



Phylogenetic Analysis of Abaza, Kaçkar, Georgian (Caucasian), Ovit Region (İspir) Native Goat Breeds Using mtDNA D-loop Sequences

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ABSTRACT

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The objective of this research was to determine genetic diversity of mtDNA D-Loop region in Abaza, Kaçkar, Georgian (Caucasian) and Ovit Region (İspir) breeds and their phylogenetic relationship with each other. The number of goat have decreased gradually due to many reasons in some regions of Turkey. Thus, non-scientific hypotheses about goats began to be produced. Therefore, the origin and gene flow of native goat breeds reared in Eastern of Turkey are needed to reveal by using DNA based methods. In this study, 200 individuals from four native breeds in eastern of Turkey were sampled for the mtDNA D-loop sequences. The haplotype and nucleotide diversities were estimated as 0.974 and 0.019, respectively. In total, 52 haplotypes were observed from 63 polymorphic sites in native goat populations. Only one haplogroup A were found in studied populations. With the participation of all sequences, an neighbor-joining tree of native breeds was constructed. The lowest genetic distances (0.011) was observed between Kaçkar and Ovit Region (İspir) which have relatively close genetic relationships.

Abaza, Kaçkar, Gürcü (Kafkas), Ovit Bölgesi (İspir) Yerli Keçi Irklarının mtDNA D-Loop Dizileri Kullanılarak Filogenetik Analizi

MAKALE BİLGİSİ

ÖZET

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Anahtar Kelimeler

Abaza Keçisi
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Haplotip
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Mitokondrial D-Loop
Ovit Bölgesi (İspir) Keçisi

Bu araştırmanın amacı, Abaza, Kaçkar, Gürcü (Kafkas) ve Ovit Bölgesi (İspir) keçi ırklarında mtDNA D-Loop bölgesinin genetik çeşitliliğini ve birbirleriyle olan filogenetik ilişkilerini belirlemektir. Türkiye'nin bazı bölgelerinde birçok nedenden dolayı keçi sayısı giderek azalmış, böylece keçiler hakkında bilimsel olmayan hipotezler üretilmeye başlanmıştır. Bu doğrultuda, Türkiye'nin Doğu ve Kuzeydoğu bölgelerinde yetiştirilen yerli keçi ırklarının kökeni ve gen akışının, DNA temelli yöntemler kullanılarak ortaya konması önemli bilgilerin elde edilmesine yardımcı olacaktır. Bu çalışmada, dört yerli ırktan 200 baş keçi mtDNA D-loop dizileri için örneklenmiştir. Haplotip ve nükleotid çeşitliliği sırasıyla 0.974 ve 0.019 olarak tahmin

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edilmiştir. Yerli keçi popülasyonlarında 63 polimorfik bölgeden toplam 52 haplotip gözlenmiştir. İncelenen popülasyonlarda sadece bir A haplogrubu bulunmuştur. Tüm sekansların katılımıyla, neighbor-joining tree (NJT) oluşturulmuştur. En düşük genetik uzaklıklar (0,011), Kaçkar ve Ovit Bölgesi (İspir) arasında gözlemlenmiştir.

Introduction

Indigenous animal breeds have grand importance in terms of factors such as the ecological balance for the world, advance breeding program for scientific studies and continuation of cultural values for social live. They have more high importance, especially, in terms of carrying local life to the global platform. In this process, the goat has still a different task. Goat play an important role not only in agriculture, economy and, culture but also provide meat, milk, and hair for local populations in the Eastern Black Sea and North-Eastern Anatolian regions of Turkey (Sezgin et al., 2010a; 2010b). The rich plant cover in the bush form and the rough terrain are among important resources of goat rearing in the Eastern Black Sea Region. This region have different morphological looking goat genotypes that have adapted to the local environment (Sezgin et al., 2010a; 2010b) existence because of sparse settlement and high mountains. However, this indigenous goat herds reared in the region suffer a serious situation, because the their's number was beigning decrease gradually. Biotechnological studies and field observations indicate that origin of the goat is likely to have based on wild goat in region (Batu, 1951; Chen et al., 2006; Naderi at al., 2007). Deviations in breeder preference, uncontrolled protection programs and lack of an

international institutionalized market for goat products may result with extinction of the goat generation. So far, over the genetic diversity, phylogenetic relationship, and maternal origin of the Eastern Black Sea and North-East Anatolia Regions goat populations not be made any research.

