



Original Research

The Identification of Novel Single-Nucleotide Polymorphisms of Equine Beta-Lactoglobulin and Lactotransferrin Genes

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ABSTRACT

Lactoferrin (*LTF*), also called lactotransferrin, is an iron-binding protein and member of transferrin family, whereas β -LG is an important milk protein and belongs to the ligand-binding protein family of lipocalins and binds retinol. Beta-lactoglobulin (β -LG) is a major whey protein and it is a member of lipocalins family. β -LG is also known as a major allergen reason of cow milk protein allergy. Yet, no information has been reported about β -LG and *LTF* genes in donkeys. In this study, the genetic variation of β -LG and *LTF* genes were analyzed via DNA sequencing in 77 donkeys, which were individuals reared in Thrace region of Turkey. Four novel SNPs are identified as g.12887G>A, g.12973G>A, g.12654G>A, and g.13057T>C in the third intron region of β -LG gene and a genetic variant g.272719G>A in the 14th intron of *LTF* gene. The DNA sequences of β -LG and *LTF* genes in donkeys are reported for the first time in this study and these sequences were deposited to NCBI GenBank database, with the accession number MK125015 and MK211165, respectively. These variations may have an effect on milk yield and content in donkeys, so further investigations are needed to prove this hypothesis and these may be candidate SNPs for donkey breeding.

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1. Introduction

The donkey or ass is a domesticated member of the Equidae family and it belongs to the order of Ungulates (*Perissodactyla*). *Equus* is represented by eight extant species: Przewalski's horse (*E. przewalskii*), domestic horse (*E. caballus*), African wild ass (*E. africanus*), kiang (*E. kiang*), mountain zebra (*E. zebra*), Asiatic wild ass (*E. hemionus*), plains zebra (*E. quagga*), and Grevy's zebra (*E. grevyi*) [1–3]. The wild ancestor of the domestic donkey is the African wild ass (*E. africanus*).

In the recent years, donkey milk has gained popularity, and it is considered as a natural remedy that can cure some diseases, such as liver problems, cold, fever, arthritis, asthma, or even cancer. This is mostly because donkey milk includes high levels of lysozyme and

lactoferrin that has anti-inflammatory, anti-tumors, and anti-microbial activity that catalyses the hydrolysis of glycosidic bonds, present in peptidoglycan heteropolymers of the cell wall [4–9]. Mao et al [10] reported that the active components of donkey milk suppressed tumor proliferation in A549 human lung cancer cells; thus, it kills tumors through activation of lymphocytes and macrophages.

Donkey milk is found very similar to human milk in terms of proteins and nutrient composition that has low casein and high lysozyme contents [11–14]. For this reason, it is recommended to be alternatively used in infant nutrition formulas by Italian Society of Pediatric Nutrition [15].

Lactoferrin (*LTF*), also called lactotransferrin, is a transferrin family member and it binds and transports iron ions (Fe^{3+}). *LTF* supports immune reactions against pathological agents such as protozoa, bacteria, and viruses etc. and also it has a function in cell cycle regulation, as a transcription factor. Mare's milk includes around 0.6–1.3 g L⁻¹ of lactoferrin [16–19]. *LTF* gene consists of 18 exons and it is about 28.611 base pairs long and is located between 253862th and 282472th bp on Guanzhong donkey whole genome shotgun sequence (Genbank Accession number NW_014638067.1). *LTF* protein has 708 amino acid residues.

Many studies have shown that in mammals, milk contains important proteins which are caseins (80%) such as α -s1, α -s2, β and κ casein, and serum proteins (20%) such as β -lactoglobulin, α -

Animal welfare/ethical statement: All procedures involving manipulation of the animals were conducted in accordance with Turkish guidelines on animal welfare. This research was approved by the Ethics Committee on the Use of Animals, University of Namık Kemal University Animal Experiments Local Ethics Committee protocol No. 2017/06 because it is in accordance with the norms issued by the National Council of Animal Experimentation of Turkey.

Conflict of interest statement: The authors declare no conflicts of interest.

