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Characterization of Bubaline Leptin Gene Polymorphism in Anatolian Buffaloes By Using PCR-RFLP Method

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Abstract: Leptin is a hormone which produces 16 kDa protein predominantly expressed in adipose tissue. Leptin gene has pleiotropic effects on several functional traits such as milk yield, carcass and meat quality, growth, feed intake and fertility. Therefore, this study was performed to identify single nucleotide polymorphism of bubaline leptin gene in Anatolian buffaloes. In this study, 513 bp of leptin DNA fragment was amplified. A PCR-RFLP analysis was used to identify bubaline leptin gene T1131G polymorphism. DdeI restriction enzyme was used to genotype bubaline leptin gene T1131G polymorphism in Anatolian buffaloes. The analysis revealed TT, GT and GG genotypes in Anatolian buffaloes. Allele frequencies of T and G were found 0.478 and 0.521, respectively. This study is the first report of identification of bubaline leptin gene T1131G polymorphism by using PCR-RFLP method in Anatolian buffaloes.

Keywords: Leptin, Anatolian Buffalo, SNP, PCR-RFLP

PCR-RFLP Metodu Kullanılarak Manda Leptin Geni Polimorfizminin Anadolu Mandalarında Karakterizasyonu

Öz: Leptin yağ dokusunda ağırlıklı olarak ifade edilen 16 kDa protein üreten bir hormondur. Leptin geni, süt verimi, karkas ve et kalitesi, büyüme, yem tüketimi ve üreme gibi çeşitli fonksiyonel özellikler üzerine pleotropik etkilere sahiptir. Bu nedenle, bu araştırma Anadolu mandalarındaki manda leptin geninde tek nükleotid polimorfizmini belirlemek amacıyla yapılmıştır. Çalışmada, Leptin geninde 513 baz çiftlik bir bölge çoğaltılmıştır. 513 baz çiftlik fragment içerisinde T1131G polimorfizmini belirlemek üzere PCR-RFLP yöntemi kullanılmıştır. Anadolu mandalarında manda leptin geni T1131G polimorfizminin belirlenmesi için DdeI restriksiyon enzimi kullanılmıştır. Yapılan PCR-RFLP analizi sonucunda ele alınan Anadolu mandalarında TT, GT ve GG genotipleri belirlenmiş ve T ve G allel frekansları sırasıyla 0.478 ve 0.521 olarak bulunmuştur. Bu çalışma, Anadolu mandalarında PCR-RFLP yöntemi ile manda leptin geni T1131G polimorfizminin tanımlanmasına ilişkin ilk çalışma sonuçlarıdır.

Anahtar Kelimeler: Leptin, Anadolu mandası, SNP, PCR-RFLP

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1. INTRODUCTION

Molecular genetics is a significant tool for characterization of genes and identification of genetic variations of individuals. However, substantial developments have been occured in molecular genetics in the last past few decades. Following these developments, there have been great interest for using molecular markers in livestock breeding programs. Therefore, using molecular data in breeding programs have made great contribution not only to improve efficiency and adaptability to environmental conditions but also to continue genetic diversity of livestock animals (Dekkers, 2002; Naqvi, 2007).

Cattle breeds have been widely studied in animal genetics studies due to their economic affects on agricultural economy. And the main concerns of these studies are to identify genetic markers associated with economical important traits in cattle. Therefore, many significant genetic marker such as Leptin (Buchanan et al., 2003), DGAT1 (Grisart et al., 2002), Growth hormone (Høj et al., 1993), IGF1 (De la Rosa Reyna et al., 2010), Calpastatin (Schenkel et al., 2006), Myostatin (Grobet et al., 1997) FABP3 (Cho et al., 2008) have been reported in dairy and beef cattle. However, buffalo and cattle have strong genotypic and phenotypic similarities. And, these species are the members of subfamily of Bovinae (Scherf, 2000; Bondoc, 2013). Surprisingly, there were very limited genetic study about identification of genetic markers associated with economical important traits in buffalo breeds.

Water buffaloes are divided two distinct classes called as the river and swamp buffalo. The river buffalo is generally raising for milk production distributed in Indian sub-continent. And the swamp buffalo which is distributed in South East Asia is raising for farming purposes (Cockrill, 1981; Albarella et al., 2017). Anatolian buffaloes are originated from Mediterranean type buffaloes which are subgroups of water buffaloes. Anatolian buffaloes are mainly distributed middle of Black Sea, North of Middle Anatolia and Thrace region. The morphological characteristics of the Anatolian buffaloes are the black in colour, frequent white switch, long hair, 138 cm height and 200-500 kg body weight (Borghese and Mazzi, 2005; Soysal et al., 2007).

