Polymorphism in Exon 7 of β- Lactoglobulin (*β-LG*) Gene and Its Association with Milk Yield in Saanen Goats

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β-Lactoglobulin (*β-LG)* is one of milk protein and has important function on technological properties of milk such as cheese making. The relations between whey protein genes and milk yield/ composition have been investigated in previous researches. *β-LG* can be utilized as a candidate gene for selection and breeding programs to increase milk yield and protein quality. The aim of this study is to investigate the *β-LG* gene polymorphism and relation between *β-LG* genotypes and milk yield. In this study, a total of 74 purebred Saanen goats originated from Australia were used to detect polymorphism with PCR-RFLP method. *SacII* digestion in 427 bp of *β-LG* exon 7 (GenBank: Z33881.1) was revealed. Two alleles (S_1, S_2) and 3 genotypes (S_1S_1, S_1S_2, S_2S_2) were determined in *β-LG*/ *SacII* locus. The *β-LG*/S_1S_1 genotype with only one restriction site revealed two bands (347 bp and 80 bp). The *β-LG*/S_1S_2 heterozygote genotype generated three bands (427 bp, 347 bp and 80 bp). An undigested product, 427 bp was β-LG/S_2S_2 genotype which was due to a single nucleotide substitution at position g.4601G>A. S_1S_2 with genotype frequency (43.3%) higher than the other genotypes. S_1 allele frequency was determined predominantly. Deviation from Hardy-Weinberg equilibrium was not identified in the Saanen breed. In *β-LG/ SacII* locus, a significant relationship was not found between genotypes and lactation milk yield. However, *β-LG*/ S_1S_1 genotype was observed to have higher lactation milk yield. It is concluded that *β-LG* gene could be used as a molecular marker for economic traits such as milk yield and composition.

Keywords: *β-LG*, PCR-RFLP, Saanen, SNP, *SacII*, milk yield

Saanen Keçilerinde β- Laktoglobulin (*β-LG*) Geni Ekzon 7 Polimorfizmi ve Süt Verimi ile İlişkisi

Süt proteinlerinden biri olan B-Laktoglobulin *(β-LG*), peynir yapımı gibi sütün teknolojik özellikleri üzerinde önemli bir fonksiyona sahiptir. Serum protein genleri ile süt verimi/bileşimi arasındaki ilişkiler daha önceki çalışmalarda araştırılmıştır. *β-LG*, süt verimi ve protein kalitesini artırmak için seleksiyon ve ıslah programları için aday bir gen olarak kullanılabilmektedir. Bu çalışmanın amacı, *β-LG* gen polimorfizmini ve *β-LG* genotipleri ile süt verimi arasındaki ilişkiyi araştırmaktır. Bu çalışmada, PCR-RFLP yöntemi ile polimorfizm belirlenmesi amacıyla Avustralya kökenli toplam 74 safkan Saanen keçi kullanılmıştır. *SacII* restriksiyon enzimi ile *β-LG* geni 427 baz çiftlik yedinci ekzonu (GenBank: Z33881.1) genotiplenmiştir. *β-LG/SacII* lokusunda iki alel (S_1, S_2) ve üç genotip (S_1S_1, S_1S_2, S_2S_2) belirlenmiştir. *β-LG*/S_1S_1, genotipi tek kesim bölgesine sahip olduğundan iki bant vermektedir (347 bç ve 80 bç). *β-LG*/S_1S_2 heterozigot genotipi üç bant oluşturmaktadır (427 bp, 347 bp ve 80 bp). *β-LG*/S_2S_2 genotipi g.4601G> A pozisyonunda tek nükleotid değişiminden dolayı 427 bç uzunluğunda kesilmemiş ürün vermektedir. S_1S_2 genotip frekansı (% 43.3) diğer genotiplerden daha yüksektir. S_1 allel frekansı predominant olarak belirlenmiştir. Saanen ırkında Hardy-Weinberg dengesinde sapma gözlenmemiştir. *β-LG/ SacII* lokusunda, genotipler ile laktasyon süt verimi arasında önemli bir ilişki bulunmamıştır. Ancak *β-LG*/ S_1S_1 genotipinin daha yüksek laktasyon süt verimine sahip olduğu belirlenmiştir. *β-LG* geninin süt verimi ve kompozisyonu gibi ekonomik özellikler için moleküler marker olarak kullanılabileceği sonucuna varılmaktadır.

