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Study of mitochondrial DNA (mtDNA) D - loop region polymorphism in Şavak Akkaraman sheep

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Abstract: This study was conducted to investigate the maternal genetic diversity using the mtDNA D - loop region 842 base pair (bp) polymorphism in Şavak Akkaraman sheep raised in the province of Erzincan. According to the results, in the mtDNA D - loop of the Şavak Akkaraman breed, 27 polymorphic sites and 20 haplotypes were identified and then, 11 of the 20 haplotypes identified in Şavak sheep were in haplogroup B (55%), 6 in haplogroup A (30%), and 3 in haplogroup C (15%). Haplogroup B was the most frequent haplogroup in Şavak Akkaraman sheep. Haplotype and nucleotide diversities were estimated to be 0.995 ± 0.004 and 0.0146 ± 0.0003 , respectively. The Şavak Akkaraman breed was compared with the mtDNA sequencing of different breeds and wild sheep, and the contribution of the breed to biodiversity was considered.

Key words: Diversity, mtDNA, haplogroup, control region

1. Introduction

The domestication of sheep occurred approximately 11,000 years ago [1,2,3]. This historical process began in the Zagros Mountains and continued down to the southeast of Anatolia and the lands known as the Fertile Crescent [2]. It has been demonstrated that the breeds in this region where domestication began, experienced genetic variation depending on their predecessors, and caused a high rate of genetic transfer to other regions in the area [2]. Since Turkey is located in the region known as the Fertile Crescent, it is one of the world's most important ovine gene pools. Therefore, identifying the genetic makeup of domestic sheep breeds raised in Turkey is important and necessary in order to understand how they have influenced the gene pools of European sheep and the process of domestication as well as for programs to protect genetic resources [4,5]. It is becoming increasingly important to protect the genetic diversity of domestic animals. It is critical that productivity and constant improvement be sustained by protecting the genetic variations in existing animal resources [6]. Protecting domestic genetic resources in Turkey, which possess rich genetic diversity in terms of domestic animal genetic resources, and identifying their genetic makeup is more important than ever before, and identifying the intra - and inter - breed differences has become a priority

[7]. As with many other animal species, the mitochondrial DNA (mtDNA) control region (D - loop) and cytochrome B gene (cytB) polymorphisms are commonly used to further illuminate the evolutionary history of domestic sheep [8]. The 13 regions that code ovine mtDNA protein consist of (Cytochrome C complex and its subunits, ATPaz complex and its subunits, NADH dehydrogenase and its subunits, as well as Cytochrome B, 2 ribosomal RNA region (12S rRNA, 16S rRNA)), control region (D - loop), and approximately 22 types of tRNA (50–75 bp) [9]. Because mtDNA (especially D - loop and Cytochrome B gene regions) has different genomic characteristics than the core DNA and is not involved in recombination, it is one of the markers commonly preferred to identify polymorphisms and differences in species based on geographical distribution [8,10,11]. Estimates of genetic diversity from the control region, which does not provide genetic coding, are said to provide more insight when identifying genetic relationships [8,11]. Phylogenetic and phylogeographic studies have shown that domesticated sheep have 5 different mtDNA haplogroups named HA, HB, HC, HD, and HE [3,9,11,12,13,14,15,16]. Domestic and wild sheep lineages are divided into 2 overarching classes, namely Asian (type HA) and European (type HB) in the mitochondrial genome analyses conducted by Wood and

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Phua [12], Luo et al. [14] and Pedrosa et al. [13] identified a third haplogroup (type HC) in domestic Chinese and Turkish sheep, respectively. Although not common, HD and HE haplogroups have been found in sheep breeds in the Middle East [11,15]. The haplogroups A, B, C, D, and E have also been identified in studies conducted on domestic sheep breeds in Turkey [3]. In domestic sheep breeds, the most common haplogroups in the mtDNA D - loop region analyses are B, A, and C, respectively [3,13]. For example, the A, B, and C haplogroup distribution ratios for the Akkaraman breed raised throughout Turkey is 19%, 43%, and 38% respectively based on the results of mtDNA D-loop region analyses [13].

