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Araştırma Makalesi

# Molecular Phylogenetic Analysis of Morkaraman Sheep in Bingol Region

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**Abstract**

In the present study, we report mitochondrial DNA (mtDNA) 16S rRNA gene sequence analysis of the Morkaraman ewe, (*Ovis aries*) was investigated. In domestic sheep, the 16S rRNA gene is *ca* 1574 bp in length. Partial fragment of 536 *bp* 16S rRNA gene of mtDNA was amplified by using designed primers from published data of Sheep sequences derived from National Center for Biotechnology Information (NCBI) web server (ncbi.nlm.nih.gov/NC_001941.1). Homology of the sequences obtained from the present work were performed by using BLAST program at NCBI. Obtained 16S rRNA gene sequences were submited to the GenBank database with accession numbers, KU686952.1, KU686953.1, KU686954.1, KU686955.1, KU686956.1, KU686957.1, KU686958.1, KU686959.1, KU686960.1, KU686961.1 and KU686962.1, respectively. According to nucleotide differences of mtDNA 16S rRNA gene region 10 polymorphic regions and 8 haplotypes were detected in Turkish domestic sheep Morkaraman (*n*=11). The nucleotide composition averages of all the sequences was 32.1% Adenine (A), 24.2% Thymine (T), 22.2% Cytosine (C) and 21.5% Guanine (G); G+C was 43.7%. DNA polymorphism based on 16S rRNA gene sequences, haplotype diversity (*Hd*) and nucleotide diversity ($\pi$) were found to be 0,9273±0,00442, and 0,00427±0,00125, respectively. Based on gene sequences information, in sheeps, mtDNA haplotypes, the phylogenetic relationship among mtDNA polymorphism, haplotypes and relationship between native sheep breeds and foreign sheep breeds were determined and discussed. Thus, sequence analysis of mitochondrial 16S rRNA gene can be used as a tool for phylogenetic analysis of Morkaraman sheep.

**Key words:** Genetic Diversity, Morkaraman (*Ovis aries*), Phylogenetic, mtDNA, 16S rRNA

## Bingöl Yöresinde Morkaraman Koyunlarının Moleküler Filogenetik Analizi

**Öz**

Bu çalışmada, Morkaraman koyunlarının (*Ovis aries*) filogenetik ilişkileri mitokondriyal DNA (mtDNA) 16S rRNA gen dizisi analizi ile gerçekleştirilmiştir. Evcil koyunlarda, 16S rRNA geni 1574 baz çifti (bç) uzunluğundadır. Çalışmamızda mtDNA 16S rRNA gen bölgesi hedeflenerek, Ulusal Biyoteknoloji Bilgi Merkezi (NCBI) alınan örnek sekansla (NC_001941.1) tasarlanan primerler ile, 536 bç'lik sekans ürünleri elde edilmiştir. Elde edilen diziler için homoloji araştırması, NCBI'de BLAST kullanılarak yapılmıştır. Çalışma sonunda elde ettiğimiz 16S rRNA gen bölgesi dizi bilgileri GenBank veri tabanına sırası ile bu erişim numaralarıyla yer almaktadır; Sırasıyla KU686952.1, KU686953.1, KU686954.1, KU686955.1, KU686956.1, KU686957.1, KU686958.1, KU686959.1, KU686960.1, KU686961.1 ve KU686962.1. Morkaraman koyun örnekleri (n=11) üzerinde yapılan analizler sonunda, mtDNA 16S rRNA gen bölgesi sekanslarından nükleotit farklılıkları bakımından 10 polimorfik bölge ve 8 haplotip bölge tespit edilmiştir. Tüm dizilerin nükleotit kompozisyon ortalamaları % 32.1 Adenin (A), % 24.2 Timin (T), % 22.2 Sitosin (C) ve % 21.5 Guanin (G); G + C % 43.7 olarak bulundu. 16S rRNA gen dizileri, haplotip çeşitliliği (*Hd*) ve nükleotit çeşitliliğine ($\pi$) dayanan DNA polimorfizmi sırasıyla 0.9273 ± 0.00442 ve 0.00427 ± 0.00125 olarak bulunmuştur. Gen dizileri bilgisine dayanarak, koyunlarda, mtDNA haplotipleri, mtDNA

polimorfizmi, haplotipleri arasındaki filogenetik ilişkiler ile belirlenen yerli ve yabancı koyun ırkları arasındaki filogenetik ilişkileri belirlenmiştir.

