# Determination of the Genetic Diversity of the Lactoferrin Gene Polymorphism in Indigenous Anatolian Goat Breeds

**I. Akisa[1,*], F.E. Gursela[2], C. Unb[2] and K. Oztabaka[2]**

[1]*Department of Biochemistry, Faculty of Veterinary Medicine, Istanbul University Istanbul, Turkey*
[2]*Department of Biology, Faculty of Art and Science, Ege University Izmir, Turkey*

**Corresponding author. Email iraz@istanbul.edu.tr*

**Abstract**

Lactoferrin (LF) gene is considered as a candidate gene for mastitis resistance due to the role of LF in defence mechanism of the mammary gland. LF gene is highly conserved among mammals. Studies have focused to analyze the diversity of LF gene polymorphisms in different breeds. There are limited data about genetic diversity, phylogeny and candidate genes in Anatolian goat breeds. In this study four indigenous Anatolian goat breeds were genotyped for 7605 C>T polymorphism in exon 4 of LF gene using PCR-RFLP method and direct sequencing. Two alleles C and T were observed, which are resulting Arginine and Tryptophan, respectively. T allele frequency was found to be higher in all the breeds, varied between 81.25 and 93.75%. CC genotype was the least observed genotype and it was absent in Hair goat breed. The populations except Hair goat were found to be in Hardy-Weinberg equilibrium. The genetic diversity of the analyzed site was found to be high except Kilis breed. Investigation of the genetic diversity of Anatolian goat breeds with different parameters would contribute the genetic database of goat breeds. Further studies on caprine LF gene and its associations with mastitis in Anatolian goat breeds should be conducted.

***Keywords:*** lactoferrin gene, PCR-RFLP, Anatolian goat breeds, genetic diversity

## Introduction

Lactoferrin (LF) is a glycoprotein with a molecular weight of 80 kDA, isolated from milk and identified in different mammalian secretions (Iyer and Lonnerdal, 1993). It is a member of the transferrin family, which is capable to bind and transfer iron ions (Metz-Boutique, 1984). Lactoferrin has many biological functions. Beside its role in iron transport, lactoferrin is also very important for immune system because of its antimicrobial, antibacterial, antiviral and antiparasitic effects (Adlerowa, 2008). Lactoferrin acts as a transcription factor and as a growth factor that stimulates cell proliferation (Yanagihara, 2000). It has been identified as an anabolic factor affecting osteocytes through osteoblast proliferation and reducing apoptosis (Cornish et al., 2004).

The most interesting qualification of lactoferrin is its role in antimicrobial defence mechanism of mammary gland. LF gene is considered as a candidate gene for resistance against mastitis infection, due to the concentration increases during infection (Kutila, 2003). In a study on goats, Saanen goat lactoferrin could not affect E. coli O111 at 7.5 mg $mL^{-1}$, whereas Korean goat lactoferrin was active at 5 mg $mL^{-1}$. These differences in antibacterial resistance might be the result of the polymorphisms in lactoferrin gene (Lee et al., 2007).

LF gene is highly conserved among species. Studies have shown that the length of the exons was

identical among cattle, goats and sheep. The caprine LF gene is located on chromosome 22 and the coding sequence is spread over 17 exons and 16 introns (Schwerin et al., 1994). LF gene has 75 bp 5' UTR (untranslated region), ORF (open reading frame) encoding a mature protein consists of 690 aminoacids and 3' UTR (Provost et al., 1994; Lee et al, 1997).

Several variable sites in caprine LF gene have been reported. A comparison of LF gene sequences among between 11 species (human, mouse, rat, chimpanzee, pig, sheep, goat, cattle, buffalo, camel and dog) 6 amino acid variation sites have been found in goats (Kang et al, 2008). A total of 19 nucleotide polymorphisms have been identified in Italian Nicastrese and Saanen goats by comparing cDNAs from French, Korean and Tibetan goat lactoferrin gene cDNAs. 11 of these nucleotide changes are responsible for 8 amino acid variation. A cytosine insertion found in promoter region of lactoferrin gene in Nicastrese goats has been suggested to increase anti-bacterial defence activity through creating a new AP2 binding site (Pauciullo et al., 2010). There are also studies on association between lactoferrin gene and trait characteristics. Guo et al. (2010) concluded that G198A polymorphism in exon II has an effect on milk composition and body traits in dairy goat breeds. Kang et al. (2010) observed 30 SNPs consist of 23 transitions and 7 transversions and one missense mutation at the 7605. position (C/T) in exon 4 resulting arginine/triptophan change.