There are 36,177,000 head of sheep in Turkey [17]. There are many breeds of sheep raised in Turkey. The Akkaraman sheep breed constitutes 44% of the sheep in Turkey [18] and there are many varieties of this breed. One of the varieties is the Şavak Akkaraman sheep raised in the provinces of Erzincan, Elazığ, and Tunceli [19]. Within the total sheep presence, Şavak Akkaraman sheep is 494,587 head [20] and it is 1.3%. According to this data, it is understood that Şavak Akkaraman sheep does not carry the risk of extinction [21,22,23]. The Şavak Akkaraman sheep is one of Turkey's domestic genetic resources. It is named for the Şavak clan that raises this breed. The Şavak clan has a seminomadic lifestyle and makes it living by raising sheep and goats [19]. Şavak Akkaraman sheep farmers have a geographical indication certificate and generate significant revenue from the production of a local bryndza - type cheese (tulum) [24]. The Şavak Akkaraman sheep are an increasingly important population of sheep not only for the cheese production but also they are raised by a clan that is loyal to its traditions. In this respect, it is important to identify the genetic makeup of the Şavak Akkaraman breed, which is valuable to the region, and identify the position of this breed among genetic resources, especially its genetic distance from domestic genotypes and its position on the phylogenetic tree. Milk production from the Şavak Akkaraman sheep is not insignificant, so its loss should be prevented and made sustainable for regional animal husbandry. This reason that identifying its genetic makeup is important.

This study was conducted to identify polymorphism in the mtDNA D - loop region of the Şavak Akkaraman sheep raised in the province of Erzincan, as well as its phylogenetic relationship with wild sheep and other domestic sheep.

2. Material and method

2.1. Collection of blood samples and DNA isolation

The animal material for this study was taken from the National Small Ruminant (Şavak Akkaraman Sheep) Breeding Project conducted in the province of Erzincan

(General Directorate of Agricultural Research and Policies, GDAR) (project no: 24SAV2011 - 01, 24SAV2012 - 02). The sheep were selected by random sampling from the herds and they were not related. Blood samples were taken from the *vena jugularis* into sterile vacuum tubes containing K3 EDTA for DNA isolation from 50 sheep. All experiments were conducted at Harran University, Faculty of Agriculture, Animal Biotechnology Laboratory.

The genomic DNA was isolated using a DNA isolation kit (GeneJET whole blood genomic DNA purification kit) in accordance with the DNA isolation manufacturer's protocol. An agarose gel (1%) was used in imaging and checking the integrity of the DNA.

2.2. PCR amplification and sequence analysis of the mtDNA D - loop gene region

Primer sets for the mtDNA D-Loop gene region (forward and reverse primers) were used in amplified with PCR. The primers used in the study were designed with the online primer design tool. The primer sequences were as follows: forward primer: 5' - CACTGAAGCATGTAGGGTATTAG - 3' and reverse primer: 5' - GCTTGATACCTGCTCCTTTTA - 3' were constructed using the reference sequence for the sheep (Accession Number: NC _ 001941). The PCR reaction mixture is a total mixture of 40.0 mL consisting of 1.0 mL of template DNA (20–30 ng/mL), 4.0 mL of 10X PCR buffer, 1.0 mL of forward primer (10 pmol/mL), 1.0 mL of reverse primer (10 pmol/mL), 1.0 ml of dNTP mix (1.0 nM), 0.5 mL of Taq DNA polymerase (5U/mL), and dH₂O. PCR reaction conditions were set to be a single cycle, 4 min at 95 °C for preliminary denaturation, 30 s at 94 °C for denaturation, 30 s at 51 °C for annealing, 1 min at 72 °C for synthesis, and 30 cycles for these steps, as well as a single cycle of 10 min at 72 °C for the final elongation. The services of the Iontek (<http://www.iontek.com.tr>) company were utilized for the gene sequencing procedure on the samples that had undergone PCR amplification. Imaging and reading of the results and evaluations of the signals (peaks) in the chromatogram were conducted using the BioEdit program.