**Anahtar kelimeler:** Filogenetik, Genetik Çeşitlilik, Morkaraman (*Ovis aries*), mtDNA, 16S rRNA.

**Introduction**

One of the important production line of livestock sector in agriculture is sheep breeding. The sheep have a special place in the agricultural structure in terms of combined yield characteristics in animal production, such as milk, meat, leather, fleece, fertilizer and fertility, which are adapted to different geographical regions. The archaeological findings show that sheep were culturally and economically used in social life as an altar in religious ceremonies in some communities (Togan et al., 2013). Moreover, archeological and genetic studies have revealed that the sheep was domesticated in Turkey, in the vicinity of eastern/south-eastern Anatolia. Turkish native sheep (one of them is Morkaraman) are considered as a separate race because they have grown up among themselves for many years (Kurt, 2017; Kul and Ertuğrul, 2010). Morkaraman sheep (*Ovis aries*), Turkish indigenous breed, are mainly distributed in mountain areas across Eastern Anatolia area at a altitude between 1500 and 2500 m (Yalçın, 1986). Total number of ovin livestock in 2020 is 55.063.391 in Turkey. Among all the livestock, sheep are 42.712.580 heads. The proportion of domestic sheep breed is 91.44 percent (TUIK, 2020). Domestic sheep has 54 (2n) of chromosomes, 26 pairs of autosomal chromosomes and 1 pair of sex chromosomes and in addition they have mitochondrial DNA (mtDNA) as well. Sheep mtDNA is a small extrachromosomal genome that is characteristic 16 to 20 kb in size with 37 genes. Sheep mtDNA is maternal inheritance. Among these 37 genes, 13 protein-coding genes and 22 transfer RNAs (tRNAs), 2 genes encode ribosomal RNAs which 12S rRNA and 16S rRNA. In domesticated sheep, the 16S rRNA gene region has a length of *ca* 1573 bp (www.ncbi.nlm.nih.gov). Mitochondrial genes were identified for various species (Saikia et al., 2015; Mane et al., 2013). Especially, mtDNA is commonly used for the study of domesticated species and mtDNA has enable information utility in the study of molecular evolution, population genetic analysis, and identification of relationship for interspecific and intraspecific. (Fan et al., 2016; Naderi et al., 2007). The 16S rRNA gene sequence is widely used as markers for analysis of phylogenetic evolution of sheep and animal species (Sanna et al., 2015; Yan et al., 2019). In addition, subpopulation phylogenetic relationships were determined in populations (Kirikci et al., 2018). Among all molecular techniques, the DNA sequencing has enabled as a widely used method for identifying species. Furthermore, the technique is regarded as one of the most efficient method in terms of detection and definition (Sarri et al., 2014). Considering these facts in view investigations of the phylogenetic analysis of *Ovis aries* and provides important annotation information for we compared 16S rRNA gene regions sequences from Morkaraman sheep in this study.

**Materials and Methods**

The animal material of the present work is Morkaraman sheep which was bred in Bingöl region. Bingöl is located between 41º - 20º and 39º - 56º east longitudes and between 39º - 31º and 36º - 28º North latitudes. The animal material used in this study was obtained from the livestock operations of the provinces of Karlıova, Bingöl city center and Solhan of Bingöl province. Animal welfare was taken into consideration when blood samples were taken from animals by the veterinarian in accordance with The Republic Of Turkey Ministry Of Agriculture And Forestry Elazığ Veterinary Control Institute directive, for which the ethical license (*2011/11-1*) of this study was obtained. Genomic DNA was extracted from vena blood (*vena jugularis*) of eleven native Turkish sheep Morkaraman's using standard commercial Kit (Promega Wizard Genomic DNA Purification Kit A1120) as recommended by the manufacturer. Mitochondrial sequences of sheep were downloaded from the GenBank database and aligned using MEGA 5.1 program (Lasergene software; DNAStar, Inc., Madison, Wisconsin, USA). The GenBank accession number for *Ovis aries* mtDNA sequences is NC_001941.1. The binding regions of the primers generated from the 16S rRNA gene sequence on the Sheep's mtDNA and their expected sequence sizes by PCR analysis are shown in the Table 1.

