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Research Article

Investigation of Genetic Structures of Coloured Horses by mtDNA D-loop Sequence Analysis in Turkey [1][2]

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Abstract

The aim of this study was to determine the genetic structure of Coloured horses in Turkey by analysis of the D-loop sequence of mitochondrial DNA (mtDNA). A total of 28 Coloured horses were examined. DNA was extracted from blood samples using DNA isolation kit; 519 bp long mtDNA D-loop region was amplified by PCR and sequenced by capillary electrophoresis system. Population parameters and phylogenetic trees were drawn by using MEGA4 software package. It was also compared with the DNA sequences of horse populations from different countries. In this study, 42 different polymorphism regions and 10 different haplogroups were detected. Additionaly, Tajima D value was found to be -0.31 and population expansion was determined. It was determined that the base differences among the horses ranged between 0.000 and 0.032. It was detected that the horses formed different clusters from each other and they were intertwined with the populations of different countries. Moreover, it was also observed that some horses formed their own populations at different points from the other countries' horses. As a result, it was observed that the genetic structures of horses which used in population were different from each other and they originated from different mothers. According to the results obtained, it is considered that Coloured horses can be a native horse breed of Turkey.

Keywords: *Coloured horse, D-loop region, Haplogroup, Mitochondrial DNA*

Türkiye'deki Alaca Atların mtDNA D-Loop Dizi Analizi İle Genetik Yapılarının Araştırılması

Öz

Bu çalışma, Türkiye'deki Alaca atların mtDNA D-Loop dizi analizi ile genetik yapılarının belirlenmesi amacıyla yapılmıştır. Araştırmada, toplam 28 Alaca at incelenmiştir. Alınan kan örneklerinden DNA izolasyon kiti kullanılarak DNA elde edilmiş ve 519 bp uzunluğundaki mtDNA D-loop bölgesi PZR ile çoğaltılmış ve kapiller elektroforez sisteminde dizileme işlemi yapılmıştır. MEGA4 paket programı kullanılarak populasyon parametreleri ve filogenetik ağaçları çizilmiştir. Ayrıca farklı ülke at populasyonlarına ait DNA dizileri ile de karşılaştırılması yapılmıştır. Araştırmada, 42 farklı polimorfizm bölgesi ve 10 farklı haplogrup elde edilmiştir. Ayrıca Tajima D değeri -0.31 elde edilmiş ve populasyon genişlemesi olduğu belirlenmiştir. Örnekler arasındaki baz faklılıklarının 0.000 ile 0.032 arasında değiştiği belirlenmiştir. Çizilen filogenetik ağaçlar sonucunda örneklerin birbirlerinden farklı kümeler oluşturduğu ve farklı ülke populasyonları ile de iç içe girdiği belirlenmiştir. Ayrıca, bazı örneklerin farklı ülke atlarından tamamen ayrı noktalarda kendi populasyonunu oluşturduğu da gözlenmiştir. Sonuç olarak çalışılan populasyondaki örneklerin genetik yapılarının birbirinden farklı olduğu ve farklı analardan köken aldıkları görülmüştür. Elde edilen sonuçlara göre; Alaca atların Türkiye'ye ait yerli bir ırk olabileceği düşünülmektedir.

Anahtar sözcükler: *Alaca at, D-loop bölgesi, Haplogrup, Mitokondrial DNA*

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INTRODUCTION

In the archaeological and genetic studies, the origins of many species were clearly stated [1]. Although there is no clear information about the time and place where the horses were domesticated, it is reported that they were domesticated in different places at different times [2]. It was stated that Eurasian steppes are belived to be an important domestication center [3]. Different horse populations were also compared in other studies, and it was found that horses were domesticated in different regions since the Iron Age [4].

As a result of the phylogenetic analysis, no relationship could be found between domestication of horses and geographical places where they were bred [5]. It was reported that at least 17 haplotypes of the oldest ancient horse breeds have become extinct in the last 5500 years [4]. In a study carried out in indigenous stallions, which were found in Europe for many years, it was reported that genetic diversity has decreased [6].