2.3. Phylogenetic analysis

The sequence number, number of polymorphic site (S), number of haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π), and average number of nucleotide differences (k) for the populations were identified using the DnaSP 5.0 [25] program.

Phylogenetic analyses and identification of haplotypes were conducted using the Kimura-2-parameter model [26] based on the UPGMA (unweighted pair group method with arithmetic mean) method in the MEGA 4.0.1 program [27]. The Bootstrap test (1000 repetitions) was used to test the reliability of the nodes [28].

The reference haplogroups identified as A, B, C, D, and E for sheep in previous studies and the haplogroups in this study were analysed using the following sequences: (HGA: DQ852286 - 852287, HGB: DQ852282 - DQ852285, HGC: DQ852283 - DQ852284, HGD: DQ852288 - DQ852289, HGE: DQ852280 - DQ852281, NC:001941) [15]. The sequencing information from previous studies conducted on domestic sheep breeds and wild sheep were obtained from the gene bank (NCBI: National Centre for Biotechnology Information). Phylogenetic trees were created using these sequences and the sequences for the Şavak Akkaraman breed.

3. Results

3.1. DNA isolation results

DNAs were successfully obtained from all blood samples used in the study.

3.2. PCR amplification and sequence analysis results of mtDNA D - loop gene region

The amplification of the mtDNA D - loop region was performed under appropriate PCR conditions. The products obtained as a result of PCR were imaged in 2% agarose gel, and the sample gel image is provided in Figure 1.

The 842 bp portion of the ovine D - loop gene region was amplified, and the 483 bp sequence information was obtained as a result of analyses of the gene sequence of this region (Iontek). Images of the sample electropherograms for the analyses are provided in Figure 2.

3.3. Phylogenetic analysis results

The mtDNA D - loop gene sequence information in the sheep were evaluated to calculate the number of polymorphic site (S), number of haplotypes (h), haplotype diversity (H_d), nucleotide diversity (π), and average number of nucleotide differences (k) (Table 1). In the mtDNA D - loop of the Şavak breed, 27 polymorphic sites and 20 haplotypes were identified.

The phylogenetic tree created with the mtDNA D - loop sequences of Şavak Akkaraman sheep and the reference sequences (HG A: DQ852286 - 852287, HG B: DQ852282

-DQ852285, HG C: DQ852283 - DQ852284, HG D: DQ852288 - DQ852289, HG E: DQ852280 - DQ852281) [15] are provided in Figure 3. The phylogenetic tree shows us that of the 20 haplotypes identified in the Şavak Akkaraman sheep, 11 are in haplogroup B, 6 in haplogroup A, and 3 in haplogroup C.

Genetic distances calculated for the haplogroups identified based on reference sequences and sequences for the Şavak Akkaraman breed are provided in Table 2.

Phylogenetic relations were determined based on the results of the sequence analysis of the Şavak Akkaraman breed and wild sheep with (AF242347: *Ovis ammon*, JX673912: *Ovis ammon hodgsoni*, AY091492: *Ovis ammon collium*, KF677288: *Ovis orientalis anatolica*, AY091489: *Ovis vignei arkal*, AY091493: *Ovis ammon nigrimontana*, AF242348: *Ovis ammon darwini*, AF039580: *Ovis vignei bochariensis*). The phylogenetic tree created based on this assessment is provided in Figure 4.

Genetic distances between wild sheep and the Şavak haplogroups obtained from the mtDNA D - loop region analysis results are provided in Table 3.

