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# INVESTIGATION OF LEPTIN GENE POLYMORPHISMS IN EAST ANATOLIAN RED ANATOLIAN AND BLACK CATTLE AND DETERMINATION OF GENETIC DISTANCE FROM BROWN SWISS CATTLE

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## ABSTRACT

The objective of the present investigation was to study single nukleotid changes genetic in leptin gene in indigenous (East Anatolian red Cattle and Anatolian Black) cattle breeds. The T and C allele frequencies were 0.54±0.06 and 0.46±0.06 for East Anatolian Red cattle, 0.48±0.05 and 0.52±0.05 for Anatolian Black respectively. All genotypes were distributed according to the Hardy-Weinberg equilibrium. Effect of leptin T allele polymorphism on the chest circumference was non-significant in East Anatolian Red breed. However, it was found significant in Anatolian Black breed ($P<0.05$). Statistical analysis results are showed that TT genotyped in Anatolian Black breed were higher than values of the other genotypes (CC and CT). These results indicate that the leptin TT genotype is associated with increased chest circumference. These observations may be economic interest. Genetic distances were determined according to Nei methods between the Brown Swiss (16) and East Anatolian Red Cattle (38) and Anatolian Black Cattle (45) breeds.

**Key words:** East Anatolian Red, Anatolian Black, Leptin gene, Chest girth

## INTRODUCTION

Determination of genetic information on the structure and role of different genes and its collection in an international data bank is an essential component of present day animal breeding. Leptin is a candidate genes for marker assistant selection (Nassiry *et. al.,* 2007). Leptin secreted by adipose tissue (Itosser, 1998), is a 16 kDa important polypeptide hormone (Zhang *et. al.,* 1994) that plays an important role in feed intake, energy balance, fertility, reproduction, milk production, immune functions and feed conversion, energy balance, metabolism and reproduction (Kadokawa *et. al.,* 2000; Block *et. al.,* 2001; Liefers *et. al.,* 2002; Lagonigro *et. al.,* 2003; Nobari *et. al.,* 2010). The leptin gene has been shown to be polymorphic in previous investigations (Pompe *et. al.,* 1997; Wilkins and Davey, 1997).

Studies in different cattle breeds have shown correlation between leptin gene polymorphism and body weight, daily live weight gain, back fat thickness, feed intake, feed efficiency and some carcass characteristics (Zwierzchowski *et. al., 2001*; Buchanan *et. al.,* 2002; Oprzadek *et. al., 2003*; Lusk, 2006; Kulig *et. al.,* 2007; Kulig and Kmiec, 2009). Buchanan *et al.*(2003) reported that the T allele of the bovine leptin gene resulted in higher milk production. Komisarek and Dorynek (2005) reported that Arg4Cys TT genotype has a highly significant effect for increasing milk yield. Similarly, Buchanan *et al.* (2002), Lusk (2006) and Kulig and Kmiec (2009) reported that TT homozygote animals were found to have higher daily weight gain than CC and CT genotypes.

In this study, polymorphisms in leptin gene of the Turkish indigenous cattle breeds such as Anatolian Black and East Anatolian Red cattle, their genetic origin, genetic structure and allele frequencies were determined. The genetic difference between these indigenous cattle breeds and Brown Swiss was also determined.

## MATERIALS AND METHODS

This study was carried out in Biotechnology and Genetic Engineering Laboratory, Department of Animal Science, Faculty of Agriculture, Sütçü İmam University, Kahramanmaraş Blood samples from 99 healthy animals (East Anatolian Red, 38: Anatolian Black, 45: Brown Swiss, 16) were collected reared at Suşehri region of Sivas province in Turkiye. For genetic analysis, blood was taken from jugulars vein in EDTA added tubes and kept at -20 °C until further analysis.

**DNA Extraction:** DNA was extracted using the Sigma Gene Elute$^{TM}$Blood Genomic DNA Kit, and with adherence to the manufacturer's recommendations. .

**Primer designing for leptin gene:** Reported sequences of leptin gene region (U50365) were used to ensure primary selections from specific regions during designing of primers to reveal mutations. The leptin gene sequenced regions were analyzed using Clone Manager software program. Two primers were (CBF:

5′TATCTGTCTTACGTG- GAGGCTGTGCggATC3′), and (CBR:5′ TACCGTGTGTGAGATGTCATTGAT3′) designed. Oligonucleotides were purchased from Iontek (Istanbul, Turkey). PCR was performed in 50 µl of mixture by using DNA polymerase (5 U) and 10X PCR buffer (Favogen Biotech Corp. Taiwan).