Anahtar Kelimeler: *β-LG*, PCR-RFLP, Saanen, SNP, *SacII*, süt verimi

Introduction

The world goat population, 95% of which is found in Asian and African countries, is estimated to be about 1 billion in 2015. In the last 10 years, world goat population has increased by 10% and similarly increased by 27% in Turkey (FAO, 2016). Especially goat breeding is localized intensively in Mediterranean, Aegean and South-Eastern Anatolia regions of Turkey. Generally, indigenous breeds are reared such as Hair, Angora, Cashmere, Norduz, Honamlı goat but Saanen and its hybrids with local breeds have become widespread in recent years. Saanen goat is used for the breeding of native breeds since the milk yield is high.

Milk is an important food for human nutrition. Milk proteins consist of 80 % casein (α-s1, α-s2, β and κ-casein) and 20% serum proteins (β-lactoglobulin, α-lactalbumin and others). serum protein occurs; β-lactoglobulin (50%), α-lactalbumin (20%), serum albumin (10%), immunoglobulins (10%) and proteose-peptones (10%) (Gür et al., 2010). *β-LG* is a protein that has dimer form and its molecular weight is 36.4 kDa in ruminant milk (Hambling et al., 1992). *β-LG* gene is localized on chromosome 11 in goat and cattle genome, on chromosome 3 in sheep genome (Hayes and Petit, 1993). The *β-LG* transcription unit consists of 7 exons (42-178 bases), 6 introns (213-1116 bases) and 4.7 kb length (Jain et al., 2012). Various investigations have been made to elucidate the variation in the gene regions coding for milk proteins (α-s1, α-s2, β- and *κ-casein*, *β-lactoglobulin*, *α-lactalbumin*) and to identify variants in these genes (Folch et al., 1993;1994; Sánchez et al., 2005). One of these milk protein, *β-LG,* was investigated on protein and DNA levels (Aschaffenburg and Drewry 1955; Eigel et al., 1984; Gaye et al., 1986; Erhardt, 1989; Özdil and Asal, 2002; Lekerpes et al., 2014). Firstly, two different variants have been identified for the goat-lactoglobulin gene at the molecular level by Pena *et al.,* (2000) in Spanish and French goats. Pena *et al.,* (2000) examined on exon 7 and 3 'flanking region by PCR-RFLP analysis and they detected the presence of a G> A base transition (5 'CCAC'GG 3') which caused the *SacII* restriction enzyme target site (5 'CCGC'GG 3'). Then, Graziano et al., (2003) identified a new transition (T>C) mutation at 341. position in the promoter region of the goat *β-LG* gene. Kumar et al., (2006) investigated *β-LG* gene polymorphism in eight different goat breeds grown in India and identified three different genotypes. Rout et al., (2010) observed two different variants of this gene, A and B, in the same breeds. Chen et al., (2005) detected a variant of the *β-LG* gene in the 5' flanking region in Xinong Saanen goats. At the same time, polymorphisms of *β-LG* gene and their effect on milk yield and composition have been studied in farm animal (Chen et al., 2005; El-Hanafy et al., 2010; El Shazly et al., 2012; Kahilo et al., 2014; Selvaggi et al., 2015; Gharedaghi et al., 2016). Also, polymorphism of *β-LG* was studied by Elmacı *et al.* (2009), Ağaoğlu et al. (2012), Yüksel and Akyüz (2014) in some Turkish local goat breeds.

The aim of this study is to investigate the *β-LG* gene polymorphism and relation between *β-LG* genotypes and milk yield by using PCR-RFLP method in Saanen goats.

Materials and Methods

Blood samples and DNA isolation

A total of 74 blood samples were collected from Saanen goats around İzmir province, Turkey. Blood samples from goats were placed into an EDTA evacuated blood collection tubes, transported to the laboratory and storaged in -20 °C until analysis. Genomic DNA was isolated from whole blood using genomic DNA Purification Mini Kit (GeneJET Whole Blood Genomic DNA Purification Mini Kit, Thermo Fisher Scientific) following the manufacturer's protocol. DNA concentration and purity were examined on 1% agarose gels and UV spectrophotometer at 260/280 nm.