**Table 1.** mtDNA 16S rRNA gene region forward (F) and reverse (R) species-specific primers sets

| Primer | Primer Sequence 5'→3' | Lenght (bp) | Position | Tm (°C) | Expected product size (bp) |
|---|---|---|---|---|---|
| MRK16S(F) | CCAAAATCTCCCACTCTCCAG | 21 | 1106-1127 | 64.71 | 1470-1573 |
| MRK16S(R) | CTCTTGTCCTTTCGTACTGGG | 21 | 2555-2576 | 62.52 | |

Reactions for mtDNA amplification were performed according to Sambrook et al. (1989). PCR reactions were carried out using a Thermal Cycler (Favorgen) and each PCR reaction was consisted of 1 µl of template DNA, 1 µl of each dNTP, 1 µl of each primer, 1 µl of MgCl2, 1 µl of Taq (5U) DNA polymerase and 4 µl of 10X buffer and 30 µl distilled water in a total volume of 40 µl. The PCR conditions were conducted as; predenaturation at 95ºC for 2 min followed by 35 cycles of 94ºC for 1 min, 60ºC for 50 sec, 72ºC for 1 min and a one cycle of final extension at 72ºC for 5 min. After the PCR amplification of 16S rRNA encoding gene, 11 individuals were ordered by İontek (Istanbul) company to perform 22 DNA sequencing for both forward and reverse chains. Pairwise sequence alignments were carried out using NCBI-BLAST. Multiple sequence alignments of 16S rRNA sequences from Morkaraman Sheep were carried out using CLUSTALW. Total number of regions for populations, number of polymorphic regions (*S*), haplotype number (*h*), haplotype diversity (*Hd*), nucleotide diversity ($\pi$), average number of nucleotide differences (*k*) and Tajima's D test statistics values were determined using the program of DnaSP 5.0 (Librado and Rozas, 2009). Phylogenetic analyzes of this study were performed by the MEGA 4.0.1 program (Tamura et al., 2007) using the Kimura-2-parameter + Gamma distribution (K2P + Γ) model according to the Neighbor-Joining (NJ) method (Saitou and Nei, 1987). Bootstrap value of 1000 replicates was used to test the reliability of nodes (Nei and Kumar, 2000). For phylogenetic analysis, Turkey native breeds and gene sequences of previously reported studies with other sheep breeds derived from GenBank (NCBI) were used and optimal phylogenetic trees were obtained.

**Results And Discussions**

The 16S rRNA gene regions of the mtDNA of Morkaraman sheep were amplified by PCR techniques using designed primers (Figure 1). The 16S rRNA gene region in domestic sheep is 1573 bp in length (https://www.ncbi.nlm.nih.gov/).

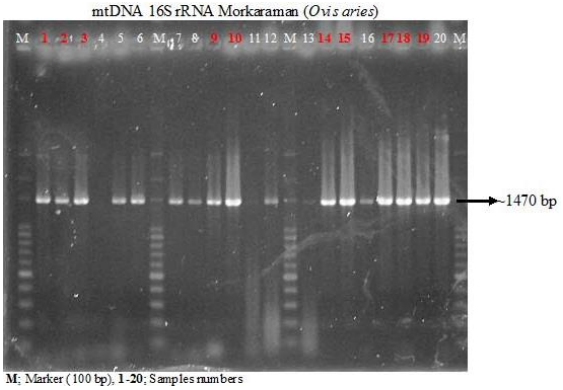


**Figure 1.** 16S rRNA gene region PCR agarose gel image in Morkaraman sheep.

The PCR products of the 16S rRNA gene region were approximately 1470 bp in length. For sequencing analysis, the DNA samples in wells 1, 2, 3, 9, 10, 14, 15, 17, 18, 19 and 20 were selected in the genomic DNA agarose gel image, respectively. As a result of the arrangement and evaluation of the 1470 bp sequences, a 536 bp segment was sequenced. Additionally, mtDNA 16S rRNA gene sequences belonging to the chromatograms were generated (Figure 2.).

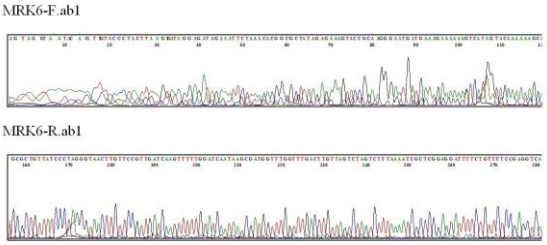


**Figure 2.** An examplified chromatogram (sample MRK6-F and R) of a section of the mitochondrial DNA 16S rRNA gene of Morkaraman sheep.