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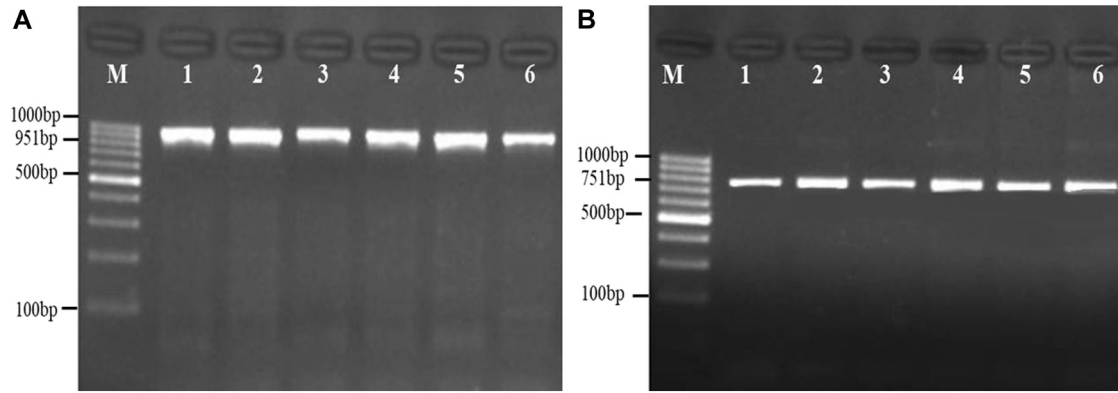


Fig. 1. Electrophoresis patterns of the β -LG (A) and *LTF* (B) genes. β -LG, beta-lactoglobulin; *LTF*, lactotransferrin; M; marker.

lactalbumin, etc. β -lactoglobulin is found in about 50% of serum proteins and the other serum proteins are α -lactalbumin (20%), serum albumin (10%), immunoglobulins (10%), and proteoseptones (10%). Equid milk protein composition is different in cow's milk and this difference alters the milk allergy in human. α -lactalbumin and β -lactoglobulin are the major whey proteins which account for 30% of the whey protein fraction in horses [11].

β -LG is a member of the ligand-binding protein family of lipocalins and binds retinol [20,21]. β -LG consists of two paralogous genes as β -lactoglobulin I and β -lactoglobulin II in donkeys [22,23]. β -LG gene is localized on chromosome 11 in goat-cattle and on chromosome 3 in sheep. But the location of β -LG gene is not exactly known in donkeys [24]. The β -LG gene consists of 5 exons between the 10753th and 15234th nucleotides, which make a total of 4482 base pairs in donkeys.

The previous studies which considered the variation in some of the gene regions coding for milk proteins on goat, sheep, and cattle have been described [25–27]. β -LG gene was firstly identified on protein level and after DNA level, and two different variants of β -LG gene were found in cattle [28–33]. Afterward the polymorphism of β -LG gene and its effect on milk yield and composition have been studied in farm animals [34–39]. However, no information has been revealed on β -LG and *LTF* genes in donkeys yet. The aim of this study is to investigate the β -LG and *LTF* genes polymorphisms in donkeys by using DNA sequencing method.

2. Materials and Methods

In this study, a total of 77 blood samples were collected from Thrace provinces of Turkey, Kırklareli (29), Tekirdağ (21), and İstanbul (Çatalca) (27) Provinces. Blood samples were taken to

10 mL of vacuum tubes, including EDTA as anticoagulant and stored at -20°C till the DNA extraction. Genomic DNA was isolated by using the phenol-chloroform extraction method.

Primer sequences of β -LG and *LTF* genes were designed, based on the donkey sequence retrieved from GenBank (Accession number NW_014637182.1 and NW_014638067.1, respectively) using Primer-BLAST algorithm (www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences of β -LG gene are F: 5'-CCGAGGACAACCTGGAGATCAT-3', R: 5'-GGTCCAACCTCCCGAAGGCGAC-3' and *LTF* gene F: 5'-GCAACAACGAGAATGAGAACAAGT-3', R: 5'-ATTT-CACCACCAGATGGCGT-3'.

The PCR reaction of β -LG gene were performed in a 25 μL reaction mix, which contains 50 ng of genomic DNA, $1\times$ PCR buffer, 0.3 μM of each primers, 200 μM dNTP, 1.5 mM MgCl_2 and 1 units of Taq DNA polymerase (Invitrogen, Life Technologies). The PCR cycling protocol duration was 5 minutes at 95°C , 35 cycles of 95°C for 45 seconds, 58°C annealing for 1 minute, 72°C for 2 minutes, with a final extension of 72°C for 10 minutes.