Milk recording and statistical analysis

Goats were reared at Ege University, Faculty of Agriculture, Department of Animal Science, Small Ruminant Animal Application and Research Unit. Milk yield was recorded 2 times per day during the lactation. Total milk yield was statistically analysed by ANOVA at significance level ($P<0.05$). Genotypes were determined by direct counting of restriction fragments observed in the gel. Genotype frequency was estimated. The allele frequency of *β-LG* and Hardy-Weinberg equilibrium of the population were calculated in the PopGene (Yeh et al., 2000). Association between *β-LG* genotypes and milk yield was estimated in SPSS program using one way ANOVA (SPSS Inc. V. 18.0, IBM, Chicago, IL, 2009).

DNA amplification and genotyping

A region of the *β-LG* gene spanning over exon 7 to 3' flanking area was amplified by Polymerase Chain Reaction according to Pena et al., 2000. The primer sequences of the β-lactoglobulin gene (accession number Z33881.1) were: forward 5'-CGG GAG CCT TGG CCC CTC TGG-3'; reverse 5'-CCT TTG TCG AGT TTG GGT GT-3'. PCR amplification of *β-LG* gene was carried out in 25 µl reaction mixture, containing 2 mM $MgCl_2$, 200 µM of each dNTPs, 0.5 µM each primer, 1 X PCR buffer, 1U Taq polymerase (i-star Taq DNA Polymerase, Intron) and 100 ng of genomic DNA template. The thermal cycling conditions were as follows: Pre-denaturation at 95°C for 5 min, 35 cycles; denaturation at 94°C for 30 sec, annealing at 65°C for 60 sec and extension at 72°C for 90 sec, followed by a final extension at 72°C for 5 min. Then, for the RFLP analysis on seventh exon which is 427 base pairs length of *ß-LG* gene was digested using *SacII* restriction enzyme (ER0201, Thermo Fisher Scientific) at 37°C

for at least 3 h. PCR products and restriction fragments were electrophoresed on 2,5 % agarose gel stained with SafeView™ Classic (abm) and visualised on UV transilluminator.

Sequencing analysis

In order to verify the *β-LG* fragments which revealed different genotypes, PCR products were sequenced on capillary electrophoresis (ABI 3130XL Genetic Analyzer, USA), Genmar Laboratories (İzmir). The sequences of three genotypes were analyzed using the Molecular Evolutionary Genetics Analysis (MEGA6) software and ClustalW sub-programme of the BioEdit Sequence Alignment Editor (BioEdit Version 7.2.5, 2013).

Results and Discussion

β-LG is one of milk protein and has important function as major whey protein technological properties of milk (such as cheese making) in ruminants and several non-ruminant species (Schaar, 1985; Perez and Calvo, 1995). After the genetic polymorphism in milk proteins was described by Aschaffenburg and Drewry (1955), researches focused on association between *β-LG* polymorphism and milk production. Polymorphisms of some protein variants have been found not only in the open reading frame of the *β-LG* encoding gene (Godovac-Zimmerman et al., 1996) but also in non-coding areas such as the 3' flanking region (Wagner et al., 1994).

In this study, genetic polymorphism of the *β-LG* gene and the relations between the *β-LG* genotype and milk yield were investigated by PCR-RFLP method in Saanen goats. Exon 7 to the 3'flanking region of *β-LG* gene (427 bp) was amplified and digested with restriction endonuclease *SacII* to detect S_1 or S_2 variants. As a result of amplification product with *SacII* digestion, two alleles (S_1 and S2) with three different restriction patterns or genotypes (S_1S_1, S_1S_2 and S_2S_2) were observed. S_1S_1, S_1S_2 and S_2S_2 genotypes have been entitled as BB, AB, AA respectively in some studies (Ağaoğlu et al., 2012; Yüksel and Akyüz 2014).