Native horse breeds of Turkey are decreasing in terms of number and genetic resources and pure breeding is interrupted as a result of the cross-mating of breeds. For this reason, it is important to take measures for preserving these breeds and to conduct molecular genetic analyses to guide these preservation programs. Due to genetic bottleneck, the population faces the danger of extinction. It is also very important to be able to maintain purebred breeding and to develop economically important traits [7].

It was reported that populations created by using haplotypes obtained from horse studies may be used to classify ancient remains, to assess haplogroup variation in modern breeds, and to evaluate possible roles of horses in race performance [8].

Recently, Y chromosome (for paternal history) and the mitochondrial DNA (mtDNA) (for maternal history) were used in the evolutionary, phylogeographic and genetic diversity studies [9-13]. In a study conducted in the United States, mtDNA sequence analyses were found to be useful in eliminating doubts about the origin of horses, other than biological characteristics, and they may also be used in solving problems based on traditional assumptions about Arabian horses with a close common ancestry [14].

In a study conducted for genetic characterization of different horse breeds in Turkey using D-loop region of mtDNA and establishing a preservation program, it was determined that haplotype diversity was high; however, there was low nuclotide diversity. In addition, no valid genetic separation was detected among the breeds. Moreover, a phylogenetic tree was created using 22 horses representing seven haplogroups previously published and indigenous horses. mtDNA analysis of Turkish horses confirmed that many ancestral mare breeds were involved in the domestication of the horses [15].

In a study conducted using the mitochondrial control region to determine the genetic variation in Ayvacık Pony, Malakan, Hınıs and Canik horses; the haplogroups showed high diversity [16]. As a result of mtDNA sequence analysis of 5 horse breeds in Turkey, 68 polymorphic regions and 151 haplotypes (Haplotype diversity, Hd: 0.9866±0.0017, nucleotide diversity, Pi: 0.021±0.00036, and average nucleotide diversity, k: 8,006) were found [17].

Coloured horses are bred in a limited region especially in Ardahan, Kars and Iğdır provinces and have a small number of population in Turkey. In the literature review, there is no study found to define genetic identification of Coloured horses in Turkey by using the mtDNA D-loop sequence analysis. The aim of this study was to reveal the genetic structure of Coloured horses by analysis of the mtDNA D-loop sequence.

MATERIAL and METHODS

Preliminary Study and Determination of Specimens

In the present study, a preliminary field works were conducted to determine the number and characteristics of the animals as research materials to be investigated. Below are some photographs of Coloured horses taken during preliminary work in the field *(Fig. 1, 2, 3, 4)*.

In the field studies conducted, it was found that the total number of Coloured horses in Turkey is 250-300, that horse owners usually have one or two horses, and that horses frequently change hands among the breeders in the region. In the field works, it was determined that some of the Coloured horses sire or dam were not Coloured, and there could be some challenges in collecting samples and data due to the difficulties in reaching some plateaus where horses are found. In samples collected for the study, attention was paid to the fact that horses were not close relatives.