The PCR reaction of *LTF* gene were performed in a 20 μL reaction, which contains 50 ng of genomic DNA, $1\times$ PCR buffer, 0.4 μM of each primers, 200 μM dNTP, 1.4 mM MgCl_2 and 1 units of Taq DNA polymerase (Invitrogen, Life Technologies). Thermal program for PCR included the following steps in 35 cycles; 4 minutes at 95°C , of 95°C for 30 seconds, 67°C annealing for 1 minute, 74°C for 2 minutes with a final extension of 74°C for 10 minutes. Polymerase chain reaction products were electrophoresed on 2.5% agarose gel stained with SafeView Classic (Applied Biological Material Inc, Canada) and photographed in Vilber Lourmat gel imaging system.

951 bp of β -LG and 751 bp *LTF* genes were sequenced on an Applied Biosystems 3500XL Genetic Analyzer System (Applied

Table 1

The comparison of the genotype and allelic frequencies of the g.12887G>A, g.12973G>A, g.13057T>C polymorphisms of the β -LG gene.

Locus (β -LG)	Genotypes			Allele Frequency		χ^2
	AA	AG	GG	A	G	
g.12887G>A						
N	0	33	44	0.21	0.79	5.64
Obs.	0	42.9	57.1			
Exp.	3.5	26.0	47.5			
g.12973G>A						
N	0	8	69	0.05	0.95	0.22
Obs.	0	10.4	89.6			
Exp.	0.2	7.6	69.2			
	TT	TC	CC	T	C	χ^2
g.13057T>C						
N	29	34	14	0.6	0.4	0.52
Obs.	37.6	44.2	18.2			
Exp.	27.5	37.0	12.5			

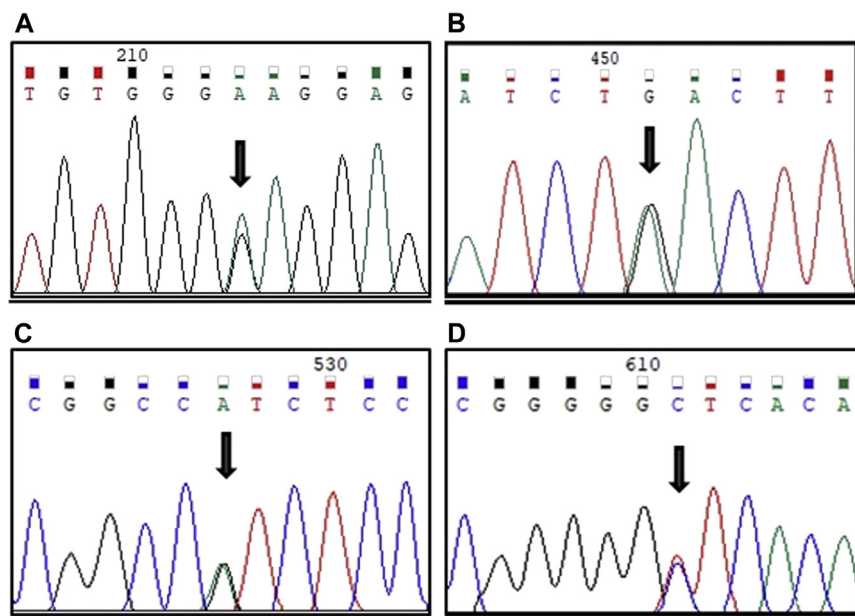


Fig. 2. Partial sequence of β -LG gene showing (A) g.12887G>A, (B) g.12973G>A, (C) g.12654G>A, (D) g.13057T>C polymorphisms. β -LG, beta-lactoglobulin.

Table 2

The GenBank accession numbers and polymorphism sites of the β -LG gene region of *Equus asinus*.

DNA nucleotide positions		12,654 ^c	12,887 ^c	12,973 ^c	13,057 ^c
Haplotypes/Reference	GenBank Accession Numbers				
Reference Sequence	NW_014637182.1 ^a	G	G	G	T
New Sequence	MK125015 ^b	—	A	A	C
	MK425197 ^d	A	A	A	C
	MK425198	—	—	A	C
	MK425199	—	A	A	—
	MK425200	—	A	—	C

^a NC_001566: 16343 bp of *Equus asinus* whole genome shotgun sequence.

^b MK125015- 951 bp of β -LG gene of Turkish donkeys found in this study. — indicates identical nucleotides at that site.