It has been thought more useful the studies of mitochondrial DNA (mtDNA) polymorphisms for describing the molecular phylogeny (Baker and Marshall, 1997) and diversity of goats (Carmelet al., 2000; Wayne et al., 2002; Smith et al., 2005; He et al., 2009) in the researchs. Because, it has faster substitution rate as compared to nuclear DNA (Brown et al., 1992) due to transfer from a single parent throughout the generation, regardless of the nuclear genome, its maternal heredity (Gyllensten et al., 1991).

Mitochondrial DNA genotypes are defined as molecular clones, mitotypes (mitotypes) or haplotypes (haplotypes). Mitochondrial DNA has different regions that evolve at different speeds. One of them is the region that hosts the replication origin of the heavy (H) yarn. The other is the mtDNA Control Region (D-loop region), which houses the promoter regions of both yarns (light and heavy yarns). This region evolves 3-5 times faster than other parts of the mitochondrial genome, depending on whether it is a region without any enzyme or protein equivalent (Brown et al., 1982; Wenink et al., 1994). The D-

loop sequence on mtDNA has been used to assess the genetic relationships among the goat breeds in geographical locations. Up to now, six different mitochondrial haplogroups (A, B, C, D, E, F and G) are known in goats by phylogenetic analysis (Naderi et al., 2007).

The area where the research material is located consists of different geographical and life styles. In this areas goat breeding is often done as a main source of income. However, there was no investigation of the genetic diversity on goat breeds of these region. In this study, it was investigate the mtDNA D-loop variability, maternal lineages (haplogroups) and genetic diversity within the region of Kaçkar, Abaza, Georgian and Ovid Region (İspir) breeds indigenous to the Northeastern Anatolian and Eastern Black Sea Regions.

Material and Methods

Sample collection and DNA extraction

In this study, 25 males and 25 females from each breed, a total of 200 goats were sampled from four different breeds; Abaza, Kaçkar, Georgian (Caucasian), and Ovit Region (İspir) reared in East Black Sea and North-East Anatolian regions. Blood samples were collected from the vena jugularis with sterile syringes into a tube containing ethylenediaminetetraacetic acid, transported to laboratory and stored at -20°C until genomic DNA extraction, which was carried out using salting-out method according to Miller et al. (1988). The DNA extraction and PCR assays were carried out in Genetic Laboratory

located at Ankara University, Faculty of Agriculture, Department of Animal Science, Biometrics and Genetics Science (AUFAAS). The sampling and handling of the goats were approved by the Animal Experimentations Local Ethics Board at Ankara University.

Extraction of DNA genome

The extraction of DNA genome was conducted using the salting-out method of Miller and Storts (1996). 500 µl of each blood sample was taken and placed in a 1.5 ml ependorph tube. 1.000 µl Erythrocyte Lysis Buffer Solution was added to the samples and kept for 10 minutes. At the end of the holding period, the samples were centrifuged at 3,000 rpm for 10 minutes. The liquid portion collected at the top of the Ependorf tubes was removed. The remaining cell part was treated with Erythrocyte Lysis Buffer Solution until the color was white. Pellets were mixed with 1.000 µl Physiological Buffer Solution for a while. These samples were centrifuged at 3,000 rpm for 10 minutes. Then 600 µl Lisis TE Buffer Solution was added and dissolution was achieved. 100 µl of 10% SDS solution and 5 µl of proteinase K (10 mg / ml) were added to the dissolved pellets and kept in a water bath at 65 ° C for 1.5 hours. It was added 200 µl of 6M NaCl solution to the samples removed from the incubation and centrifuged at 11.000 rpm for 10 minutes. At the end of the centrifuge, the liquid part containing the above DNA molecules was centrifuged at 10.000 rpm for 5 minutes. To the samples was added 99.9% ethyl alcohol twice as much as the sample volume. The tube was

centrifuged at 10.000 rpm for 5 minutes to accumulate the clustered DNA strands into the bottom of the tube. At the end of the centrifuge, ethyl alcohol was removed and 1.000 µl of 70% ethyl alcohol was added to the DNA pellet, which collapsed to the bottom of the tube, and was centrifuged for 5 minutes at 10.000 rpm. 100 µl of pure water was added on the completely dried samples and the DNA pellet was left overnight at +4 ° C to dissolve. In result of spectrophotometer measurements, DNA molecules of each sample, which were found in 260/280 wavelengths of 18-20 purity and above 50 ng / µl and also found to be one piece in 2% agarose gels, were stored at +4 °C until PCR was made.