Fig 1. Coloured horses in Ardahan-Turkey

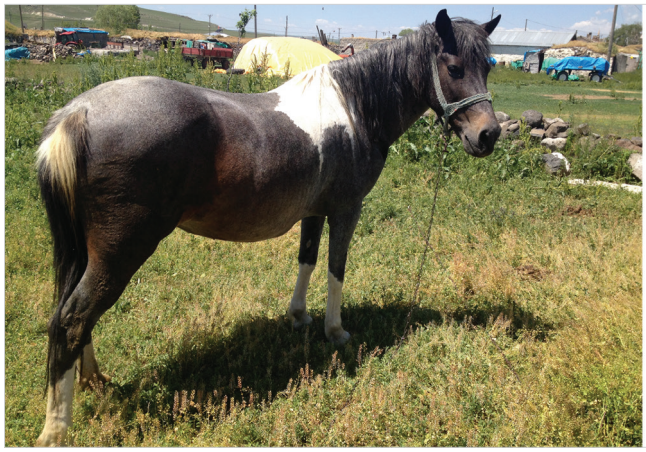

Fig 2. Coloured horse in Kars-Turkey

Fig 3. Coloured horses in Malatya-Turkey

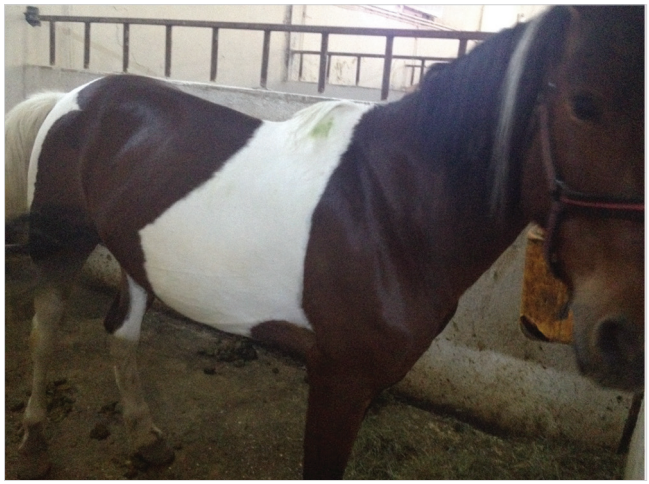

Fig 4. Coloured horse in Erzurum-Turkey

Collection of Blood Samples and Isolation of DNA

Blood was drawn from the *V. jugularis* of 28 Coloured horses (bred in Ardahan, Kars, Iğdır, Erzurum and Malatya provinces) and added into the anticoagulant (K_3EDTA) tubes. Blood samples were kept in the cold chain and delivered to the laboratory. In samples collected for the study, we paid attention to the fact that horses were not close relatives. DNA isolation was performed by using an automated Qiagen Biorobot M48 DNA isolation system and MagAttract DNA Mini M48 kit (Catalog No. 953336).

Amplification and Genotyping of Control Region (D-loop) in Mitochondrial DNA

The control region (D-loop) in mitochondrial DNA (mtDNA) was amplified using the forward primer F7 (5'-CCA TCA ACA CCC AAA GCT GAA-3') and the reverse primer R525 (5'-GTG AGC ATG GGC TGA TTA GTC-3'). Primers were designed using reference sequence (GenBank accession number AF064628.1) with FastPCR Professional 6.2.1 software [18]. A 25 µL PCR mixture consisted of 40 ng DNA, 6 µM of each primer, 2.4 mM $MgCl_2$, 1×PCR buffer, 0.2 mM dNTP mix and 1 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, U.S.A.). Pre-denaturation phase of PCR was programmed to be at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, and extension at 72°C for 1 min by 35 cycles and final extension at 72°C for 10 min.

For DNA sequencing analysis, a total of 20 µL reaction mixture was prepared containing 2 µL of Big Dye 3.1, 11 µL of 1X Sequencing Buffer, 5 µL of forward or reverse primer (1 pmol) and 2 µL of PCR product. The PCR was programmed for 30 sec at 96°C for pre-denaturation, 10 sec at 96°C for separation, 15 sec at 50°C for annealing and 4 min at 60°C for extention by 30 cycles. PCR products were cleaned with Bigdye XTherminator and sequencing was performed by Genetic Analyzer (ABI 3500).

Statistical Analysis

As a result of DNA sequencing analysis, D-loop region of mtDNA sequences were edited and assembled using Sequencher 5.4.6 (Gene Codes Corporation, Ann Arbor, MI, USA). Subsequently, all of sequences were aligned in BioEdit 7.0.9 Sequence Alignment software [19].