4. Discussion

Sheep have been divided into 2 major groups, namely Asian (A) and European (B) types based on studies of the mtDNA D - loop region in different breeds of sheep [9,12]. However, the existence of a third lineage (C) was later identified in research conducted by Luo et al. [14] on domestic Chinese sheep breeds and Pedrosa et al. [13] on domestic Turkish breeds (Akkaraman, Karayaka, Hemşin, Morkaraman, and Tuj). Pereira et al. [29] reported that there is a low frequency of the C lineage in domestic Portuguese sheep. Tapio et al. [11] identified a fourth maternal lineage named D in the Karachai sheep in the northern Caucasus region, which is separate from the 3 lineages already identified. Meadows et al. [15], on the other hand, were the first to identify a fifth lineage (E) in the Tuj and Ivesi sheep breeds in a sample population consisting of Israeli Ivesi sheep and the Karakaş, Norduz, Morkaraman,

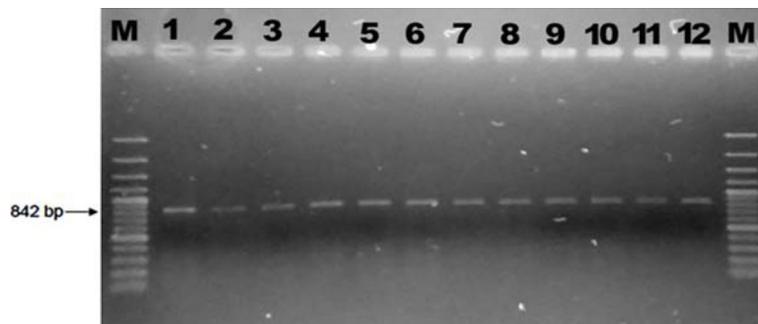


Figure 1. D - loop region in sheep (sample numbers 1–12): 842 bp (M: marker, ladder: 100 bp).

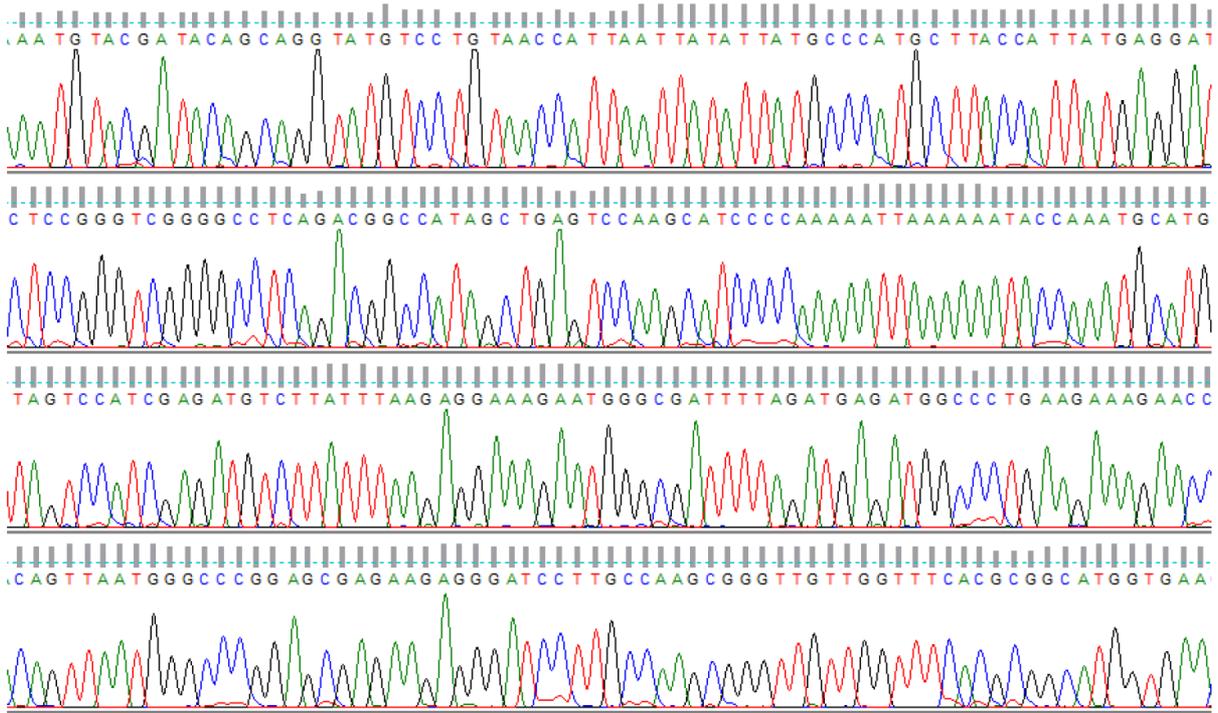


Figure 2. Chromatogram image of the sample sequence analysis of the Şavak Akkaraman breed mtDNA D - loop region.