There are few studies conducted on genetic diversity, phylogenetic relationship and candidate genes affecting trait qualities in Anatolian goat breeds. The aim of the present study was to determine the distribution of the allele and genotype frequencies of the 7605 C>T polymorphism in exon 4 of LF gene in four indigenous Turkish goat breeds and analyze the genetic diversity and phylogeny of these breeds.

## Materials and Methods

### Animals

Blood samples were taken from a total of 152 individuals of four indigenous goat breeds in Turkey consist of Hair goat, also called Anatolian Black goat (n:45), Angora goat (n:41), Kilis goat (n:42) and Damascus goat (n:24). Genomic DNA samples were isolated by using standard salt-out method (Miller et al., 1998).

### PCR-RFLP Analysis

Animals were genotyped for 7605 C>T polymorphism in exon 4 of goat LF gene. PCR reaction was performed in a reaction volume of 25 µL using 2 U Taq DNA polimerase (Fermantas Life Sciences, Canada), 2-2.5 mL 10X PCR buffer, 1.5 mM $MgCl_2$, 50-100ng genomic DNA, 100 µM dNTP (Takara, Biotechnology Co, Ltd, Japan) and 10 pmol of each primer. The primer sequences used for the 430 bp fragment, containing a polymorphic NlaI restriction site, were F: 5'- TGT CCC TGG GCT CTT TAG -3' and R: 5'- CCG AAG TGG CTT GTG AA -3'. Amplification was carried out for 94$^{o}$C for 5 min; 35 cycles of 94 0C for 1 min, 56$^{o}$C for 1 min, 72$^{o}$C for 1 min; and a final extension at 72$^{o}$C for 5 min.

For RFLP analysis 10 ml of the PCR products were digested with 10 units of NlaIII (Fermantas Life Sciences, Canada) fast digest restriction enzyme at 37$^{o}$C for 10 minutes. The digested DNA fragments were separated by electrophoresis in 2% agarose gel including ethidium bromide and visualized under UV light.

### Sequence Analysis

Some of the samples were also sequenced to check the correct position of the SNP. Sequencing performed by using an ABI-3100 sequencer (PE Biosystems, Germany) and the BigDye$^{TM}$ terminator cycle sequencing kit, after the purification of the PCR products. Forward prime was used to sequence the PCR products.

### Statistical Analysis

PopGene32 software program (Yeh et al., 2000) was used to calculate genotype and allele frequencies, to test Hardy-Weinberg equilibrium and to do a neutrality test. Heterozygosity (observed, expected) and Nei's heterozygosity (Nei, 1973), effective number of alleles, Shannon's Index and Wright's fixation index, which indicate the genetic diversity of the populations, were also calculated using PopGene32 software.

## Results and Discussion

The missense mutation at 7605 position revealed two alleles, C and T. The restriction site of NlaIII enzyme is CATG and it digested the 430 bp fragment into 264 and 166 bp fragments, if C existed at 7605. position. The existence of T base at this position changes the sequence to CACG and resulted with the absence of restriction site and the fragment remains undigested. The sequences of CC, TT homozygotes and heterozygote CT genotypes were shown in Figure 1. Genotype and allele frequencies were given at Table 1. In all of the four breeds T allele had a higher frequency varied between 81.25 to 93.75%. CC genotype was the least observed genotype in Kilis, Angora and Damascus breeds and it was absent in Hair breed. The populations except Hair breed were found to be at Hardy-Weinberg equilibrium. The difference between observed and expected genotypes was significantly high in Hair goat population ($P<0.001$). Values for heterozygosity, Shannon's index, effective alleles and Wright's fixation index indicating genetic diversity are showed in Table 2. The highest and lowest genetic diversity for the 7605 T>C site analyzed in the study is found in Hair and Kilis breeds, respectively.

The phylogenetic tree based on the Nei's (1973) genetic distance of 7605 T>C between four Anatolian goat breeds was constructed using UPGMA method (Figure 2). Kilis breed was found to be the most distant breed from other three breeds.

The candidate gene researches are improving in goats up to date. The most analysed candidate genes are affecting milk quality in goats are milk protein genes. Investigations of several genes are only at the beginning (Moioli, 2007).