In the Morkaraman sheep, 10 polymorphic regions and 8 haplotypes were identified according to their nucleotide sequence differences for 16S rRNA gene. It is divided into 8 haplotypes. UPGMA genetic haplotype are shown in tree form (Figure 4.). The haplotype difference (*Hd*) was 0.9273 (±0.00442) and the nucleotide difference ($\pi$) was 0.00427 (±0.00125). Tajima's D value (-1.40051) was negative and statistically significant (Table 2).

**Table 2.** Basic molecular characteristics of 16S rRNA gene region of Morkaraman sheep breed.

| | |
|---|---|
| Number of sequence | 536 |
| G +C | 0.437 |
| Variable sites (*S*) | 10 |
| Number of haplotype (*h*) | 8 |
| Haplotype diversity (*Hd*) | 0.9273±0.00442 |
| Nucleotide diversity ($\pi$) | 0.00427±0.00125 |
| Average number of nucleotide differences (*k*) | 2.29091 |
| Tajima's *D* test statistics (P>0.1) | -1.40051 |

Findings of current study show the remarkably high haplotype diversity of Morkaraman sheep. Furthermore, these results indicate the effects of the current population expansion with the addition of new mutations in previous generations of morkaraman sheep race. Some of the haplotypes were also found to be partner as stated in tabulated form below (Table 3.).

**Table 3.** Haplotypes partner to Morkaraman sheep

| Haplotype | Number of haplotype | Sample code |
|---|---|---|
| Hap_5 | 3 | [MRK05, MRK08, MRK09] |
| Hap_8 | 2 | [MRK10, MRK11] |

Obtained from mismatch distribution analysis the value (k=2.29091) of the average pairwise differences. The results of Raggedness Statistics (r) are determined as 0.1864.

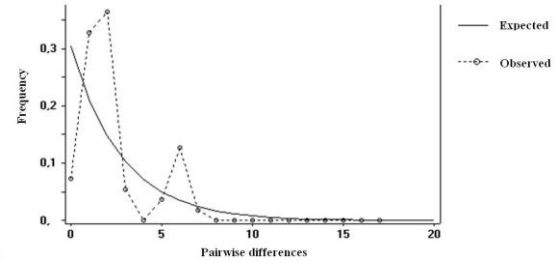


**Figure 3.** Mismatch distribution of haplotypes.

When the analysis is carried out for 8 haplotypes in this investigation group, multi-crowned bell curves were presented in Figure 3. German native sheep of the Merinolandschaf race was used as a reference sequence (NC_001941.1) in the comparison of the genetic difference of the haplotypes found for Morkaraman in current work (Hiendleder, 1998). Morkaraman haplotypes were analyzed in order to see their genetic distances to each other and to compare the genetic differences between them with reference gene sequencing (NC_001941.1). As a result of analysis, it was found that the genetic distance was the highest between MRK06 and MRK01 with the highest value of 0.01325. On the other hand, the maximum genetic distance between the reference sequence and MRK06 was found to be 0.00944. (Table 4.).

**Table 4.** Genetic distance between Morkaraman haplotypes and the German native sheep of the Merinolandschaf (RefSeq).

| Haplotypes | RefSeq | MRK01 | MRK02 | MRK03 | MRK04 | MRK05 | MRK06 | MRK07 | MRK08 | MRK09 | MRK10 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| RefSeq | | | | | | | | | | | |
| MRK01 | 0.00374 | | | | | | | | | | |
| MRK02 | 0.00187 | 0.00563 | | | | | | | | | |
| MRK03 | 0.00187 | 0.00563 | 0.00375 | | | | | | | | |
| MRK04 | 0.00187 | 0.00187 | 0.00375 | 0.00374 | | | | | | | |
| MRK05 | 0.00187 | 0.00187 | 0.00375 | 0.00374 | 0.00374 | | | | | | |
| MRK06 | 0.00944 | 0.01325 | 0.01135 | 0.01134 | 0.01134 | 0.01134 | | | | | |
| MRK07 | 0.00187 | 0.00563 | 0.00375 | 0.00375 | 0.00375 | 0.00375 | 0.01135 | | | | |
| MRK08 | 0.00187 | 0.00187 | 0.00375 | 0.00374 | 0.00374 | 0.00000 | 0.01134 | 0.00375 | | | |
| MRK09 | 0.00187 | 0.00187 | 0.00375 | 0.00374 | 0.00374 | 0.00000 | 0.01134 | 0.00375 | 0.00000 | | |
| MRK10 | 0.00000 | 0.00374 | 0.00187 | 0.00187 | 0.00187 | 0.00187 | 0.00944 | 0.00187 | 0.00187 | 0.00187 | |
| MRK11 | 0.00000 | 0.00374 | 0.00187 | 0.00187 | 0.00187 | 0.00187 | 0.00944 | 0.00187 | 0.00187 | 0.00187 | 0.00000 |