Table 1. Some statistical data calculated based on the mtDNA D - loop gene sequences of the Şavak Akkaraman sheep.

	Şavak Akkaraman sheep
Total number of sites	483
G + C (guanin + sitozin)	0.435
number of polymorphic site (S)	27
number of haplotypes (h)	20
haplotype diversity (H_d)	0.995 ± 0.004
nucleotide diversity (π)	0.0146 ± 0.0003
average number of nucleotide differences (k)	7.068

Tuj, Çine Çaparı, Sakız, Karya, and Karayaka sheep breeds. In the Şavak breed that we investigated in this study, the D and E haplogroups were not found, but the A, B, and C haplogroups were identified, which are consistent with other similar studies [3,13,15,30,31]. The nucleotide diversity values identified in the Şavak Akkaraman breed were similar to those found by Pedrosa et al. [13], but were lower than the 3 different domestic Chinese sheep breeds [32] and 13 domestic sheep breeds [3]. While the haplotype diversity found in the Şavak Akkaraman sheep is similar to that found by Zhao et al. [32] in 3 different domestic Chinese sheep breeds, by Demirci et al. [3] in 13 domestic Turkish sheep breeds, by Liu et al. [33] and Guangxin et al. [31] in Tibetan sheep, by Kuseniuk and Slota, [34] in

Poland sheep, by Alonso et al. [35] in Mexican Creole sheep, and by Nigussie et al. [36] in Ethiopia sheep, the study that Chen et al. [37] conducted in domestic Chinese sheep is lower than the Şavak Akkaraman breed. In other words, the haplotype diversity in the Şavak Akkaraman breed is higher. From a historical perspective, the reason for this is thought to be gene transfer connected with migration. The haplotype and nucleotide diversity found in the study that Öner et al. [5] conducted in domestic Turkish sheep breeds (Dağlıç, Kıvrıcık, İmroz, Sakız, Morkaraman, İvesi, Hemşin, Karayaka and Akkaraman) were consistent with the results of the Şavak Akkaraman breed. The distribution of haplogroups in Şavak Akkaraman sheep was determined using the sequences