Amplification of mtDNA D-Loop

In processing amplification the D-loop region of goat mtDNA, a pair of primers was designed using the known goat mtDNA sequence (GenBank: KP776461.1). The fragment of 590 bp in length from the most changeable region HVR1 of the D-loop region in goats mtDNA was amplified by polymerase chain reaction (PCR). It were prepared that was amplification reactions a final volume 50 µl. Its containing was as follows: 10 X PCR buffer (5 µl), 1 mM

dNTPs (1 µl), 5 U/ng Taq DNA Polymerase (0.4 µl), 25 mM MgCl₂ (5 µl), 10 pmol of forward (table 1, 1 µl) and reverse (table 1, 1 µl) primers suggested by supplier's instructions (AUFAAS, Ankara), dH₂O (35.6 µl) and 100 ng DNA (1 µl). Amplification was performed using (Applied Biosystems, USA) an initial denaturation of 4 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 2 min at 72°C, and a final extension step of 7 min at 72°C. PCR products were controlled by electrophoresis on 1% agarose gels. Electrophoresis process was completed at 80 V / cm approximately after 30-40 minutes. After gel electrophoresis, the amplicons were purified using a Qiamp Mini Kit (QIAGEN, Valencia, CA, USA). Than, the photographs of the gels were taken in the Gel Imaging System (Kodak gel Logic 200) and the images obtained were transferred to the computer environment. The nucleotide sequences of the D-loop HVR1 region in goats were determined using the automated DNA Sequence Analysis System. The DNA sequence analysis was carried out by using the ABI PRISM 3130 Automatic DNA Sequence Analyzer. The primers used are shown in Table 1.

Table 1. Primer bases D-Loop.

Tablo 1. D-Loop bazlı primerler

Locus	Primer sequences	Fragment Size
D-loop HVR1	Forward: 5' AAGTACATTACACCGCTCGC3' Reverse: 5' GGAAGAGTGGGCGATTTTA3'	590 bp

Analysis of data

The DNA sequence analysis results were displayed in FinchTV program. The sequences of 590 bp in length from mtDNA D-loop region were aligned with MEGA 4.1 software (Kumar et al., 2008). The position and number of polymorphic sites as well as corresponding haplotypes were calculated using DNASP software (Librado and Rozas, 2009). We used ARLEQUIN software (Excoffier et al.,

2005) to calculate haplotype diversity (h), nucleotide diversity (π), and analysis of molecular variance. We constructed an unrooted neighbor-joining (NJ) tree of native goat breeds under study by using Splits Tree4 software (Huson and Bryant, 2006). The NJ dendrogram based on pairwise FST values calculated from mtDNA haplotype frequencies for Turkish breeds. Several GenBank sequences of previous studies were used for these analyses (Table 2).

Table 2. Haplotype names and GenBank accession numbers of goat mtDNA sequences used as a reference sequences in this study

Tablo 2. Bu çalışmada referans sekans olarak kullanılan keçi mtDNA sekanslarının haplotip isimleri ve GenBank erişim numaraları

Haplogroup	Geographic origin	Gen Bank Cod	Referans
A	India	AY155721	Joshi et al. (2004)
A	İtaly	EF618134	Naderi et al. (2007)
A	France	EF617779	Naderi et al. (2007)
A	Jordan	EF618200	Naderi et al. (2007)
A	Iranian	EF617945	Naderi et al. (2007)
A	Iranian	EF617965	Naderi et al. (2007)
B	Laos	AB044303	Mannen et al. (2001)
B	Azerbaijan	EF617706	Naderi et al. (2007)
B	Mongolia	AJ317833	Luikart et al. (2001)
B	China	DQ121578	Liu et al. (2006)
C	India	AY155708	Joshi et al. (2004)
C	Switzerland	AJ317838	Luikart et al. (2001)
C	Spain	EF618413	Naderi et al. (2007)
C	China	DQ188892	Liu et al. (2006)
D	India	AY155952	Joshi et al. (2004)
D	Austria	EF617701	Naderi et al. (2007)
D	China	DQ188893	Liu et al. (2006)
F	Sicily	DQ241349	Sardina et al. (2006)
F	Sicily	DQ241351	Sardina et al. (2006)
G	Iranian	EF618084	Naderi et al. (2007)
G	Turkey	EF618535	Naderi et al. (2007)
G	Egypt	EF617727	Naderi et al. (2007)