Because of the limited DNA polymorphism of the phylogenetic tree formed according to the sequences of the 16S rRNA gene and the genetic distance between the lower value is the number of haplotypes was preferred UPGMA tree.

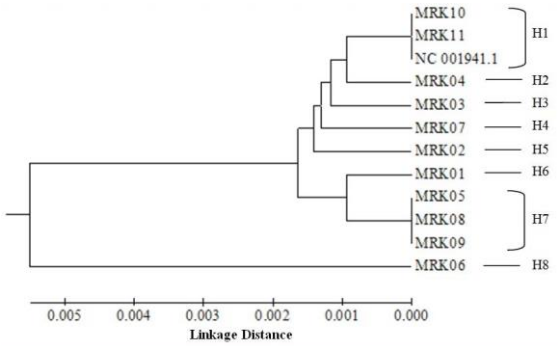


**Figure 4.** The UPGMA phylogenetic tree constructed for the mtDNA 16S rRNA gene sequences for 11 Morkaraman sheep together with the reference sequence (NC 001941.1). The tree was drawn to scale and individual substitutions are indicated (H1-H8; Haplotypes group).

Differences in the nucleotide sequences of the haplotypes when the resulting DNA sequences were compared. Merinolandschaf German native sheep (NC 001941.1) was taken for the basis for comparison as outgroup control. The first sequence on the table belongs to the reference sequence (NC_001941.1) (Table 5.). Nucleotide substitutions in the 10 polymorphic regions are observed to be in the form of transitions.

**Table 5.** The haplotypes determined according to the 16S rRNA gene region for Morkaraman Sheep when comparing to the reference sequence (NCBI: NC_001941.1)

| Haplotypes | Positions * | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2151 | 2168 | 2188 | 2284 | 2294 | 2320 | 2359 | 2442 | 2481 | 2508 |
| NC 001941.1 | A | T | T | G | A | C | G | C | A | T |
| MRK01 | - | - | - | - | - | - | - | T | - | C |
| MRK02 | - | - | - | - | - | - | - | - | G | - |
| MRK03 | - | C | - | - | - | - | - | - | - | - |
| MRK04 | - | - | - | - | - | - | - | T | - | - |
| MRK05 | - | - | - | - | - | - | - | - | - | C |
| MRK06 | G | - | C | - | G | T | A | - | - | - |
| MRK07 | - | - | - | A | - | - | - | - | - | - |
| MRK08 | - | - | - | - | - | - | - | - | - | C |
| MRK09 | - | - | - | - | - | - | - | - | - | C |
| MRK10 | - | - | - | - | - | - | - | - | - | - |
| MRK11 | - | - | - | - | - | - | - | - | - | - |

The "-" sign in the table shows the same nucleotide distribution as the reference sequence. Otherwise nucleotides with initials (ATGC) are represented. Using the model for transformational and transversion nucleotide substitution rates by the Kimura 2-Parameter model (Kimura, 1983). Transformational nucleotide substitution rates are shown in bold and transverse nucleotide substitution in italic font (Table 6.).

**Table 6**. Nucleotide substitution rates according to the 16S rRNA gene region for Morkaraman Sheep.

| | **A** | **T** | **C** | **G** |
|---|---|---|---|---|
| **A** | - | *0.03* | *0.03* | **14.88** |
| **T** | *0.04* | - | **30** | *0.03* |
| **C** | *0.04* | **32.63** | - | *0.03* |
| **G** | **22.22** | *0.03* | *0.03* | - |

The nucleotide difference is Adenine with the highest 32.1%. The second high rate is in Timin with 24.2% (Table 7). As apparent, the nucleotide composition of the 536 bp region of the 16S rRNA gene is richer in A+T comparing to the rate of G+C. The rate of cytosine and guanine were found to be 22.2% and 21.5% respectively (Table 7.).