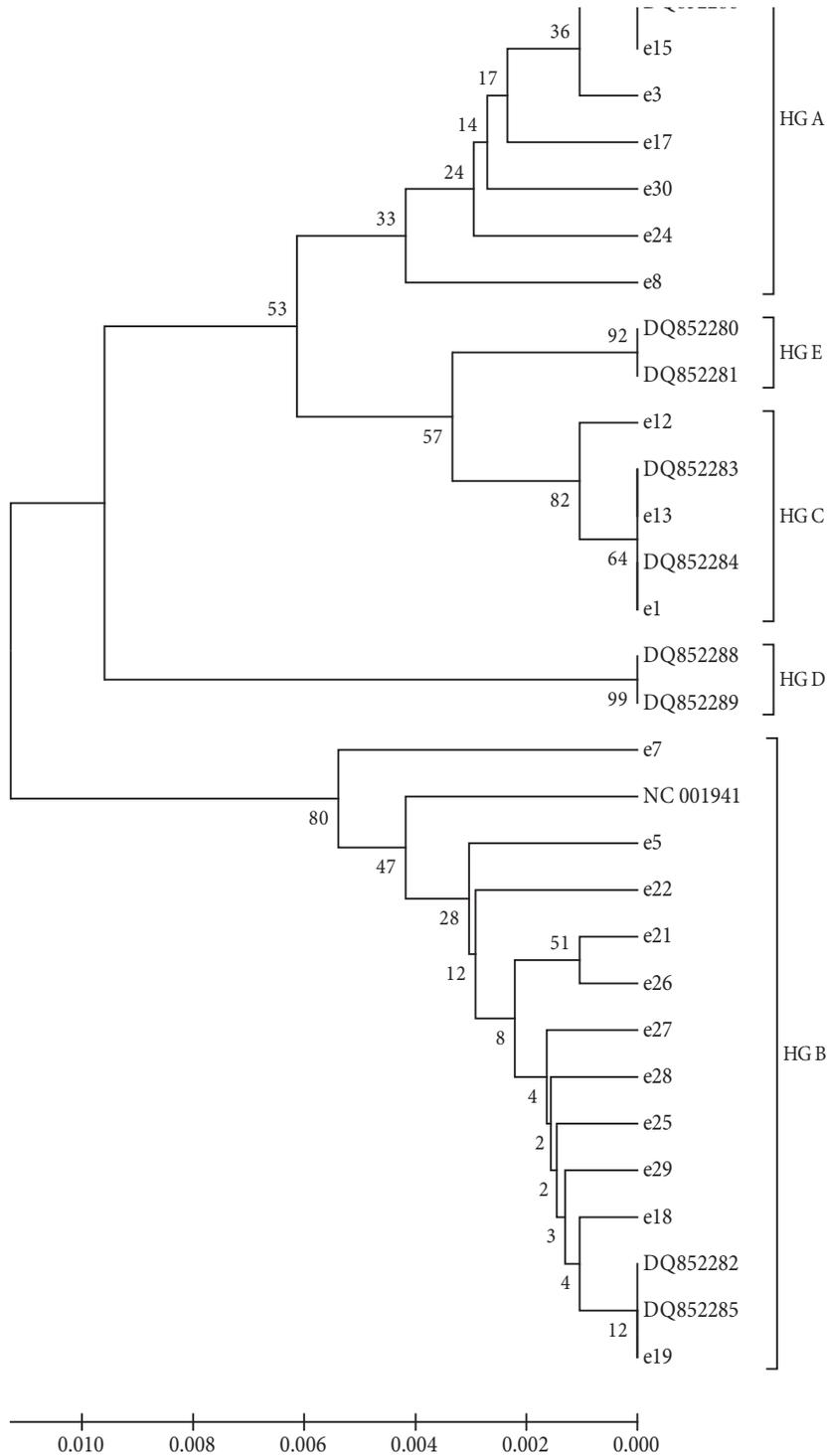


Figure 3. UPGMA tree created based on the mtDNA D - loop region in sheep.

for the haplogroups identified in the study conducted by Meadows et al. [15] as a reference. Of the 20 haplotypes identified in the Şavak Akkaraman sheep, 11 are in lineage B, 6 in lineage A, and 3 in lineage C. Examination of the phylogenetic trees created with breeds that contain A,

B, and C haplogroups based on the results of the studies conducted by Meadows et al. [15] and Demirci et al. [3] indicates that Şavak Akkaraman and the other breeds partially diverge in lineage A, but Şavak Akkaraman and the other breeds cluster in lineages B and C. The Bootstrap

Table 2. Genetic distances between the Şavak Akkaraman and Reference haplogroups

Haplogroups	HG E	HG C	HG D	HG A
HG E				
HG C	0.007			
HG D	0.023	0.021		
HG A	0.010	0.013	0.017	
HG B	0.025	0.026	0.027	0.019

Table 3. Genetic distances between Şavak Akkaraman haplogroups and wild sheep.