Results

Polymorphic site and sequencing analysis

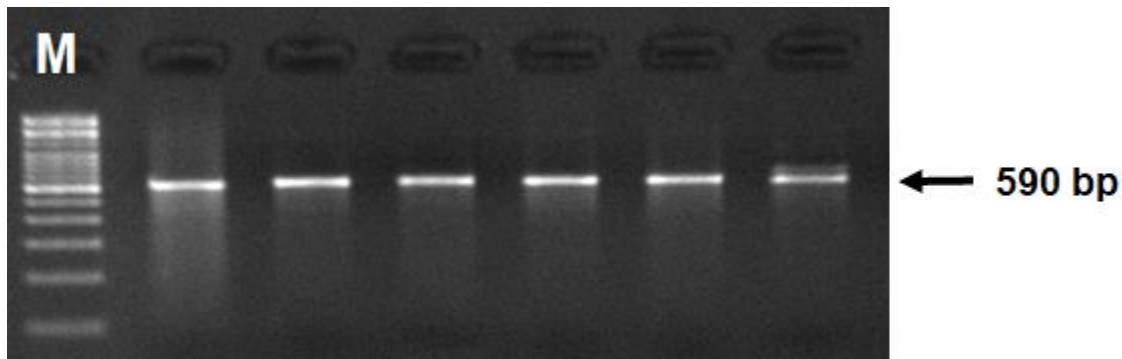
Abide by the metod stated by Naderi et al. (2007) were obtained PCR products with a length of 590 bp as a result of PCR process (Figure 1).

The forward and reverse primers are designed by AUFAAS Genetics Laboratory to amplify the region containing the most variable 481 base region (HVR1) took part on the mtDNA D-loop region. Based on the reference

sequences 32 different haplotypes were obtained from the four different goat breed in the study. Considering the reference sequences, the total different haplotypes number was 52. It was detected 63 different polymorphic nucleotide regions. The average and standard error of the isolated DNA molecules was 154.03 ± 6.70 ng / μ l. The values of haplotype diversity (Hd) and nucleotide diversity (Pi) were 0.974 and 0.019 respectively, thus was saw the genetic diversity in the 4 goat breeds.

Figure 1. Image on 2% agarose gel of the 590 bp PCR product obtained (M, 50 bp Fermentas® GeneRuler DNA marker)

Resim 1. Elde edilen 590 bp PCR ürününün% 2 agaroz jeli ile ilgili resim (M, 50 bp Fermentas® GeneRuler DNA markörü)