Due to antibacterial, anti-inflammatory and immunomodulatory fuctions of lactoferrin, LF encoding gene of several species has been analyzed in recent years (Kang et al., 2008). The comparison of the LF gene revealed very similar intron-exon organizations among ruminants (sheep, goats, cattle) and also in other mammals like mouse, rat, hamster (Kang et al., 2010). The researches on LF gene focus on two different areas. One of them is the linkage analysis between mutations and

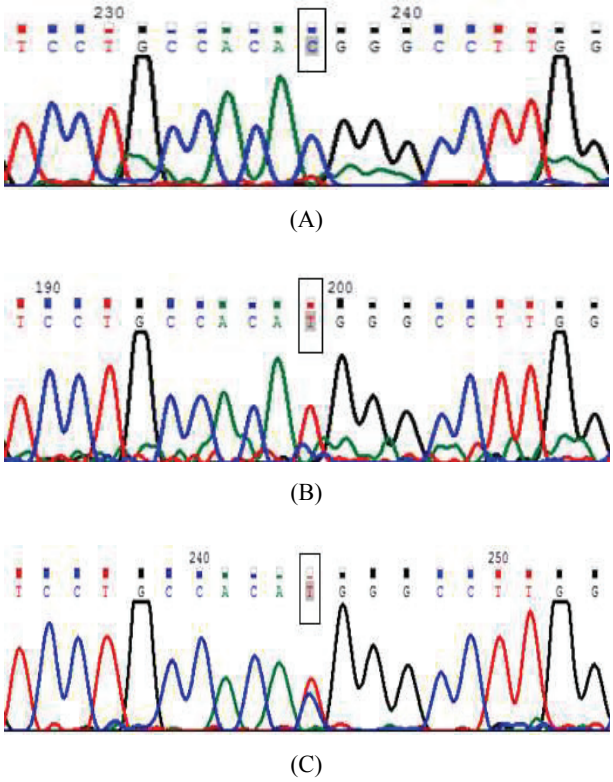


**Figure 1** Sequencing maps of the caprine lactoferrin gene. (A) CC homozygote genotype, (B) Thomozygote genotype, (C) CT heterozygote genotype

function of the lactoferrin protein and resistance to diseases, especially mastitis. The SNP's in LF gene is also analyzed to determine the genetic relationship between species and breeds and genetic variation in populations.

Kang et al. (2010) determined LF gene genotypes in ten indigenous Chinese goat breeds by PCR-RFLP. In their study, Kang et al. (2010) have stated that they have used the HindIII enzyme, which recognises the CATG sequence. They have also stated that when the C base is found at the 7605. position of the lactoferin gene, CATG sequence is formed and thus, the fragment is digested by the HindIII enzyme. Therefore, when two fragments of 264 bp and 166 bp are formed, they called this the C allele. They have argued that when the C base at the 7605. position is replaced by the T base, the CATG sequence is transformed into a TATG sequence and therefore, the 430 bp fragment is left without being cut.

**Table 1** Distribution of genotypes and allele frequencies of 7605 C>T polymorphism of the *LF* gene in Anatolian goat breeds

| Breed | Genotype | | | | | | | AlleleFrequency (%) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | CC | | | CT | | TT | | C | T | $(\chi^2)$[1/] | P |
| | n[2/] | Ob[3/] | Ex[4/] | Ob | Ex | Ob | Ex | | | | |
| Kilis | 42 | 4 | 2.7832 | 14 | 16.4337 | 24 | 22.7831 | 26.19 | 73.81 | 0.9575 | ns |
| Hair | 45 | 0 | 4.8876 | 30 | 20.2247 | 15 | 19.8876 | 33.33 | 66.67 | 10.8136 | *** |
| Angora | 41 | 3 | 4.3333 | 21 | 18.3333 | 17 | 18.3333 | 32.93 | 67.07 | 0.8951 | ns |
| Damascus | 24 | 2 | 2.5532 | 12 | 10.8936 | 10 | 10.5532 | 33.33 | 66.67 | 0.2612 | ns |

[1/] test of Hardy-Weinberg equilibrium
[2/] number of animals
[3/] observed values
[4/] expected values
ns: not significant, *** P<0.001

**Table 2** Genetic diversity of the 7605 C>T polymorphism of *LF* gene in Anatolian goat breeds.

| Breed | Heterozygosity | | | I[2/] | $N_e$[3/] | $F_{is}$[4/] |
|---|---|---|---|---|---|---|
| | Observed | Expected | Nei's[1/] | | | |
| Kilis | 0.3333 | 0.3913 | 0.3866 | 0.5750 | 1.6303 | 0.1378 |
| Hair | 0.6667 | 0.4494 | 0.4444 | 0.6365 | 1.8000 | - 0.5000 |
| Angora | 0.5122 | 0.4472 | 0.4417 | 0.6337 | 1.7912 | - 0.1596 |
| Damascus | 0.5000 | 0.4539 | 0.4444 | 0.6365 | 1.8000 | - 0.1250 |

[1/] Nei's (1973) expected heterozygosity
[2/] Shannon's Index
[3/] effective number of alleles
[4/] Wright's fixation index

However, the CATG sequence is not recognised by HindIII enzyme, it is recognised by NlaIII (Hin1II). The CATG sequence is formed when the T base is found at the 7605. position. When the T base is replaced by the C base, a CACG sequence is formed and it cannot be recognised by NlaIII.

