, S., Bhatt, S. M., Bhong, C. D., Rank, D. N., and Joshi, C. G. (2009). Genetic  
216 polymorphism study of IGF-1 gene in buffaloes of Gujarat. *Buffalo Bulletin*, 28, 159-164.

217 Ferreira J. B., Guilhermino M. M., Leite J. H. G. M., Sousa J. E. R., Araujo B. V. S.,  
218 Vasconcelos A. M., Lara M. A. C., Facanha D. A. E. (2019). Polymorphisms of leptin,  $\beta$ -  
219 lactoglobulin and pituitary transcription factor have no effect on milk characteristics in  
220 crossbred cows. *Arquivo Brasileiro de Medicina Veterinariae Zootecnia*, 71(2), 715-719.

221 Flisikowski K., Strzałkowska N., Słoniewski K., Krzyzewski J. and Zwierzchowski L. (2004).  
222 Association of a sequence nucleotide polymorphism in exon 16 of the STAT5A gene with  
223 milk production traits in Polish Black and White (Polish Friesian) cows. *Animal Science*  
224 *Papers and Reports*, 22, 515–522.

225 Hines, H. C., Ge, W., Zhao, Q., and Davis, M. E. (1998). Association of genetic markers in  
226 growth hormone and insulin-like growth factor I loci with lactation traits in Holsteins.  
227 *Animal Genetics*, 29, 69.

228 Kadokawa, H., and Martin, G. B. (2006). A new perspective on management of reproduction in  
229 dairy cows: the need for detailed metabolic information, an improved selection index and  
230 extended lactation. *Journal of Reproductive Immunology*, 52, 161-168.

231 Kiyici, J. M., Korhan, A., Bilal, A., Mahmut, K., Esma, G. A., and Mehmet, U. C. (2019).  
232 Relationships between polymorphisms of growth hormone, leptin and myogenic factor 5  
233 genes with some milk yield traits in Holstein dairy cows. *International Journal of Dairy*  
234 *Technology*, 72(1), 1-7.

235 Kononoff P. J., Defoor P. J., Engler M. J., Swingle R. S., Gleghorn J. F., James S. T. and  
236 Marquess F. L. (2017). Impacts of a leptin SNP on growth performance and carcass  
237 characters in finishing steers studied over time. *Journal of Animal Science*, 95(1), 194–200.

238 Kulig, H., and Kmiec, M. (2009). Association between leptin gene polymorphisms and growth  
239 traits in Limousin cattle. *Genetika -Belgrade*, 45, 838-841.

240 Laron, Z. (2001). Insulin-like growth factor 1 (IGF-1): a growth hormone. *Molecular Pathology*,  
241 54, 311-316.

242 Lende, T., Te Pas, M. F. W., Veerkamp, R. F., and Liefers, S. C. (2005). Leptin gene  
243 polymorphisms and their phenotypic associations. *Vitamins and Hormones*, 71, 373-404.

244 Liefers, S. C., Veerkamp R. F. and Vanderlene T., 2002. Associations between leptin gene  
245 polymorphisms and production, live weight, gene balance, fed intake and fertility in holstein  
246 heifers. *Journal of Dairy Science*, 85, 1633-1638.

247 Liefers, S. C., Veerkamp, R. F., Te Pas, M. F. W., Chilliard, Y. and Van Der Lende, T. (2005).  
248 Genetics and physiology of leptin in periparturient dairy cows. *Domestic Animal*  
249 *Endocrinology*, 29, 227–238.

250 Lynch, C. O., Mullen, M. P., Waters, S. M., Howard, D. J., O'Boyle, P., and Berry, D. P., *et al.*  
251 (2010). Single nucleotide polymorphisms in the growth hormone and insulin-like growth  
252 factor 1 genes are associated with production and fertility traits in dairy cows.

253 Maskura, Armana, C., Sumantrib, C., Gurnadib, E. and Muladno, (2012) A Novel Single  
254 Nucleotide Polymorphism in Exon 4 of Insulin-Like Growth Factor-1 Associated with  
255 Production Traits in Bali Cattle. *Media Peternakan*, 96-101.