^c Variable sites that are newly found in this study.

^d Sequences that are newly found and submitted NCBI GenBank.

Biosystems) to identify the β -LG and *LTF* gene sequences. Sequences were aligned with BioEdit sequence alignment editor with Clustal W multiple alignment modules [40]. Hardy–Weinberg equilibrium was also checked by using the POPGENE software [41].

The nucleotide sequences of β -LG gene in different species were retrieved from GenBank. The sequences were aligned by Clustal X and phylogenetic tree was constructed by the UPGMA method using MEGA 7. The evolutionary divergence between the nucleotide sequences of β -LG were estimated [42]. The probability of substitution from one base to another base was also determined [43].

Table 3

The comparison of genotype and allelic frequencies of the g.272719G>A polymorphism of the *LTF* gene.

Locus (<i>LTF</i>)	<i>LTF</i> Genotypes			<i>LTF</i> Allele Frequency		χ^2
	AA	AG	GG	A	G	
g.272719G>A						
N	15	39	23	0.45	0.55	6.9
Obs.	19.48	50.65	29.87			
Exp.	15.59	38.12	23.29			

3. Results and Discussions

The genetic characterization of β -LG gene and *LTF* genes were investigated by DNA sequencing and the comparison with the DNA sequences retrieved from GenBank were conducted (accession number NW_014637182.1 and NW_014636647, respectively). β -LG gene sequence is located between 10,753–15,234 bp at NCBI GenBank database (accession number NW_014637182). In our study, the studied β -LG gene region has spanned between the third and fourth exon and fourth intron in donkeys and it includes 35 amino acids. The β -LG gene sequence which was determined in this study was deposited to NCBI GenBank database, with the accession number MK125015.

951 bp of β -LG and 751 bp *LTF* genes were amplified (Fig. 1) and four novel genetic variants are identified at position 12654, 12887, 12973, 13057 of β -LG gene sequence in donkeys (NW_014637182.1). The SNPs which are found g.12887G>A, g.12973G>A, g.12654G>A, and g.13057T>C were localized in the third intron region. Table 1 shows the nucleotide changes and the genotype, allelic frequencies of the g.12887G>A, g.12973G>A, and g.13057T>C polymorphisms of the β -LG gene. For g.12887G>A cannot calculate genotype and allele frequency, because only three animals have this genetic variation. Partial sequence of β -LG gene polymorphism has

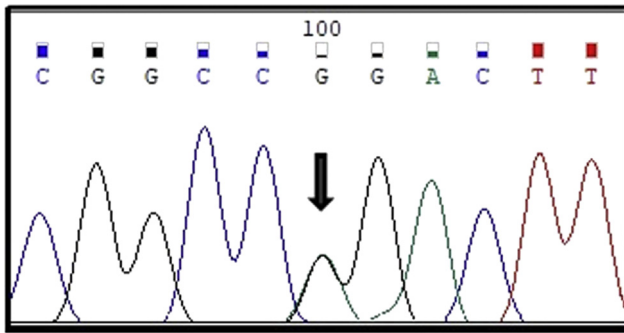


Fig. 3. Partial sequence of *LTF* gene showing g.272719G>A polymorphism. *LTF*, lactotransferrin.

been showed in Fig. 2. The GenBank accession numbers and polymorphism sites of the β -LG gene region of *Equus asinus* are revealed in Table 2.

The studied *LTF* gene region has spanned between partial 14th exon and 14th intron and it includes 23 amino acids between 750 base pairs. A novel genetic variation as g.272719G>A in the 14th intron was determined in *LTF* gene, and it was compared with the GenBank record (accession number NW_014638067.1). The genotype and allele frequencies of the g.272719G>A polymorphism of the *LTF* gene is shown in Table 3. The *LTF* gene sequence, which was determined firstly in this study, was deposited to NCBI GenBank database, with the accession number MK211165. The partial sequence of *LTF* gene g.272719G>A polymorphism has been revealed in Fig. 3.

The β -LG gene was sequenced and analyzed by the UPGMA method using MEGA 7. The pairwise distances calculated among nucleotide sequences of different species and the evolutionary distances computed by using the Maximum Composite Likelihood method are in the units of the number of base substitutions per site. The analysis involved 9 published GenBank nucleotide sequences. There were a total of 347 positions in the final data set. The phylogenetic tree based on the sequences of β -LG from donkeys and other species retrieved from GenBank are given in Fig. 4. According to Maximum Likelihood phylogenetic analysis, which was

constructed on the basis of the distance matrix, *Equus asinus* was clustered in the same clade with *Equus caballus*.