The evolutionary relationship between the horses studied was carried out using the UPGMA method [20] with the 1000-iterations Bootstrap test [21]. In addition, D-loop region of mtDNA reference sequences belonging to these populations were obtained from the National Center for Biotechnology Information (NCBI) databases and a phylogenetic tree was drawn in order to examine the relationship among horse populations in different countries. To determine whether the population has undergone mutation or natural selection, Tajima's neutrality test [22] was conducted.

The positions of the polymorphic nucleotides of Coloured horses, the evolutionary relationship among the horses studied and the neutrality test results were analyzed using the Maximum Composite Likelihood method [23] of the MEGA4 software [24]. All positions, including missing data and spaces, were removed from the data set and all sequences were brought to the same size. Analyses

Table 1. *The position of polymorphic nucleotides*

Horse No	15450	15494	15496	15521	15532	15534	15538	15542	15585	15596	15597	15601	15602	15603	15604	15617	15649	15650	15651	15657	15666	15667	15703	15709	15720	15737	15740	15770	15771	15773	15776	15777	15787	15806	15809	15811	15826	15827	15838	15869	15870	15871
1	C	T	A	G	C	C	A	C	G	A	A	T	T	T	G	C	A	A	G	T	G	A	T	C	A	T	A	C	T	A	T	A	A	T	A	C	A	G	C	C	C
2	.	C	G	.	.	T	.	.	.	.	.	.	.	C	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	T	T	T
3	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	T	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.
4	.	C	G	.	.	T	.	.	A	.	.	.	.	C	A	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	T	.
5	T	C	G	.	.	T	.	.	.	.	.	.	.	C	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	T	.
6	.	.	.	.	.	.	G	.	A	.	.	C	.	.	.	T	.	G	A	.	.	.	C	T	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	C	T	.
7	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	T	.	G	.	.	.	G	C	.	.	.	.	.	.	.	.	G	.	.	G	.	.	A	.	.	.	.
8	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
9	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.
10	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	T	.	G	.	.	.	G	C	.	.	.	.	.	.	.	.	G	.	C	G	.	.	.	.	C	.	.
11	.	C	G	.	.	T	.	.	A	.	.	.	C	C	.	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.
12	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	T	.	.	.
13	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	.	.	.	.	.	.	C	.	.
14	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.
15	.	.	.	.	.	.	.	T	.	.	G	.	.	.	.	T	.	G	A	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	T	.
16	.	.	.	.	.	.	G	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.
17	.	.	.	.	.	.	.	.	.	.	G	.	.	.	A	T	.	.	.	.	.	G	C	.	.	.	.	.	.	.	.	G	.	C	G	.	.	A	.	C	.	.
18	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.
19	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	G	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	.	.	C	T	.
20	.	.	.	.	.	.	G	.	A	.	.	.	.	.	.	T	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	.	.
21	.	C	G	.	.	T	.	.	A	.	.	.	C	C	A	T	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	C	T	.
22	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	C	.	.	.	.	.	C	T	.
23	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	C	.	.	.	.	.	C	.	.
24	.	.	.	A	-	.	.	.	.	G	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	C	.	T	C	G	.	.	.	C	.	.	.	.	.	C	T	.
25	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	T	.	.	.	.	.	.	.	.	G	.	.	.	C	.	.	.	.	C	.	.	.	.	.	C	.	.
26	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.
27	.	.	.	.	.	.	.	.	.	.	.	.	.	.	A	T	.	.	.	C	.	.	C	.	.	.	G	.	.	.	.	G	.	C	.	T	.	A	.	.	.	.
28	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	T	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.	.	T	.	.	.

were performed over a total of 519 bases. Haplogroups were determined according to the mtDNA terminology reported by Achilli et al.[8].

The Ethics Committee Approval for the study was obtained from Inönü University Experimental Animals Local Ethics Committee (2017/A-30).