256 Mehmannah, Y., Amarinia, C., Bonyadi, M., and Vaez Torshizi, R. (2010). Association of  
257 IGF-1 gene polymorphism with milk production traits and paternal genetic trends in Iranian  
258 Holstein bulls. *African Journal of Microbiology Research*, 4, 110-114.

259 Morrison, C. D., Daniel, J. A., Holmberg, B. J., Djiane, J., Raver, N., and Gertler, A., *et al.*  
260 (2001). Central infusion of leptin into well-fed and undernourished ewe lambs: effects on  
261 feed intake and serum concentrations of growth hormone and luteinizing hormone. *Journal  
262 of Endocrinology*, 168, 317-324.

263 Mullen, M. P., Berry, D. P., Howard, D. J., Diskin, M. G., Lynch, C. O., and Giblin, L. *et al.*  
264 (2011). Single Nucleotide Polymorphisms in the Insulin-Like Growth Factor 1 (IGF-1)  
265 Gene are Associated with Performance in Holstein-Friesian Dairy Cattle. *Frontiers in  
266 Genetics*, 2, 3.

267 Nicolini, P., Mariana, C., and Ana, M. (2013). Polymorphism in the insulin-like growth factor 1  
268 gene is associated with postpartum resumption of ovarian cyclicity in Holstein-Friesian  
269 cows under grazing conditions. *Acta Veterinaria Scandinavica*, 55, 11.

270 Nobari K., Mojtaba T., Shokoufe G., Mohammad R. N., and Eisa J. (2011). Relationship  
271 between leptin polymorphism with economical traits in Iranian Sistani and Brown Swiss  
272 cows. *Journal of animal and Veterinary Advances*, 10(1), 1-5.

273 Petersson, K. J., Berglund, B., Strandberg, E., Gustafsson, H., Flint, A. P., Woolliams, J.A., and  
274 Royal, M.D. (2007). Genetic analysis of postpartum measures of luteal activity in dairy  
275 cows. *Journal of Dairy Science*, 90, 427–434.

276 Reyna, X. F., Montoya, H. M., Castrellon, V. V., Rincon, A. M., Bracamonte, M. P. and Vera,  
277 W. A. (2010). Polymorphisms in the IGF1 gene and their effect on growth traits in Mexican  
278 beef cattle. *Genetics and Molecular Research*, 9(2), 875–883.

279 Ruprechter, G., Carriquiry, M., Ramos, J. M., Pereira, I., and Meikle, A. (2011). Metabolic and  
280 endocrine profiles and reproductive parameters in dairy cows under grazing conditions:  
281 effect of polymorphisms in somatotropic axis genes. *Acta Veterinaria Scandinavica*, 53, 35–  
282 44.

283 Sahin C., and Akyuz B. (2017) Detection of MYF5 gene polymorphism with PCR-RFLP method  
284 in five cattle breeds breeding in Turkey. *Mediterranean Agricultural Sciences*, 30, 35–38.

285 Sambrook, J., and Russell, D. W. (2001). Molecular cloning: A Laboratory Manual III, Cold  
286 Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

287 Siadkowska, E., Zwierzchowski, L., Oprzadek, J., Strzalkowska, N., Bagnicka, E., and  
288 Kryziewski, J. (2006). Effect of polymorphism in IGF-1 gene on production traits in Polish  
289 Holstein-Friesian cattle. *Animal Science Papers and Reports*, 24:225–237.

290 Singh U., Deb R., Alyethodi R. R., Alex R., Kumar S., Chakraborty S., Dhama K., and Sharma  
291 A. (2014). Molecular markers and their applications in cattle genetic research: a review.  
292 *Biomarkers and Genomic Medicine*, 6, 49–58.

293 Stone, R. T., Kappes, S. M., and Beattie, C. W. (1996). The bovine homolog of the obese gene  
294 maps to chromosome 4. *Mammalian Genome*, 7, 399-400.

295 Trakovicka, A., Moravcikova, N., and Kasarda, R. (2013). Genetic polymorphisms of leptin and  
296 leptin receptor genes in relation with production and reproduction traits in cattle. *Acta  
297 Biochimica Polonica*, 60, 783-787.