Haplogroups	Wild sheep
HG B	0.034
HG D	0.038
HG C	0.027
HG E	0.026
HG A	0.026

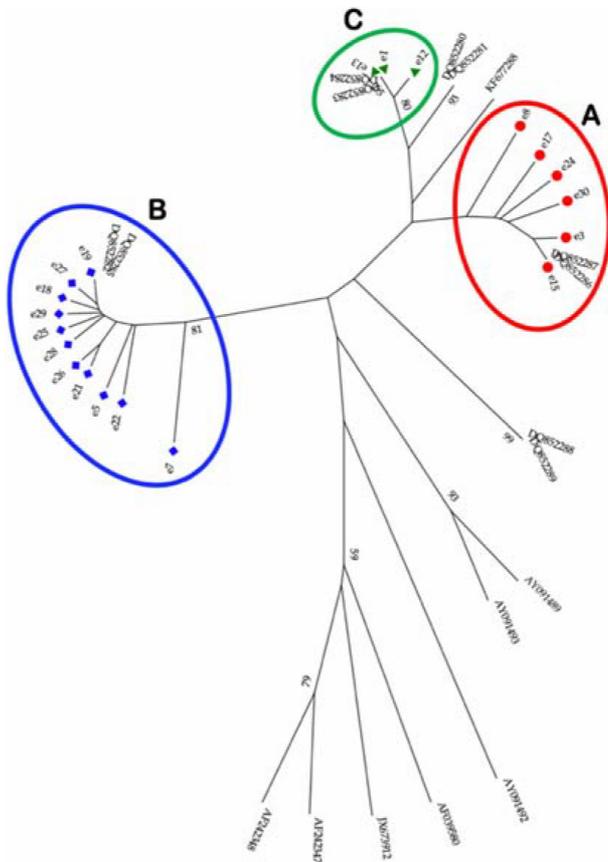


Figure 4. NJ phylogenetic tree created with the Şavak haplogroups and wild sheep.

test values were high in the phylogenetic trees' primary branches that generally separate haplogroups and low in the subbranches that indicate the individual members of the breed and the sequences used as a reference (<50%). As a result, there was no noticeable distinction between the individual members of the breed. At the same time, it was clear that there was a high degree of genetic similarity on the phylogenetic trees that were created with animals included in different haplogroups containing domestic

Turkish breeds, which can be explained by the fact that the Şavak Akkaraman breed is raised in a specific area and the fact that the sample size was small.

The distribution of each haplogroup in the Şavak Akkaraman breed is generally consistent with the results of previous studies conducted on domestic sheep breeds, where the most common haplogroup is B [3,13,15,30]. In the study conducted by Pedrosa et al. [13], haplogroup A was only predominant in the Morkaraman breed. Guangxin et al. [31] reported that haplogroup A is dominant in Tibetan sheep. However, Pedrosa et al. [13] reported that haplogroup B is generally dominant. In their study of the Dağlıç, Kıvrıkcık, İmroz, Sakız, Morkaraman, İvesi, Hemşin, Karayaka, and Akkaraman breeds, Öner et al. [5] reported that haplogroup A, which is dominant in Chinese breeds [37], was dominant in all except for İmroz breed. They reported that this situation could be related to the fact that the Silk Road trade, which was the historic caravan route that made it possible to trade products and animals between Turkey, Iran, India and China, had come to an end. It has been claimed that the weak genetic makeup among domestic sheep breeds in Turkey may be related to the strong long-term genetic diversity caused by migration. The inconsistency in the results might be explained by sampling differences. In other words, different results might be obtained in studies that include a greater population. Similar differences are also observed between phylogenetic trees created from the analyses of mtDNA regions of different lengths.

Analysis of the mtDNA gene sequences in the Şavak Akkaraman sheep raised in the Erzincan region of Turkey identified haplogroups A, B, and C, which means that this breed from this particular region is divided into 3 primary haplogroups with a high level of diversity. We think that entering the results of the sequence analysis from this study into the Gene Bank will provide data for future studies conducted with other breeds of sheep, especially in connection with the mtDNA D - loop region. The data for the Şavak Akkaraman sheep raised in this specific region of Turkey are also expected

to contribute to genetic studies and studies to protect the breed. The study was the first research conducted on the Şavak Akkaraman breed, and made it possible to compare domestic breeds as well as those from other countries. Research of the different gene regions for this breed raised in a specific location will also help breeding programs. Analysis of this breed in terms of polymorphic microsatellite markers that are common across different breeds, as well as the paternal Y chromosome will also contribute to the study of the breed's genetic makeup, not only maternally but also paternally. In addition, future studies through the whole genome sequencing of mtDNA

in sheep will help to further expand the phylogenetic analysis.

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