**PCR Amplification and Restriction Digest:** Polymorphism sequence containing region was amplified using CBF and CBR primers. The primer CBF sequence was design to detect single amino acid mutation (Arg25Cys). Also CBF will create *Bam*HI restriction site (GGATCC) when C73T mutation occur. The CBF and CBR primers were used to amplify 106 bp region. PCR was performed in 50 µl using DNA polymerase (5U $\mu l^{-1}$) 10X buffer (Favorgene, Taiwan). PCR amplifications were carried out in a DNA thermal cycle for 35 cycles (each consisting of 1 min at 95°C, 30 sec at 55°C, 30 sec 65°C and 2 min at 72°C). Two steps in 5 min at 95°C and 7 min at 72°C steps were added before and after main amplification step. 5µl PCR product was mixed in total volume of 10µl using 0.1 volume buffer, 0.1 volume BSA and 1µl *Bam*HI (5 U $\mu l^{-1}$) restriction enzyme. Restriction reaction was performed at 37 °C for 3 hours and run on 2% agorose gel. TT genotypes create no restriction site but CC genotype create *Bam*HI restriction site in 73 bp length.

**Calculation of Gene Frequency:** Calculation of allele and genotypes frequencies was based on direct counting method by

Frequency of leptin C gene; $P(C) = p = \frac{2N_{CC} + N_{CT}}{2N}$,

Frequency of leptin T gene; $P(T) = q = \frac{2N_{TT} + N_{CT}}{2N}$,

The standard error of gene frequencies was calculated as;

$$\sigma_q = \sqrt{\frac{q(1-q)}{2N}}$$

where, N is sample size, p is the frequencies of the C allele, q is the frequencies of the T allele, and $N_{CC}$, $N_{CT}$ and $N_{TT}$ are numbers of CC, CT and TT types, respectively (Vanlı *et al*. 2009).

The frequencies of the alleles and genotypes were estimated, and chi-square analysis was performed to test whether the genotype distributions obtained were in accordance with the Hardy-Weinberg equilibrium. Allel frequencies between breeds were compared using chi-square analysis (SAS, 2002).

**Calculation of standard genetic distance and the normalized equivalence:** The data were further analyzed with a similarity matrix and a dendrogram using Nei's similarity indices. The Popgen software (version 1.44) was used to construct the dendrogram and similarity matrices.

For the estimation of genetic distance among populations, original genetic distance (D) method was used.

Normalized equivalence: $I_{XY} = \frac{J_{XY}}{\sqrt{J_X * J_Y}}$

Standard genetic distance: $D_{XY} = - ln (I_{XY})$

Where; $J_X$: average homozigothy in population, $J_Y$: average homozigothy in population y , $J_{XY}$: represents the average homozigothy between populations (Vanlı *et. al.,* 2009).

**Statistical Analysis:** Effects of leptin genotype on the chest circumference measurements were analyzed using PROC GLM of SAS (2000), using the following model;

$Y_{ijkl} = \mu + A_i + L_j + C_k + e_{ijkl}$

where

$Y_{ijkl}$ is the chest circumference measurements on individual, µ is the overall mean for the trait, $A_i$ is the effect of the $i^{th}$ age, $L_j$ is the effect of the $j^{th}$ leptin genotype, $C_k$ is the effect of $k^{th}$ sex, $e_{ijkl}$ is the residual error.

Differences between mean values of the traits were tested with Duncan's multiple range test.

## RESULTS AND DISCUSSION

**PCR Analysis of leptin gene:** PCR reaction started with 5, 10, 20, 30, 50, 75 and 100 pmol (forward and reverse primers) to determine the optimum primer concentration. The best results were observed at 20 pmol. After cutting three different results were observed in the gel photographs. First band was observed at 106 bp length in which cutting did not take place whereas the second cutting occurred at bands with 84 and 19 bp length, and the third at 106, 84 and 19 bp in length (Figure 1).

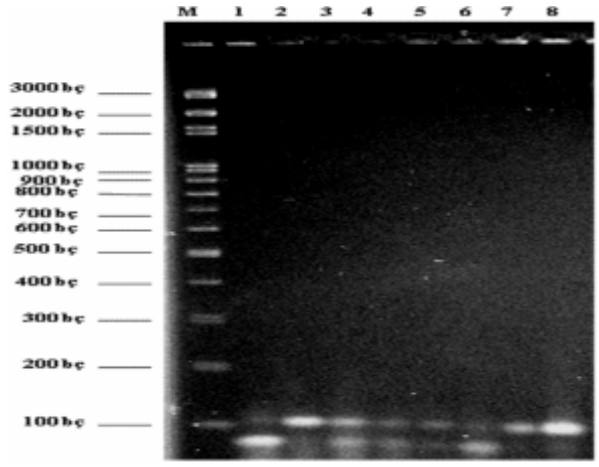


**Figure 1. The gel analysis after cutting reaction, M: 100 bp marker**

**Allele and genotype frequencies**: The number of individuals with genotypes and allele frequencies for this polymorphism of leptin gene are shown in Table 1.