Thus, contrary to what Kang et al. (2010) have stated, in the present study, the fragment which possesses a T base at the 7605. position and which is cut by the NlaIII enzyme (264 and 166 bp) is labelled as the T allele. The fragment with a C base at the 7605. position and which remains uncut (430 bp) is labelled as the C allele.

In 10 Chinese indigenous breeds C allele frequency varied between 29.63 and 75.00 with a average of 50.12 %. C allele frequency was found to be slightly higher than T allele frequency (Kang et al., 2010). In Anatolian breeds C allele frequencies were found to be lower than T allele frequencies in all of the breeds in a range between 26.19 and 33.33 %.

The heterozygosity observed in Anatolian breeds ranged from 0.3333 to 0.6667, from Kilis to

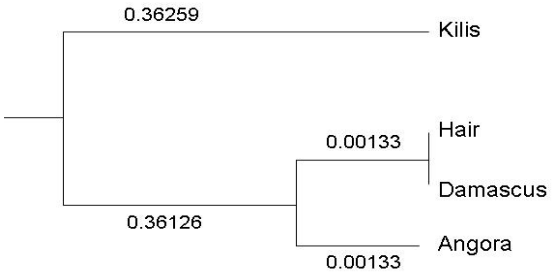


**Figure 2** Phylogenetic tree based on Nei's genetic distance of 7605 C>T polymorphism among Anatolian breeds.

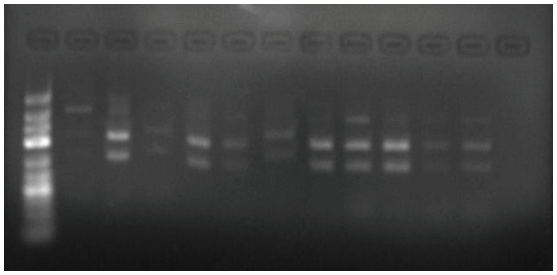

**Figure 3** *Nla*III enzyme digestion products of different *LF* gene genotypes; Lane 1: Low range ladder, Lane 2: TT genotype, Lane 3: CC genotype, Lane 9: TC genotype.

Hair breed, respectively. The average observed heterozygosity of 10 Chinese breeds was calculated as 0.5400 (Kang et al., 2010). Four indigenous breeds analyzed in this study seem to be less diverse than the most of the Chinese breeds. The most diverse breed is Hair breed and these goats are spread in all of the regions of Turkey. In a study on mt-DNA diversity of Turkish indigenous goat breeds, Hair goat breed was found to have the highest haplotype and nucleotide diversity (Cınar Kul and Ertugrul, 2011). Some features of Hair goat breed like high genetic variation, geographical distribution all over Anatolia and adaptation to different conditions (TAGEM, 2009), makes one think that it is more ancestral than other Anatolian goat breeds. The lowest values for genetic diversity were found to be in Kilis breed. It can be suggested that the isolated breeding area of Kilis goats could have decreased the genetic variance. But contradictory to these results, the mt-DNA analyses show that the Kilis breed exhibits the highest diversity among all indigenous goat breeds (Cınar Kul and Ertugrul, 2011). The low genetic variation of LF gene polymorphism might be a result of a selection process in the Kilis breed.

Kilis breed is the most distant breed to other breeds according to the phylogenetic tree constructed using UPGMA method. This result is contradictory to the suggestion that Kilis breed is a Hair goat crossbreed (Yalcin, 1986; Porter, 1996). The origin and breeding history of Kilis goat breeding should be investigated further.

CC genotype is observed with very low numbers in all breeds and it is absent in Hair breed and the Hair breed population was found to be in Hardy-Weinberg disequilibrium. The difference between observed and expected genotypes were found significantly higher ($P<0.0001$) in Hair goat population. The association between CC genotype and mastitis susceptibility should be investigated. This genotype could have result a disadvantageous health status for individuals and therefore an elimination of this genotype could have appeared.

## Conclusions

The present study showed that the Hair goat breed is the most diverse breed in Anatolia according to the lactoferrin gene diversity. The genetic relationship between Kilis and Hair goat breeds, which is defined in terms of 7605 T>C polymorphism of lactoferrin gene, conflicts with the data showing that the Kilis breed is a crossbreed of Hair goat breed. In conclusion we might say that the phylogenetic relationship between Anatolian goat breeds should be investigated further. Studies on different polymorphisms of caprine lactoferrin gene and their associations with production traits and disease susceptibility should be conducted with bigger number of breeds. The results obtained from the present study would contribute the genetic database of Anatolian indigenous goat breeds.

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