Leptin is a hormone which produces 16 kDa protein predominantly expressed in adipose tissue (Frühbeck, 2001). Leptin gene was first clonned in human and mouse in 1994 (Zhang et al., 1994). Leptin gene has pleiotropic effects on several functional traits such as milk yield (Komisarek et al., 2005), carcass and meat quality (Schenkel et al., 2005), growth (Kulig and Kmieć, 2009) feed intake (Lagonigro et al., 2003) and fertility (Clempson et al., 2011). Buffalo leptin sequence have significant homology with Ovis aries, Bos taurus, Capra hircus, Bos indicus and Homo sapiens as 99%, 97%, 98%, 97% and 80% respectively. (Datta et al., 2012). Therefore, genetic characterization of Buffalo leptin gene can also provide valuable informations for other species. So, the main aim of this study was to characterise the leptin gene polymorphism in Anatolian buffaloes.

2. MATERIAL AND METHODS

Tissue Samples

A total of 70 Anatolian Buffalo muscle tissue samples were collected after slaughtering and stored at -20 °C in a deep freezer until molecular genetic studies are performed.

DNA isolation and amplification

The genomic DNA was isolated within the muscle tissue using the GeneMatrix tissue DNA purification kit (Eurx, E3551) as the manufacturer's instructions. The primer pair (Table 1) amplifying the 513 bp DNA fragment (containing the exon 2 and part of the intron 1, intron 2) was designed using the Primer 3 (NCBI) program PubMed (accession AH013754.2). PCR amplifications were performed with the PCR master mix (Thermo, K0171) in accordance with the manufacturer's instructions. The PCRs were carried out in volumes of 25 µl using; 12,5 µl Pcr Master Mix, 50 ng (5 µl) genomic DNA, 1 µl (5 pmol) each primer, and the rest was ddH2O. The amplification was performed at 95 °C for 3 min, followed by 35 cycles at 95 °C for 30 sec, annealing at 66 for 30 sec, 72 °C for 45 sec and a final extension of 72 °C for 10 min on T100 Thermal Cycler (Biorad). The PCR products were subjected to electrophoresis on 2 % agarose/ethidium bromide gel (Aga003R, Bioshop, Canada) in 1× TBE buffer (TBE-001, New Bioscience). Gels were visualized under UV light and documented in WGD30S Molecular Imager apparatus (Wisd).

Table 1. Primer pair

Primer Code	Primer Sequence
Lep F	TCATGCCCTGGCTTACTGC
Lep R	AGGCTGCACAGCTTCCTC

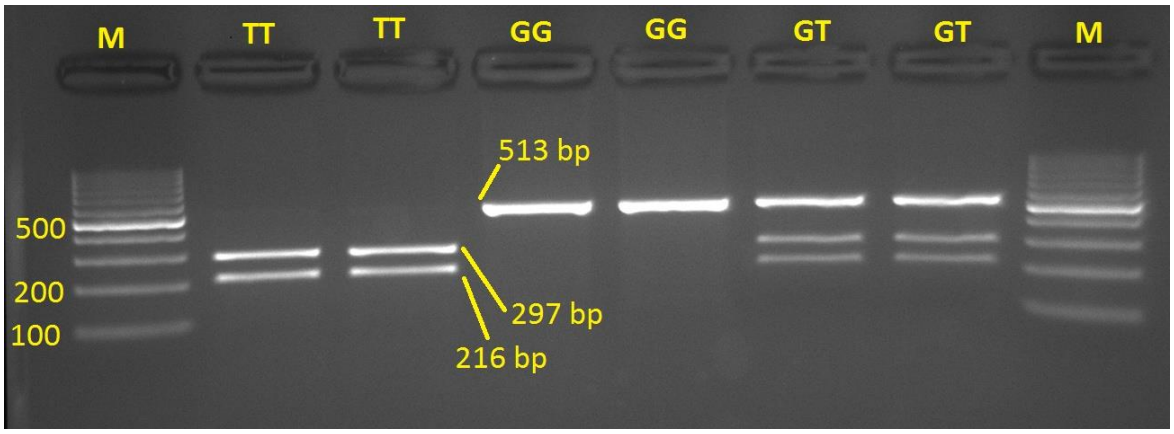
PCR-RFLP analysis

For Lep/DdeI genotyping, 20 µl of PCR product were digested with 0,5 µl (5 U) of DdeI (R0175S-New England Biolabs) restriction enzymes at 37°C for 6 hours. DdeI enzyme cuts the T allele, not the G allele. This enzyme was detected using the NEBcutter V2.0 program (Biolabs). The restriction fragments were subjected to electrophoresis on 2% agarose/ethidium bromide gel in 1× TBE buffer. Gels were visualized under UV light and documented in WGD30S Molecular Imager apparatus (Figure 1). Restriction enzyme, the size of PCR products and genotypes are given in Table 2.

Table 2. Restrictions enzyme, PCR product and genotypes

Gene	PCR Products (bp)	Restriction Enzyme	Restriction Product (bp)	Genotypes
			297, 216	TT
LEP	513	DdeI	513, 297, 216	GT
			513	GG

Figure 1. The restriction products on agarose gel electrophoresis.



Statistical Analysis

In this study, the Chi-square test was used to determine whether genotype frequencies of all polymorphism were in Hardy Weinberg equilibrium estimated by (Yeh et al., 1999).