The *β-LG* genotype S_1S_1 with only one restriction site revealed two bands of sizes 347 bp and 80 bp. An undigested product of size 427 bp termed as *β-LG* S_2S_2 genotype was also obtained (Fig. 1). The small restricted fragment at 80 bp do not appear on agarose gel. The presence of 427 bp and 347 bp bands makes it possible to identify heterozygous individuals.

The results of sequence analysis of a heterozygous genotype (S_1S_2) and homozygous genotypes (S_1S_1 and S_2S_2) were shown in Figure 2.

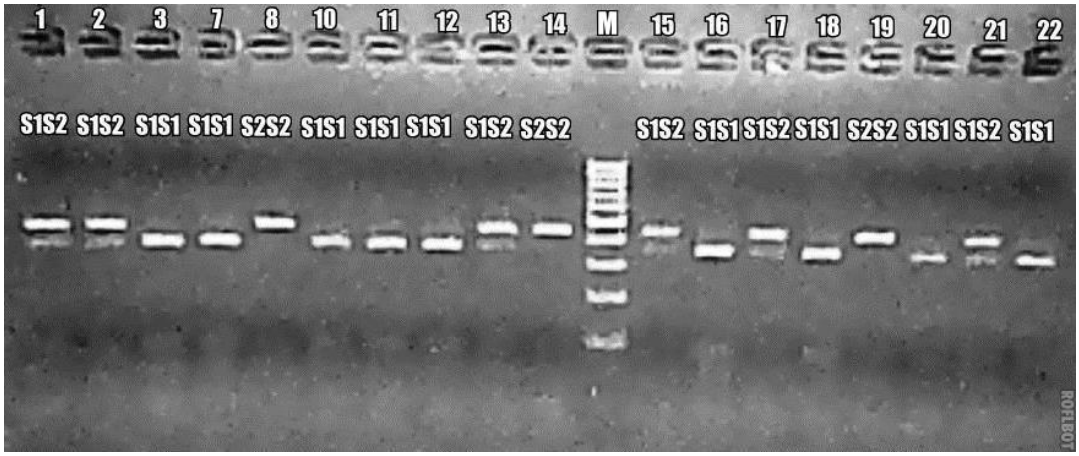

Fig 1. Electrophoresis of RFLP of caprine *β-LG* gene after digestion by *SacII* of animals with S_1S_2 (Lane 1,2,13,15,17,21; 427bp/347bp/80bp), S_1S_1 (Lane 3,7,10,11,12,16,18,20,22; 347bp/80bp), S_2S_2 (Lane 8,14,19; 427bp) genotypes. Lane M, molecular size marker (100 bp DNA ladder)

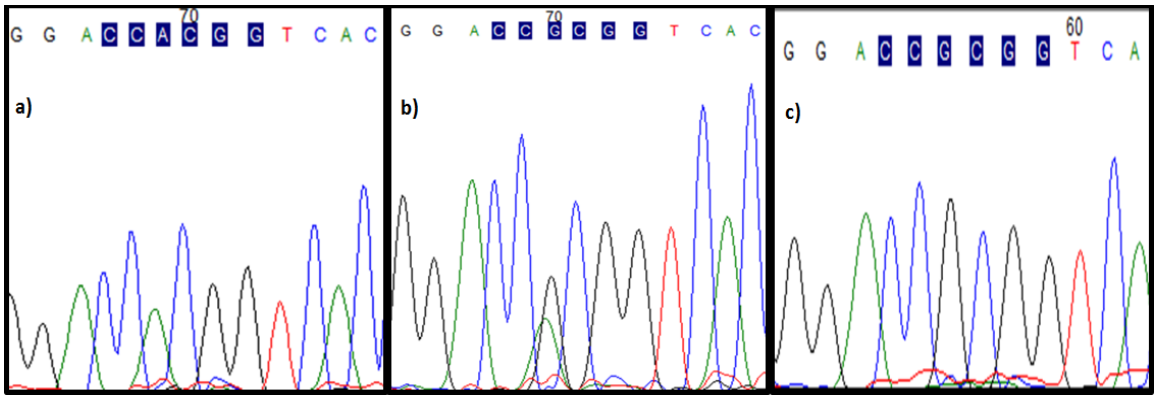

Fig 2. *SacII* restriction enzyme cleavage site a) S_2S_2: Sequence analysis of a homozygous genotype b) S_1S_2: Sequence analysis of a heterozygous genotype c) S_1S_1: Sequence analysis of a homozygous genotype