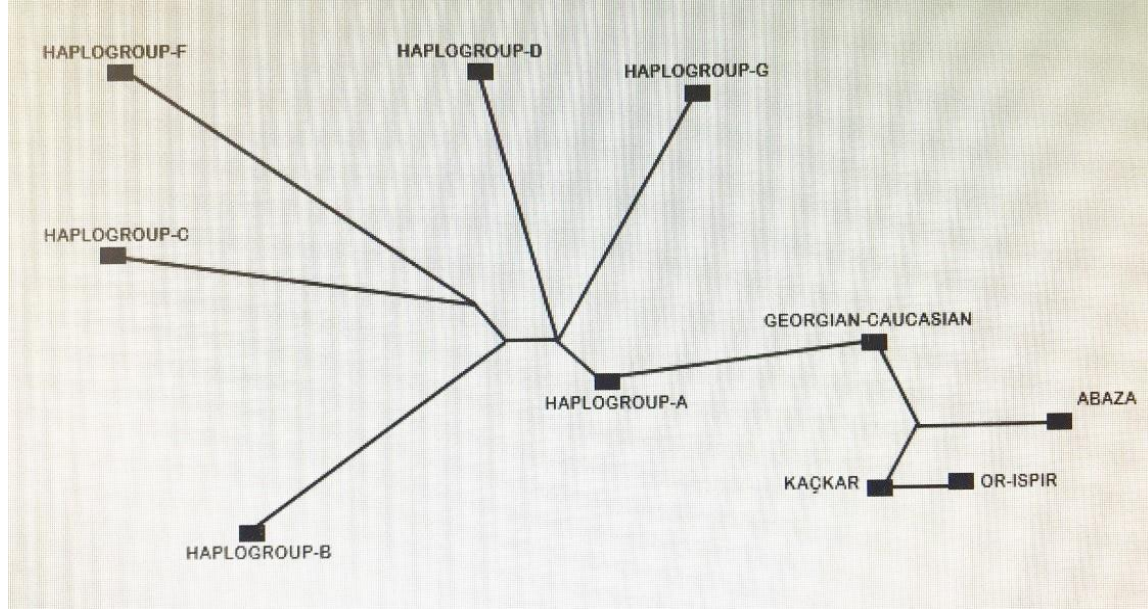
Phylogenetic status

For the phylogenetic status of the goat breeds reared in Eastern Black Sea and North-Eastern Anatolian Regions of Turkey, a phylogenetic tree was constructed using NJ metod based on the complete mtDNA D-loop sequences of 200 individuals and total 52 (20 reference). In the study, it was used six reference cluster haplogroups: A, B, C, D, F and G (Naderi et al., 2007) (Table 2). A haplotype was common haplotype in terms of 52 haplotypes belonging to 200 goats. According to the results of the

analysis, it can be clearly stated that Abaza, Georgian (Caucasian), Ovit Region (İspir) and Kaçkar goat breeds belong to the haplogrup A. On the other hand, the maximum composite likelihood method was used to analyze the genetic distance among herds. Particularly, the NJ phylogenetic tree (Figure 2) of the mtDNA D-loop sequences, based on units of the number of base substitutions per site divided the four goat breeds into four different groups effectively.

Figure 2. A neighbour-joining (NJ) Phylogenetic tree based on pairwise F_{ST} values in studied goat breeds and reference haplogroups

Resim 2. Çalışılan keçi ırklarında ve referans haplogruplarda ikili F_{ST} değerlerine dayalı bir komşu birleştirici (NJ) Filogenetik ağaç



The coefficient of genetic differentiation between Ovit Region (İspir) and Kaçkar breeds populations was calculated to be very low. Hence, it was observed that to each other the closest populations were these two breeds. The most distant populations were the Ovit Region (İspir) and Abaza breeds.

The AMOVA was conducted analysis for details. The results for the total genetic variation were given in Table 3. The AMOVA revealed a variation of 9.95% among the populations and of 90.05% within the populations significantly ($P < 0.05$).

Table 3. Molecular variance analysis of the four goat breeds studied (AMOVA)

Tablo 3. İncelenen dört keçi ırkının moleküler varyans analizi (AMOVA)

Variation source	SD	Squares mean	Variant components	Rate of variation
Among populations	3	29.819	0.44800 Va	9.95
In populations	50	202.737	4.05474 Vb	90.05
Total	53	232.556	4.50274	

Table 4. Pairwise F_{ST} values of four goat breeds studied*Tablo 4. İncelenen dört keçi ırkının ikili F_{ST} değerleri*

	Abaza	Georgian	OR (İspir)	Kaçkar
Abaza	***			
Georgian	0.160	***		
OR (İspir)	0.194	0.060	***	
Kaçkar	0.147	0.056	0.011	***

OR: Ovit Region

The F_{ST} values of the four goat breeds discussed in the study are given in Table 4. Binary F_{ST} values ranged from 0.011 to 0.194. The lowest (0.011) value between Ovit Region (İspir) and Kaçkar breeds, the highest binary F_{ST} values between Ovit Region (İspir) and Abaza breeds (0.194).

Discussion

Georgia (Caucasian), Abaza, Kaçkar and Ovit Region (İspir) goats were not classified as closely related species that was indicated by this work carried out. Especially, it was determined that Ovit Region (İspir) and Kaçkar breeds differ vary genetically from each other. The phylogenetic neighbor-joining tree indicated four different groups, where these two groups were certainly divided and partial resembled with others. Sultana et al. (2003) in 13 different goat breeds, in the phylogenetic tree created with mtDNA Cyt b gene sequence information, some strains had been shown to separate from wild goats and form clusters together. It was stated by Azor et al. (2005), that there was a weak phylogenetic relationship between the Spanish goat breeds and the Iberian Peninsula goat breeds. Chen et al. (2005) showed that

Chinese native goat breeds divide into four mtDNA strains (A, B, C, D). Chen et al. (2006) shown to be divided into three main groups (A, B, C) of Tibetan and Chinese native goat breeds. It was obtained different groups for haplotypes belonging to Chinese native goat breeds (Wu et al., 2009; Liu et al., 2007; Fan et al., 2007). Lin et al. (2013) showed that South Asian goats divide into four different mtDNA strains (A, B, C, D).