298 Vohra V., Chakravarty A. K., Singh A., Gupta I. D., Chopra A., Dubey P. P., and Kumar D.  
299 (2011). Association of leptin gene polymorphism with 305 days milk yield in Karan Fries  
300 cattle. *Indian Journal of Animal Sciences*, 81 (4): 388–390

301 Yeh, F. C., Yang, R., and Boyle, T. (1999). POPGENE VERSION 1.31: Microsoft Window-  
302 based free Software for Population Genetic Analysis. Available from:  
303 <ftp://ftp.microsoft.com/Softlib/HPGL.EXE>.

304 Zhang, Y., Basinski, M. B., Beals, J. M., Briggs, S. L., Churgay, L. M., and Clawson, D. K., *et  
305 al.* (1997). Crystal structure of the obese protein leptin-E100. *Nature*, 387, 206-219.

306

307

308

309

310

311

312

313

314

315

316

317

318

319

320

321

322 **Table 1** Primer sequences, targeted region, and amplicon sizes of bovine leptin and IGF-1 gene

| Gene   | Sequence (5'-3')        | No. of base | Targeted region on gene         | Annealing Temp. | Amplicon size (bp) |
|--------|-------------------------|-------------|---------------------------------|-----------------|--------------------|
| Leptin | F-CCCTCTCTCCCACTGAGCTC  | 20          | Exon-3                          | 57.3°C          | 496                |
|        | R-TAAAGGATGCCACATAGGC   | 20          |                                 |                 |                    |
| IGF-1  | F-CAGACAGGAATCGTGGATGAG | 21          | Exon 3 +<br>partial intron<br>3 | 57.9°C          | 226                |
|        | R- TGCTGCTAAGTTGCTACAGG | 20          |                                 |                 |                    |

323

324

325

326

327 **Table 2** Nucleotide changes in leptin and IGF-1 gene in Karan Fries cattle as compared to *Bos taurus* (NCBI Gene ID AC\_000161.1 & AC\_000162.1, respectively)

| Gene   | SNP         | Location | Nucleotide Change | Type of Variation | Amino Acid Substitution |
|--------|-------------|----------|-------------------|-------------------|-------------------------|
| Leptin | rs29004508  | Exon 3   | (C>T)             | Transition        | p.80A>V                 |
|        | rs29004509  |          | (C>T)             | Transition        | -                       |
|        | rs29004510  |          | (T>C)             | Transition        | -                       |
|        | rs29004511  |          | (T>C)             | Transition        | -                       |
| IGF-I  | rs133251968 | Intron 3 | (C>A)             | Transversion      | -                       |
|        | rs137289661 |          | (C>T)             | Transition        | -                       |

329

330

331

332

333 **Table 3 Genotypic and allelic frequencies of leptin and IGF-1 gene using sequencing in Karan Fries cattle**

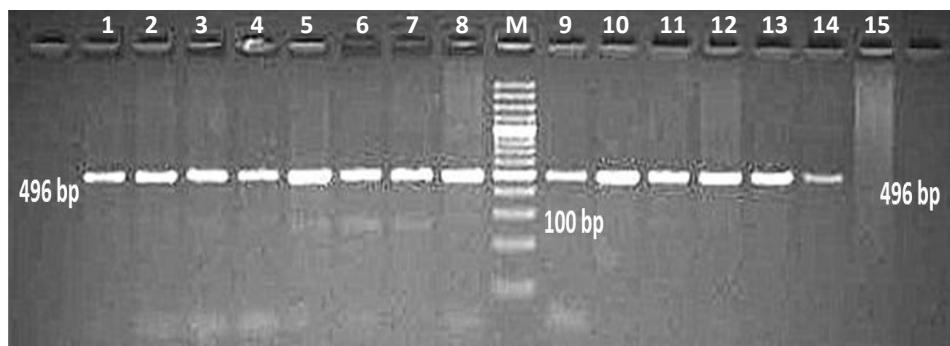
| SNP               | Gene   | Genotype | Genotype frequency | Allele | Allele frequency |
|-------------------|--------|----------|--------------------|--------|------------------|
| rs29004508 (C>T)  | Leptin | TT       | 0.1                | T      | 0.2              |
|                   |        | CT       | 0.2                | C      | 0.8              |
|                   |        | CC       | 0.7                |        |                  |
| rs29004509 (C>T)  | Leptin | CT       | 0.2                | T      | 0.1              |
|                   |        | CC       | 0.8                | C      | 0.9              |
|                   |        | TT       | 0.65               | T      | 0.8              |
| rs29004510 (T>C)  | Leptin | CT       | 0.3                | C      | 0.2              |
|                   |        | CC       | 0.05               |        |                  |
|                   |        | TT       | 0.05               | T      | 0.2              |
| rs29004511 (T>C)  | Leptin | CT       | 0.30               | C      | 0.8              |
|                   |        | CC       | 0.65               |        |                  |
|                   |        | AA       | 0.06               | A      | 0.28             |
| rs133251968 (C>A) | IGF-1  | AC       | 0.44               |        |                  |
|                   |        | CC       | 0.5                |        |                  |
|                   |        | CT       | 0.13               | T      | 0.06             |
| rs137289661(C>T)  | IGF-1  | CC       | 0.87               | C      | 0.94             |