**Table 6**. Nucleotide Compounds of 16S rRNA Gene Regions of eleven Morkaraman sheep investigated in present study.

| | T(U) | C | A | G | **Total** |
|---|---|---|---|---|---|
| NC 001941.1 | 24.3 | 22.2 | 32.1 | 21.5 | 536 |
| MRK01 | 24.3 | 22.2 | 32.1 | 21.5 | 536 |
| MRK02 | 24.3 | 22.2 | 31.9 | 21.6 | 536 |
| MRK03 | 24.1 | 22.4 | 32.1 | 21.5 | 536 |
| MRK04 | 24.4 | 22.0 | 32.1 | 21.5 | 536 |
| MRK05 | 24.1 | 22.4 | 32.1 | 21.5 | 536 |
| MRK06 | 24.3 | 22.2 | 31.9 | 21.6 | 536 |
| MRK07 | 24.3 | 22.2 | 32.3 | 21.3 | 536 |
| MRK08 | 24.1 | 22.4 | 32.1 | 21.5 | 536 |
| MRK09 | 24.1 | 22.4 | 32.1 | 21.5 | 536 |
| MRK10 | 24.3 | 22.2 | 32.1 | 21.5 | 536 |
| MRK11 | 24.3 | 22.2 | 32.1 | 21.5 | 536 |
| **Average** | 24.2 | 22.2 | 32.1 | 21.5 | 536 |

Kimura 2 parameters (K2P) model and Neighbor-Joining (NJ) method was performed to form the phylogenic trees showing the phylogenetic relations between the varieties (Figure 5.).

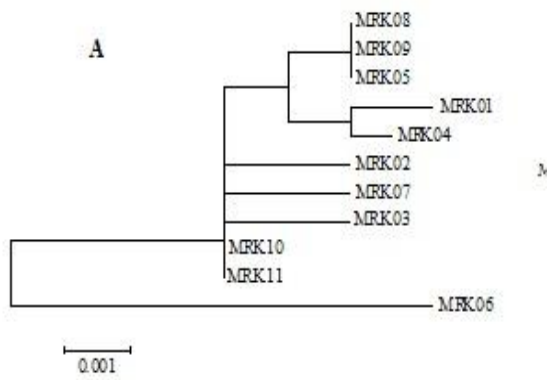


**Figure 5.** The UPGMA phylogenetic tree constructed for the mtDNA 16S rRNA gene sequences for 11 Morkaraman sheep.

MRK04 and MRK01 varieties are tightly linked to each other as shown in the phylogenic tree. Additionally, MRK05, MRK08 and MRK09 varieties are formed a separate clade whilst MRK10 and MRK11 are closely related to te each other with minimum differences. MRK06 is located significantly distinct in phylogenic tree from the all other breeds analyzed in current work. The sequence information of the 16S rRNA gene region as a genetic marker for the differentiation of species is indicated to be potential (Ramadan, 2011). The nucleotide sequences of the haplotypes of the 536 bp fragment of the 16S rRNA gene region of the Morkaraman sheep breed were first reported in this study. (The GenBank database with accession numbers, KU686952.1, KU686953.1, KU686954.1, KU686955.1, KU686956.1, KU686957.1, KU686958.1, KU686959.1, KU686960.1, KU686961.1 and KU686962.1). Sequence information of mitochondrial gene regions is generally used to explain their evolutionary relationships (Meadows et al., 2007; Tapio ve ark., 2006; Resende et al.., 2016). The sequence information of the mtDNA gene regions have also been used in many studies to identify the animal species, rather than sheep, such as poultry (Girish et al., 2007). In particular, the sequence information of the 16S rRNA gene region was also used to investigate the morphological similarity and genetic relationships between bovidae (Kuznetsova et al., 2002). Among Turkish native sheep breeds Tuj (NCBI: HM236183.1) and Karakaş (NCBI: HM236178.1) sheep were also investigated for 16S rRNA sequence using Kimura 2 parameters (K2P) model and Neighbor-Joining (NJ) method and resulted trees showed the phylogenetic relationship between the Morkaraman and these animals (Figure 6.). Tuj (HM236183.1) and Karakaş (HM236178.1) did not form separate groups from the Morkaraman varieties but were found to be so close to eachother.