The allele and genotype frequencies at the exon 7 to the 3'flanking region of *β-LG* gene in the Saanen goats were given in Table 1. The allele frequencies were determined as 0.59 and 0.41 for S1 and S2. Genotype frequencies of S1S1, S1S2 and S2S2 were 37.8 %, 43.3 % and 18.9 %, respectively. A significant deviation from Hardy-Weinberg equilibrium was not observed in the investigated breed. The result of Chi-square statistics reflected that in the investigated breed was in Hardy-Weinberg equilibrium.

Table 1. Allele and genotype frequencies of *β-LG* gene for *SacII* site in Saanen goats.

		B-LG Genotype Frequency			*B-LG* Allele Frequency (%)		χ2
		S_1S_1	S_1S_2	S_2S_2	S_1	S_2	
Saanen	Observed	28 (37.8)+	32 (43.3)+	14 (18.9)+	0.59±0.04	0.41±0.04	0.89
	Expected	26.04 (35.2)+	35.92 (48.5)+	12.04 (16.3)+			

+ Observed and expected genotype frequency (%), χ2: Chi-square

Goat β-LG polymorphism has been investigated with 28 Saanen goat breeds by Elmacı et al. (2008) and 41 Saanen goat breeds by Ağaoğlu *et al.* (2012). Elmacı et al. (2008) revealed that the frequency of S_2S_2 (0.14) genotype was found to be lower than S_1S_1 (0.41) similar with present study. In paralell with these results, observed genotype frequency of S2S2 (AA, 0.12) was found to be lower than S1S1 (BB, 0.39) by Ağaoğlu et al. (2012). In the present study, the S_1 allele frequency was 0.59 and higher than the S2 allele frequency (0.41). A similar observation has been revealed by Elmacı et al., (2008) and by Agaoglu *et al.,* (2012) in Saanen goat (0.64, 0.63) respectively. Lekerpes et al. (2014) have identified the S_1 allele frequency (between 0.76-0.86) as predominant in two native goat breeds of Kenya, similar to our study. In contrast to our study, Kumar et al., (2006) reported that of the 13 indigenous goat breeds reared in India, the S_1 allele frequency ranged from 0.03 to 0.41 and was lower than the S_2 allele frequency. Similar to Kumar et al. (2006), the frequency of the S_2 allele (0.81) was observed to be high in the study performed by Yüksel and Akyüz (2014) on Hair goat in Turkey.

In recent years, many researches have been conducted to investigate association *β-LG* polymorphism in DNA level with milk yield and composition in farm animal (Chen et al., 2005; Selvaggi et al., 2015; El Shazly et al., 2012; Kahilo et al., 2014). Investigations conducted to study the polymorphism in different goat breeds showed significant association between *β-LG* genotypes and milk yield/composition. Lactation milk yields of goats were 812.09 kg, 777.42 kg and 791.60 kg for S1S1, S1S2 and S2S2 genotypes, respectively. No

significant association was established between β-LG genotype and milk yield. But S1S1 genotype had higher milk yield (812.09 kg) than the other genotypes (p>0.05) in Saanen goats under the study (p>0.05). El-Hanafy et al. (2015) reported that S_1 allele frequencies ranged from 0.74 to 0.57 and were higher than the S2 allele frequency in three goat races reared in Saudi Arabia. The relationships between genotypes and milk yields were examined and it was reported that milk yield of S_2S_2 genotypes significantly higher than other genotypes (p<0.05).

Conclusions

The results of this study indicate that the relationship between *β-LG* polymorphism and milk yield is very important for enhancing the productive and genetic performance of farm animals. There were conflict results about association between *β-LG* polymorphism and milk yield, these results may depend on the breed and number of animal under the study. As it is obvious from the current study, more studies including large number of animals must be carried out. It is suggested to determine the relationship between *β-LG* polymorphism in the promoter and other exon regions and milk yield especially in native goat breeds.

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