**Table 1. Calculation of Allele and genotype frequencies**

| Breed | Genotype | | | | Gene frequency | |
|---|---|---|---|---|---|---|
| | CC | CT | TT | Total | p (C) | q (T) |
| East Anatolian Red | 8 | 19 | 11 | 38 | 0.46±0.06 | 0.54±0.06 |
| Anatolian Black | 11 | 25 | 9 | 45 | 0.52±0.05 | 0.48±0.05 |
| Brown Swiss | 3 | 9 | 4 | 16 | 0.47±0.09 | 0.53±0.09 |

In case of the leptin, the CC, CT and TT genotype frequency was 0.21CC and 0.50CT and 0.29TT in the East Anatolian Red cattle, 0.24CC, 0.56CT and 0.20TT in Anatolian Black Cattle, 0.19CC, 0.56CT and 0.25TT in Brown Swiss Cattle. The C and T allel frequency was 0.46 and 0.54 for East Anatolian Red cattle, 0.52 and 0.48 for Anatolian Black Cattle, 0.47 and 0.53 for Brown Swiss Cattle.

In East Anatolian Red cattle allele frequency for T (p=0.54) was higher than values 0.32 and 0.34 reported by Buchanan *et al.*(2002) for Simental and Charolois breeds,0.06, 0.11, 0.45 and 0.46 reported by Buchanan *et al.* (2002) for Guernsey, Canadienne Brown Swiss and Holstein breeds, 0.46 reported by Komisaret *et al.* (2005) for Holstein breeds, 0.29 and 0.45 reported by Nassiry *et al.*(2007) for Golpayegani and Taleshi breeds, 0.29, 0.31, 0.32, 0.43 and 0.45 reported by *Nassiry et al.*(2008) for Golpayegani, Sistani, Sarabi, Holstein, Taleshi and Brown Swiss breeds, 0.28 reported by Kulig *et al.*(2009) for Jersey breeds, 0.25 reported by Anton *et al.*(2011) for Angus breeds,

On the other hand, present results agree with Buchanan *et al.*(2002,2003) who had reported T allele frequencies of 0.53, 0.55 and 0.58 in Jersey, Hereford and Angus cattle.

In this study, p = 0.48 of value for the T allele frequency of leptin for Anatolian Black breed was higher than values 0.32 and 0.34 reported by Buchanan *et al.*(2002) for Simental and Charolois breeds, 0.06 and 0.11 reported by Buchanan *et al.* (2002) for Guernsey and Canadienne, 0.29 reported by Nassiry *et al.*(2007) for Golpayegani breeds, 0.29, 0.31, 0.32, reported by Nassiry *et al.*(2008) for Golpayegani, Sistani, Sarabi, breeds, 0.25 reported by Anton *et al.*(2011) for Angus breeds.

On the other hand, the T allele frequency of leptin in this study, was found to be similar to the findings of Buchanan *et al.* (2003) reported that 0.45 and 0.46 for Brown Swiss and Holstein breeds, Komisarek *et al.*(2005) reported that 0.46 for Holstein breeds, Nassiry *et al.*(2007, 2008) reported that 0.45 for Taleshi breeds.

The frequency for T allele of leptin gene was comparatively higher in East Anatolian Red and Anatolian cattle breeds than reported for other native and exotic cattle breeds. This might be explained breeding method, which is used in these region.

Sample collected region is a transition region. Crossbreeding with other race and migration are very common. So frequency of T alleles was found very close to common races.

**Hardy-Weinberg Equilibrium Test:** In a diploid population with two alleles, to check Hardy-Weinberg equilibrium, it was found firstly observed numbers of individuals with CC, CT and TT genotype ($N_{CC}$, $N_{CT}$, and $N_{TT}$), respectively.

In this study, the observed and expected values for T and C alleles were close to each other. Values of $\chi^2$ test for hypothesis of the fitness to Hardy-Weinberg equilibrium were calculated to be 0.0, 0.56 and 0.25 for East Anatolian Red, Anatolian Black and Brown Swiss populations respectively (Table 2). The genotype frequencies were insignificant distributed according to Hardy-Weinberg equilibrium proportions in every three herd.