RESULTS

In the study, 519 bp of the mtDNA D-loop region for 28 Coloured horses from Turkey were analysed and 42 polymorphic sites were determined *(Table 1)*.

The haplogroups determined according to the polymorphism regions obtained in individuals are shown in *Table 2*. Ten different haplogroups were identified and the frequency of haplogroup N was observed a high (21.43%) in this study.

The evolutionary history was inferred using the UPGMA method *(Fig. 5)*. The total branch length in the evolutionary relationship was measured as 0.1026. It was observed that the horses were genetically separated from each other, but they were clustered in 4 groups, and 1 horse (sample no. 24) was placed alone in a separate place from the other horses.

The relationship between horses used in the study and the horse populations of different countries was also examined *(Fig. 6)*. It was observed that the horses in the population studied were intertwined with very different horse populations, but a certain number of Coloured horse samples were not mixed with other populations and remained in their own populations.

Estimates of evolutionary differentiation and standard errors between the calculated DNA sequences according to the paired comparison of the base differences in the DNA sequences of Coloured horses studied are shown in *Table 3*. It was determined that the base differences between horses ranged from 0.000 to 0.032. The highest base difference was observed between horses 7, 9, 10, 14 and 28 with 5, and between horses 7 and 10 with 11. It was observed that there was no base difference among horses 9, 14 and 28, horses 13, 23 and 25, and between horses 20 and 18, horse 15 and 3, and horse 21 and 4.

Table 2. *Haplogroups identified*

Haplogroups	N	Frequences (%)
A	3	10.71
C	2	7.14
D	1	3.57
E	2	7.14
I	4	14.29
L	5	17.86
M	1	3.57
N	6	21.43
P	3	10.71
Q	1	3.57