334 **Table 4 Effect of polymorphism of leptin and IGF-1 gene on average milk yield in different genotypes**

| SNP               | Genotype | Average milk yield (Kg)       | p-value |
|-------------------|----------|-------------------------------|---------|
| rs29004508 (C>T)  | TT       | 3587.19 ± 153.04              | 0.899   |
|                   | CT       | 3882.20 ± 290.08              |         |
|                   | CC       | 3746.27 ± 289.59              |         |
| rs29004509 (C>T)  | CT       | 3827.06 <sup>a</sup> ± 145.39 | 0.021   |
|                   | CC       | 3280.22 <sup>b</sup> ± 242.99 |         |
|                   | TT       | 3642.67 ± 528.71              |         |
| rs29004510 (T>C)  | CT       | 3599.12 ± 193.18              | 0.162   |
|                   | CC       | 3753.37 ± 169.62              |         |
|                   | TT       | 3753.33 ± 169.62              |         |
| rs29004511 (T>C)  | CT       | 3599.12 ± 193.18              | 0.162   |
|                   | CC       | 3642.67 ± 528.71              |         |
|                   | AA       | 3836.63 ± 201.80              |         |
| rs133251968 (C>A) | AC       | 3211.43 ± 277.11              | 0.521   |
|                   | CC       | 3073.50 ± 465.40              |         |
|                   | CT       | 3551.92 ± 180.98              |         |
| rs137289661(C>T)  | CC       | 3539.57 ± 296.18              | 0.453   |

335 Values with different superscripts <sup>a, b</sup> differ between genotypes along with their average milk (P<0.05)

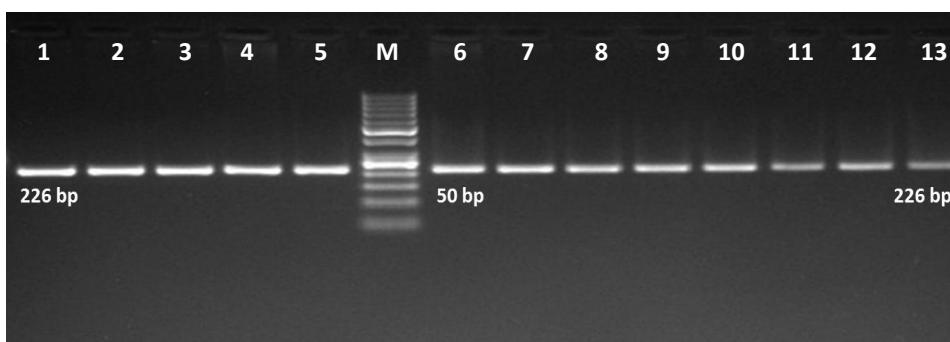
336  
337  
338  
339  
340



341  
342  
343

**Figure 1: PCR amplification of Exon 3 of leptin gene in Karan Fries cow (M=100bp)**  
Lanes 1-15 = 496 bp,  
Lane M = 100 bp