**Table 2. Observed and expected homozygous and heterozygous genotype frequencies in East Anatolian Red, Anatolian Black and Brown Swiss populations**

| | Observed | | | Expected | | | Total | $\chi^2$ |
|---|---|---|---|---|---|---|---|---|
| | CC | CT | TT | CC | CT | TT | | test |
| East Anatolian Red | 8 | 19 | 11 | 8 | 19 | 11 | 38 | $0.0^{ns}$ |
| Anatolian Black | 11 | 25 | 9 | 12.15 | 22.5 | 10.35 | 45 | $0.56^{ns}$ |
| Brown Swiss | 3 | 9 | 4 | 3.5 | 8 | 4.5 | 16 | $0.25^{ns}$ |

n.s : non-significant

**Calculation of normalized equivalency and standard genetic distance:** Blood samples from 16 Brown Swiss cows were taken to calculate the normalized equivalence and standard genetic distance in this study.

Calculation of normalized equivalency and standard genetic distance between East Anatolian Red and Brown Swiss and between Anatolian Black and Brown Swiss is presented in Table 3.

The normalized equivalency with respect to leptin gene polymorphism, between East Anatolian Red and Brown Swiss, and between Anatolian Black and Brown Swiss were found relatively high (0.9998 and 0.995).

For many years, at the region Suşehri of Sivas province, Brown Swiss bulls were used intensively. As a result of cross breeding with Brown Swiss and indigenous breeds such as Eastern Anatolian and Anatolian Black, standard genetic distance between the races were close to one. In addition, in this reason

heterozygosity level was also found higher than the expected level.

**Table 3. Calculation of normalized equivalency and standard genetic distance**

| | East Anatolian Red and Brown Swiss | Anatolian Black and Brown Swiss |
|---|---|---|
| Normalized equivalency | 0.9998 | 0.995 |
| Standard genetic distance | 0.0002 | 0.005 |

It is suggested that, crossbreeding have been done between these breeds or with culture breeds to increase the frequencies of the favorable genotype. For example, crossing of the Eastern Anatolian or Anatolian Black with the Brown Swiss stock has increased T allele.

**Relationship between Body measurements and polymorphism of leptin:** Relationship between leptin polymorphism and chest circumference was also examined by measuring chest circumference (cm) of 34 East Anatolian Red cattle and 34 Anatolian Black cattle. Means of genotypes, standard errors and significance of test results are given in Table 4.

**Table 4. Chest circumference measurements according to Leptin genotype**

| | East Anatolian Red | | Anatolian Black | |
|---|---|---|---|---|
| | N | Mean±SE | N | Mean±SE |
| Total genotype | 34 | 139.47±3.53 | 34 | 138.000±18.855* |
| CC | 9 | 134.71±7.91 | 8 | 133.125±5.975[a] |
| CT | 18 | 138.56±4.92 | 20 | 137.450±4.384[a] |
| TT | 7 | 145.00±6.97 | 6 | 153.000±7.831[b] |

SE = Standard Error, means bearing the different superscripts in a column differ significantly (P<0.05)

As shown in the Table 4, effect of leptin T allele polymorphism on the chest circumference was non-significant in East Anatolian Red breed. However, it was found significant in Anatolian Black breed (P<0.05). Although order of genotypes is similar to as reported by Kulig and Kmiec (2009), there was no statistical relationship between different genotypes of leptin gene in this study.

Heterozygous or homozygous genotypes carrying T-allele has a wider chest circumference. Chest circumference values of animals with TT genotype in Anatolian Black breed were higher than values of the other genotypes (CC and CT). These results indicate that the leptin TT genotype is associated with increased chest circumference. These results were similar to results reported in Limosin cattle breed (Kulig *et. al.,* 2007; Kulig and Kmiec, 2009). However, the small size of data makes it difficult to do a true assessment and these results are to be confirmed through studies with larger dataset.

It is therefore suggested that selection on the basis of TT genotype in Anatolian Black breed will bring improvement in body weight. However, further research on associations between the leptin genotype and growth traits in cattle in necessary before using the results in selection program.

In this study, allele frequencies, genetic structure and genetic polymorphisms in leptin gene of East Anatolian Red, and Anatolian Black cattle breeds the most common local breeds in Turkey were investigated. These preliminary results will be helpful for future studies not only these breeds but also on other breeds of the region genetic classification and genetic definition.

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