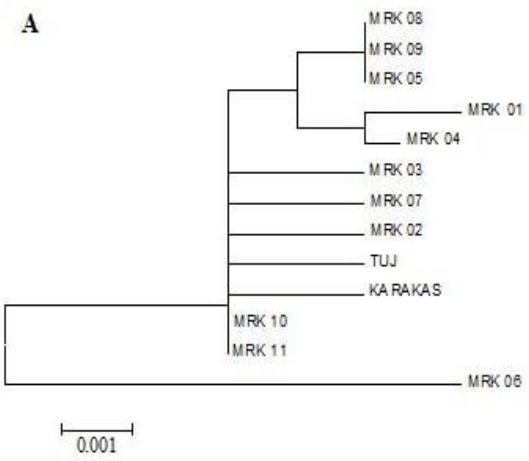


**Figure 6.** The UPGMA phylogenetic relations the Morkaraman sheep and Turkish native sheep breeds, Tuj and Karakaş sheep.

Findings of current study was also compared with the report of Lv et al. (2015) who studied 4 domestic (Pramenka-Serbia, Karakul-Moldova, Swiniarka-Poland and Mountain Carpathian-Ukraine) and 4 commercial sheep breeds (Comisana, Merinizzata Italiana, Lancaune ve Merinos) from Eastern Eurasian and Phylogenetic relationship trees are presented below (Figure 7).

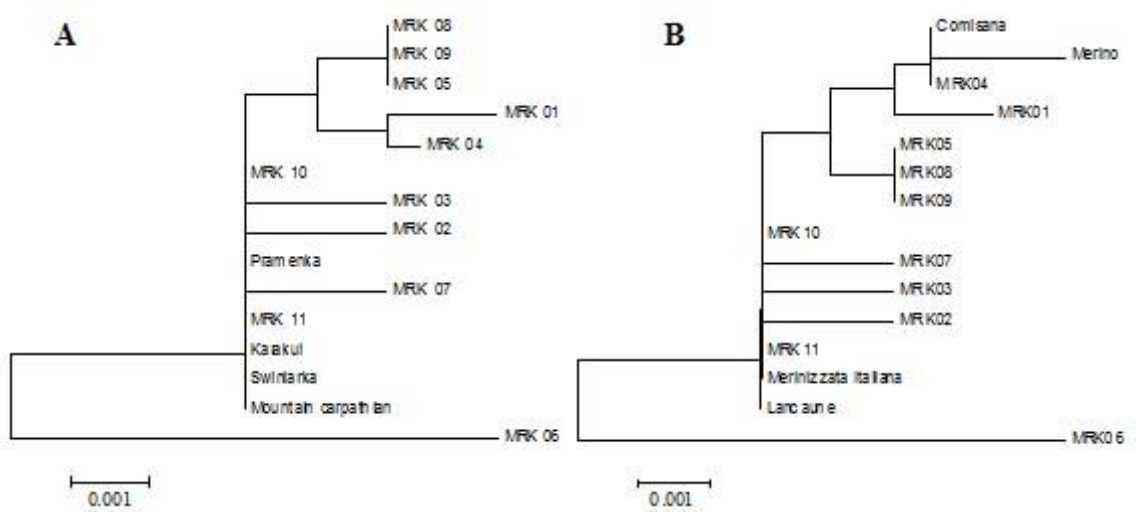


**Figure 7.** The UPGMA phylogenetic relations the Morkaraman sheep and Eastern Eurasian sheep.

Both trees are shown in the MRK06 seems to be more distinct from the others whilst other haplotypes appear to be cladded in Eastern Eurasian sheep.

**Conclusions**

Morkaraman sheep constitute an important group of Turkish domestic small ruminants. Especially, it is a small ruminant breed grown in family farms. Research and development of genetic resources and biodiversity of this native bred will be an important issue for conservation of small ruminant livestock of the region. As a result, 16S rRNA gene sequences from Morkaraman sheep of Bingöl region were determined. By depositing the gene sequence information in the Gene Bank (NCBI), it is expected to contribute to phylogenetic studies on sheep. In addition, the results of the study are thought to be contributed to genetic polymorphism, biodiversity and animal breeding studies and national gene conservation strategies.

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**Conflict of Interest Statement:** The manuscript's authors declare that, they do not have any conflict of interest.

**Researchers' Contribution Rate Statement Summary:** The authors declare that, they have contributed equally to the manuscript.

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