4. Conclusions

In the recent years, with increase of hypersensitivity to casein allergy, studies have been conducted on donkey milk as an antiallergic feed resource. In some cases, people have also shown hypersensitivity to the β -lactoglobulins and to the α -lactalbumins of milk. For this reason, it is important to identify the genetic structure and polymorphism of β -LG and *LTF* genes in donkey milk.

In the present study, we have used DNA sequencing method to validate the genetic polymorphism in endangered donkey populations from Thrace region of Turkey. In this study, we have identified β -LG and *LTF* genes in donkeys which are found in association with milk traits in farm animals. Four novel SNPs are identified as g.12887G>A, g.12973G>A, g.12654G>A, and g.13057T>C in the third intron region of β -LG gene and a novel SNP g.272719G>A in the 14th intron in *LTF* gene. The β -LG and *LTF* gene sequences were firstly analyzed in this study and the DNA sequencing results were firstly deposited to NCBI GenBank database, with the accession number MK125015 and MK211165, respectively.

The phylogenetic tree based on the sequences of β -LG from donkeys in the studied populations and other species retrieved from GenBank, showed that *Equus asinus* and *Equus caballus* clustered together. The estimates of evolutionary divergence between the sequences of β -LG revealed that *Bos taurus* is far apart from *Equus asinus*. This analysis showed that the sequence of β -LG gene within the species reveal the conducted high divergence with other species of animals.

In addition to previous findings of β -LG and *LTF* gene in different species, we report here a comprehensive analysis of these genes in donkey populations. The β -LG and *LTF* genes were firstly analyzed in this study and the sequences were deposited to GenBank. These revealed results are the preliminary results, which should be used for further studies because it is important to identify the genetic structure of endangered native animal population and to investigate their quantitative trait loci.

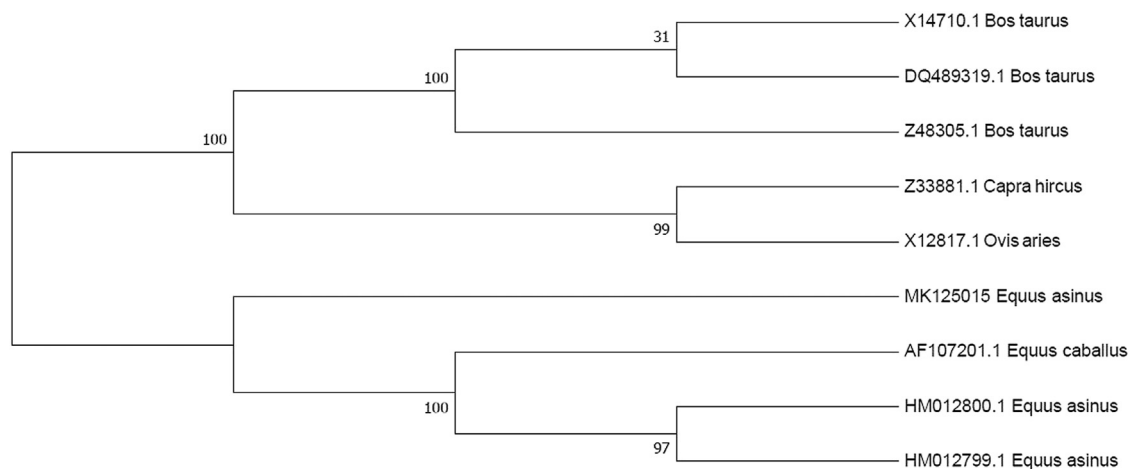


Fig. 4. Phylogenetic tree of sequences of β -LG gene of equine species indicating genetic relationship with β -LG gene sequences of different species retrieved from GenBank database (*Bos taurus*; *Ovis aries*; *Capra hircus*; *Equus asinus*; *Equus caballus*). Distances and groupings were determined using the MEGA 7 software by UPGMA method. The veracity of these trees was studied using the bootstrapping method by executing 1000 replicates. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 1.27211545 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. β -LG, beta-lactoglobulin; *LTF*, lactotransferrin.

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