344  
345  
346  
347  
348  
349  
350

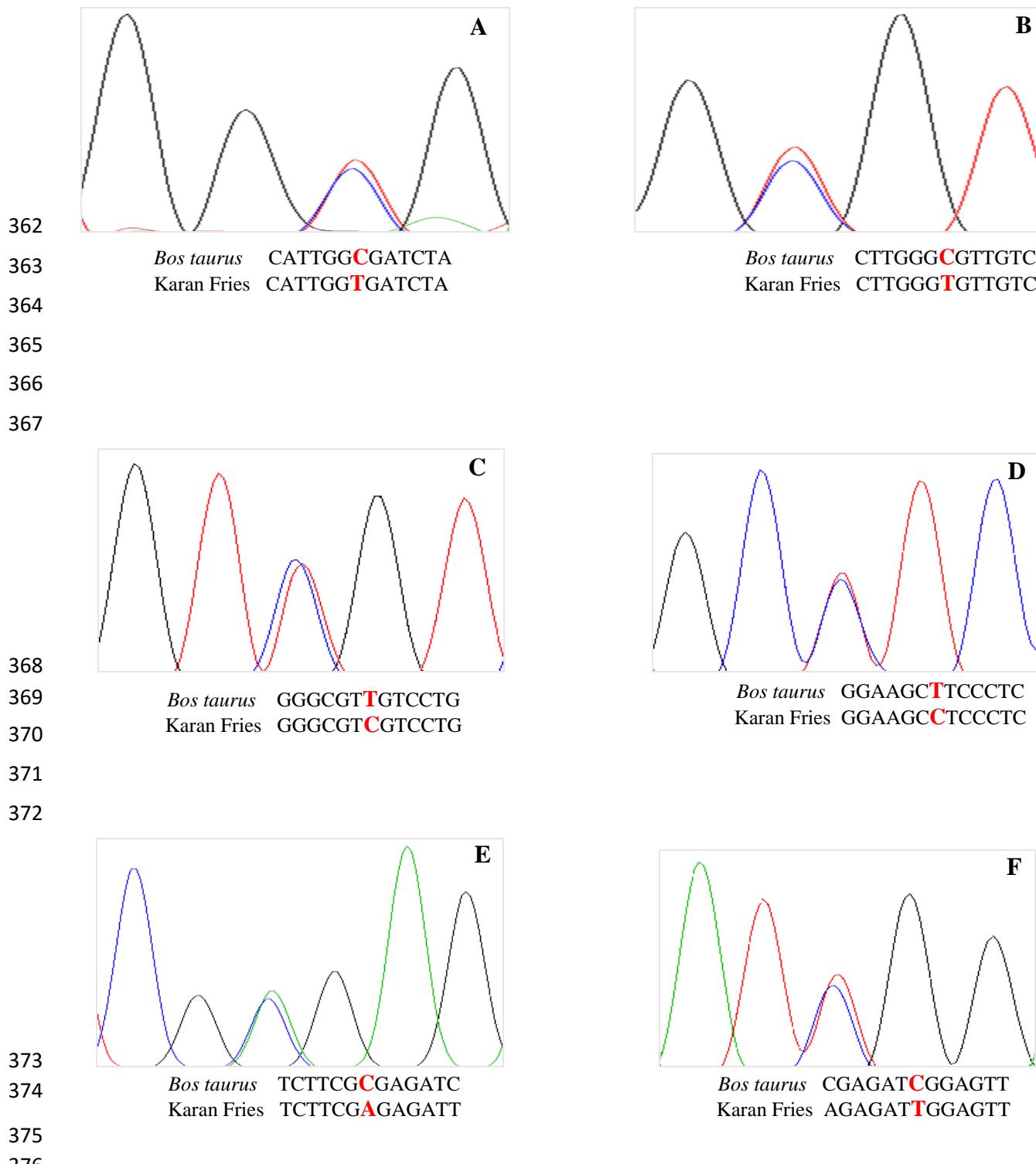


351  
352  
353  
354  
355  
356

**Figure 2: PCR amplification of Exon 3 + some part of intron 3 of IGF-1 gene in Karan Fries cow (M=50bp)**

357  
358  
359  
360  
361

**Lanes 1-15 = 226 bp**  
**Lane M = 50 bp**



378 **Figure 3. Chromatogram and Clustal Omega alignment showing variation at position (A)**  
379 **rs29004508 (C>T), (B) rs29004509 (C>T), (C) rs29004510 (T>C), (D) rs29004511 (T>C) for**  
380 **Leptin gene and (E) rs133251968 (C>A), (F) rs137289661(C>T) for IGF-1 gene in Karan Fries**  
381 **cattle.**

382