3. RESULT AND DISCUSSION

This study was aimed to identify the bubaline leptin gene polymorphism rs119028128 (T1131G) in Anatolian buffalo breeds (accession GenBank). Therefore, the 513 bp DNA fragment (containing the exon 2 and part of the intron 1 and intron 2) of bubaline leptin gene in Anatolian buffaloes were amplified. And this PCR product was genotyped with PCR-RFLP method by using DdeI restriction enzyme. In the current study, three genotypes were identified by using PCR-RFLP method (GG, GT and TT) in Anatolian buffaloes. The allele frequencies of Lep/DdeI polymorphism were calculated according to Hardy-Weinberg equilibrium. Statistical analysis revealed that the bubaline leptin gene polymorphism rs119028128 (T1131G) was not in agreement with Hardy Weinberg equilibrium in Anatolian buffaloes ($P<0.05$). (Table 3). It is estimated that inbreeding, population size and structure may be the reasons of this deviation.

Table 3. Statistic analysis of bubaline leptin gene (T1131G) loci in Anatolian buffaloes

			Heterozygosity		
Loci	**Allele Frequencies**		**Observed H.**	**Expected H.**	**Chi-square**
T1131G	0.478 (T)	0.521 (G)	0.1286	0.5027	39.334874[S]

[1] $\chi^2_{0.05;1}$; 3,84 test of Hardy-Weingberg equlibrium, S; Deviation from Hardy-Weinberg equlibrium is significant

In the current study, allele frequencies of T and G (leptin T1131G polymorphism) in Anatolian buffalo were determined as 0.478 and 0.521, respectively. However, there was only one study about bubaline leptin gene (T1131G) polymorphism in buffalo breeds (Orru et al., 2007). Orru et al. (2007) reported bubaline leptin gene T1131G polymorphism allele frequencies of T and G in Italian and Egyptian Buffaloes (0.360-0.640), respectively. This result is in agreement with the current study. The researchers were also revealed eleven single nucleotide polymorphisms (G3333A, C1221T, G3195A, C1221T, G3434A, T1015C, C1071T, G1072A, T1081C, T1143C, T1145G) in Italian and Egyptian buffalo breeds. In Mehsana buffalo, Jhala et al. (2009) determined three SNP in the nucleotide positions of 42, 44 and 250 of exon 3 of bubaline leptin gene. Tanpure et al. (2012) stated that there were five SNP (98, 111, 172, 209. 266) in intron 1 region of bubaline leptin gene in Mehsana buffalo. In swamp and river buffalo, the researchers reported a SNP (137(G/A)) in the promoter region and two SNP the nucleotide positions of (276 and 384) exon 3 region of bubaline leptin gene (Vallinoto et al., 2004). Scatà et al. (2012)studied the 5' flanking and exon 1 region of bubaline leptin gene in Mediterranean Italian water buffaloes. They determined eight polymorphism in nucleotide positions of A83G, A90G, A121G, G256T, A283G, G959T, A1010C, G1254A. Adikari, (2006) studied the intron region of leptin and detected the polymorphic sites at position 11, 365, 369 and 371 in Murrah buffaloes. There were also several reports related to the single nucleotide polymorphisms in different regions of the leptin gene in cattle breeds. Schenkel et al. (2005) have genotyped 1111 crossbred bulls to identify the variations of leptin gene. They reported five SNPs (UASMS1, UASMS2, UASMS3, E2JW, E2FB) in exon 2 and promoter region of leptin gene. The other study researchers reported two new mutations in exon 2 of bovine leptin gene that caused Alanine-Valine and Glutamine-Arginine aminoacid substitution (Haegeman et al., 2000). Lagonigro et al. (2003) studied the exon 2

and exon 3 region of bovine leptin gene. They demonstrated five polymorphism in these region. And the polymorphism in exon 2 (the nucleotide position at 252) have been found significantly associated with feed intake. Konfortov et al. (1999) indicated that there were twenty single nucleotide polymorphism in 1788 bp sequence of bovine leptin gene. Liefers et al. (2003) investigated the exon 2 and exon 3 region of bovine leptin gene in Holstein cattle. And four polymorphic site (R4C, A59V, RFLP1, BM1500) of bovine leptin gene have been identified in this study.

4. CONCLUSION

The main goal of animal genetic studies are to identify genetic markers associated with the economically important traits of livestock animals. Therefore, candidate gene researches are providing new insights for animal genetic studies. Leptin gene has many important biological functions that effects the economically important traits of livestock animals. Oving to having key biological features leptin is one of the most significant candidate gene for animal genetic studies. Using candidate genes such as leptin in animal breeding programs not only can provide opportunity to select the animals in early age but also increase the accuracy within breeding value estimation of animals. Therefore, this study was designed to reach new genetic information related to bubaline leptin gene in Anatolian buffaloes. Taken together, this study was firstly identified the bubaline leptin gene T1131G polymorphism in Anatolian buffaloes.

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