Recently, native goat breeds in region have becoming to a limited level in especially North-Eastern Anatolian Region, due to the increasing economical preferences. Now it is time for a genetic assessment of the native goat breeds of region in wide rearing areas. In the study, haplotype diversity (H_d) tended to be nucleotide diversity higher than statements of Kiraz, (2009). Our findings were within the limits reported by Wu et al. (2009), Wang et al. (2008), Liu et al. (2007), Naderi et al. (2007), Chen et al. (2005), Joshi et al. (2004). The haplotype value belonging Sicily goat breeds was lower than our findings, but results for nucleotide value were high (Sardina et al. 2006). A similar situation was found for the results of Amills et al. (2009). The haplotype and nucleotide diversity values for Chinese domestic goat breeds

(Liu et al. 2009 and Zhao et al. (2011) were higher than our findings. The haplotype diversity (Hd) and nucleotide diversity (Pi) of populations are the main indices for evaluating genetic diversity of species or populations.

Luikart et al. (2001) identified three mtDNA haplogroups (A: 316, B: 8 and C: 7) in 331 haplotypes in domestic goats according to the mtDNA D-loop and Cyt b gene region sequences. Accordingly, they explained that there could be three separate domestication events. They reported that the taming of the haplogroup A dates probably back to 10.000 years ago. The haplotype group A, that can be an total of the Abaza, Georgian (Caucasian), Ovit Region (İspir) and Kaçkar goat breeds, contained the majority of haplotypes in the Turkey (Naderi et al., 2007). However, some researchers were also reported different results that were observed haplotype A out in 2 of 31 haplotypes in Hair Goat and Kilis Goat (Kiraz, 2009), in 7 of 252 haplotypes in Honamlı, Ankara and Kilis Goats (Kul, 2010). It was reported as haplogroup A the 82 of 118 haplotypes in China goat breeds (Wu et al., 2009), 163 of 164 haplotypes in Portuguese goat breeds (Pereira et al., 2005), 117 of 146 haplotypes in China goat breeds (Chen et al., 2005).

The variability of the region of 481 bp caused the observed variation in the length of the mtDNA D-loop sequences of the Abaza, Kaçkar, Georgian (Caucasian) and Ovit Region (İspir) Goat breeds. The 4 goat populations in our study showed a medium level of genetic diversity. The

haplotype numbers was lower than that found in a some studies, and the nucleotide polymorphic regions was higher compared with the data in a some studies. In researches with different goat breeds in different geographies were obtained such as results 38 haplotypes and 129 polymorphic regions in 44 sequences (Sultana et al., 2003), 200 haplotypes in 363 sequences (Joshi et al., 2004), 118 polymorphic regions and 164 different haplotypes in 288 sequences (Pereira et al., 2005), 119 polymorphic regions and 146 haplotypes in 368 sequences (Chen et al., 2005), 44 polymorphic regions and 46 haplotypes in 84 sequences (Chen et al., 2006), 6 haplotypes and 13 polymorphic regions in 19 sequences (Odahara et al., 2006), 33 haplotypes and 84 polymorphic regions in 67 sequences (Sardina et al., 2006), 49 haplotypes and 85 polymorphic regions (Fan et al., 2007), 135 haplotypes and 144 polymorphic regions (Liu et al., 2007), 1540 mtDNA haplotype (Naderi et al., 2007), 77 haplotypes and 112 polymorphic regions (Wang et al., 2008), 54 haplotypes (Amills et al., 2009), 327 haplotypes and 163 polymorphic regions in 795 sequences (Liu et al., 2009), 123 haplotypes and 170 polymorphic regions in 145 sequences (Wu et al., 2009), 148 different haplotypes (Zhao et al., 2011), 192 different haplotypes and 141 polymorphic regions (Zhong et al., 2013). Our finding demonstrated that populations of research goat possess on a certain scale mtDNA diversity, and therefore, there is a origin of their maternal lineages. However, in the shape of the phylogenetic tree was determiner

factors duo to genetic diversity and geographic distribution.

Conclusions

mtDNA genetic diversity in the goat in the Eastern Black Sea and North-Eastern Anatolian Regions are a important resource for Turkey. Based on the research of mtDNA D-Loop region, it is shown that Ovit Region (İspir) goat have more a close relationship to Kaçkar and have been influenced from geographic location. It was determined that goat breeds studed in these regions are including in haplogroup A.

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Conflict of interest

This study is devoted to goat breeds protection that endangered or protected to be reared region. The authors acknowledge that this study is a national and important task. The authors declared that there is no conflict of interest.

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