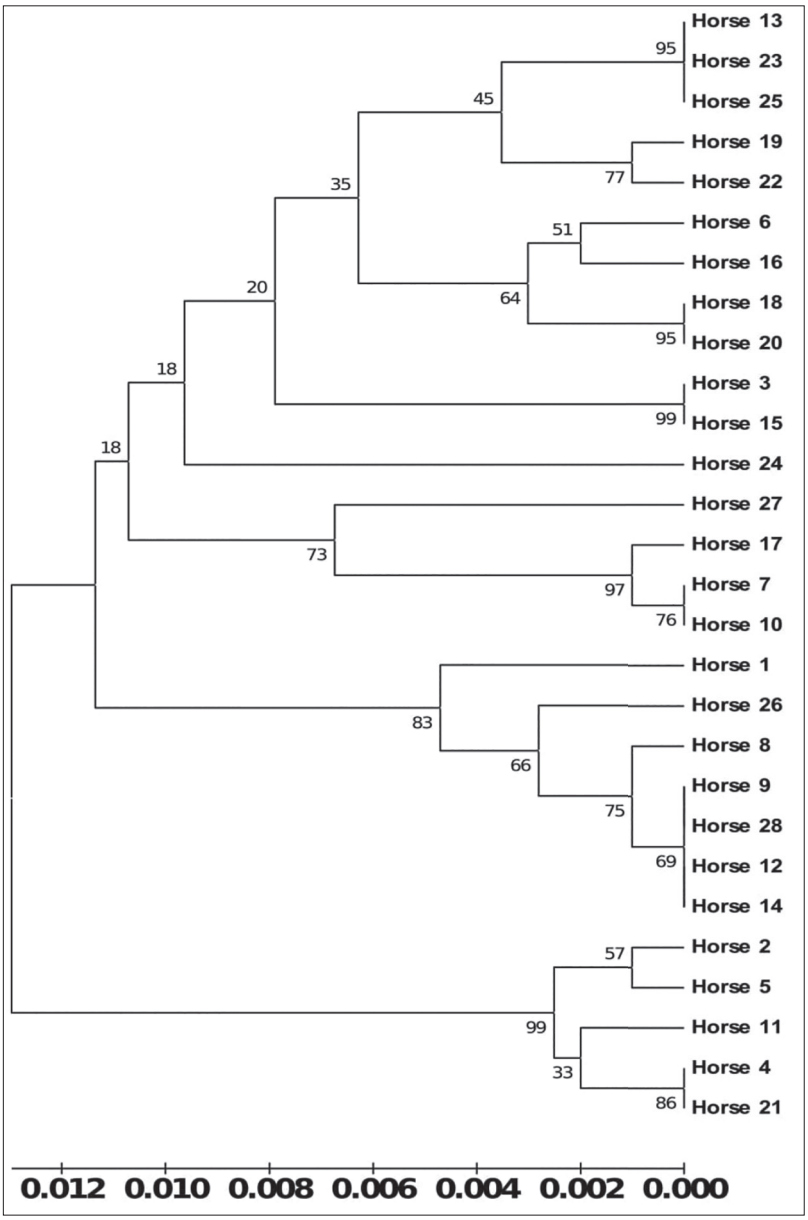

Fig 5. UPGMA dendogram showing the relationships among Coloured horses

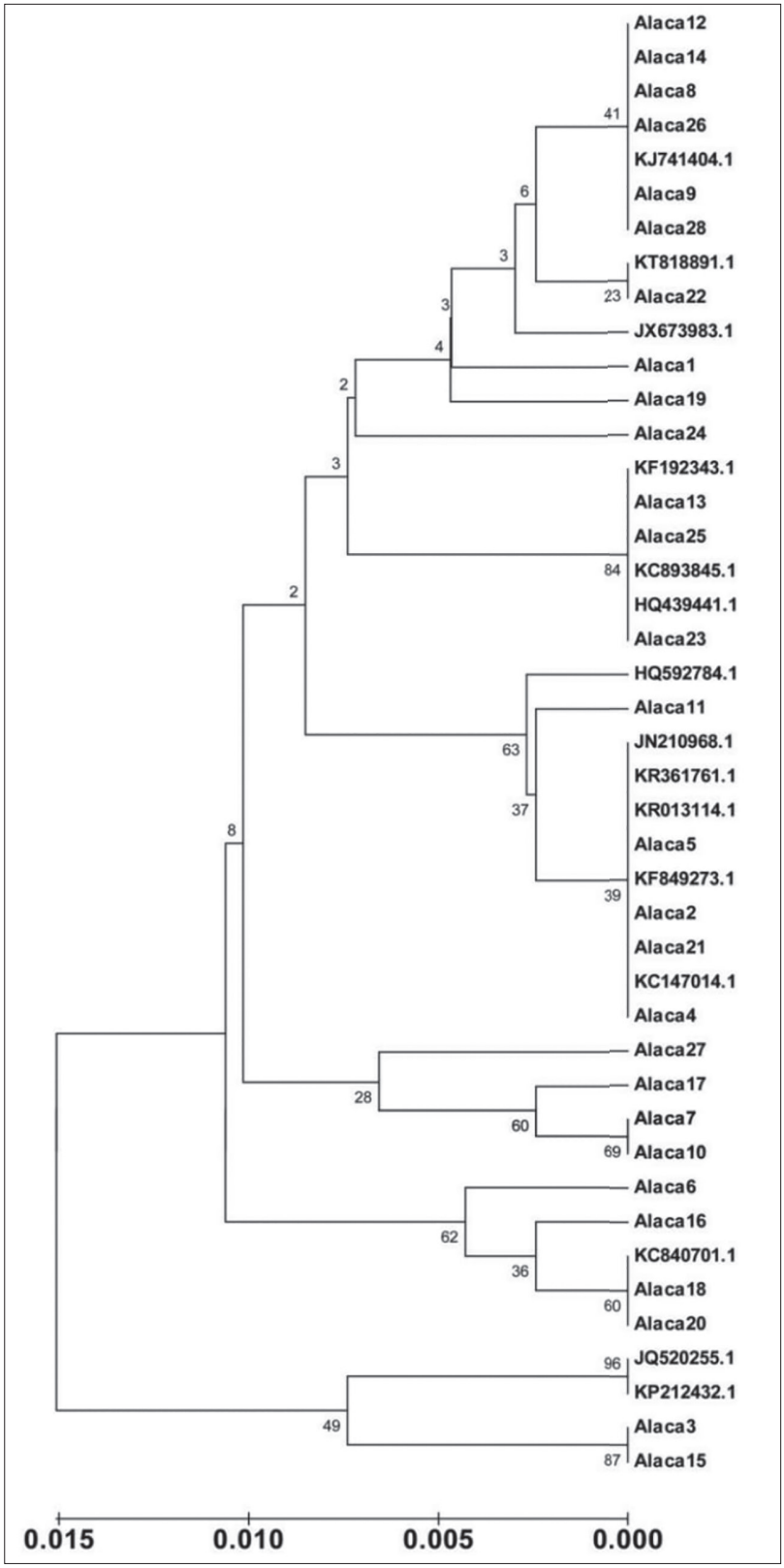

Fig 6. Evolutionary relationships of 45 taxa
Alaca: Coloured Horse; JX673983.1: Ethiopian horse; HQ439441.1: Akal Teke; HQ592784.1: Native Canadian Horse; JN210968.1: Iranian native horse; JQ520255.1: Noriker Horse; KC147014.1: Franches-Montagnes; KF192343.1: Italian Ventasso horse; KC840701.1: Arabian horse; KF849273.1: Vladimir heavy draught; KJ741404.1: Icelandic horse; KC893845.1: Celtic horse; KP212432.1: Draft horse; KR013114.1: Italian Salernitano horse; KR361761.1: Welsh Pony, section A; KT818891.1: Hungarian Gidran horse

Tajima neutrality D test results are shown in *Table 4*. It was determined that the nucleotide diversity was 1.89% and the Tajima D value was -0.31.

DISCUSSION

It is reported that mtDNA represents ancestral genetic diversity in horse populations [4,5]. Studies conducted on native horse breeds reported 23 to 43 different polymorphic regions, 2 to 164 haplotypes and 6 to 14 haplogroups in D-loop region of mtDNA [25-32].

In the studies where samples from many different regions were evaluated together, between 31 to 39 different polymorphic regions and haplogroups ranging from 17 to 68, and 19 to 33 haplotypes were identified [33-35]. In addition, a total of 99 polymorphic regions and 97 haplotypes were found as a result of the entire D-loop of mtDNA sequence analysis of the Arabian horse breed in Middle Eastern countries [36]. Although the number of horses in this study is low unlike other studies, the number of regions showing polymorphism (42) and the number of haplogroups (10) are high and this suggests that these horses may have many ancestral origins.

As a result of mtDNA sequence analysis conducted in 5 native horse breeds of Turkey, 68 polymorphic regions were identified; 54 haplogroups and 151 haplotypes were detected [17]. In this study, although a small number of horses were studied from a single population, similar results were obtained. In the study where many horse breeds of different countries were compared, distance of base differences of Anatolia and Çukurova horse breeds of Turkey was found to be 0.005 [33]. In this study, it was determined that the base differences in the population ranged between 0.000 and 0.032. According to the results obtained, it was observed that the base differences within the same population are larger than the base differences among the different populations. As a result of these findings, it was considered that the Coloured horse population has more different genotypes and may have different ancestral origins.

In a study where 18 different haplogroups were obtained in horses from different continents, haplogroups outside of D and E were reported to be located in the Middle

Table 3. Estimates of evolutionary differentiation between DNA sequences (vertical triangles) and standard errors (inverse right triangle)

Horse No	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
1		0.007	0.006	0.007	0.007	0.006	0.006	0.004	0.004	0.006	0.007	0.005	0.006	0.004	0.006	0.006	0.006	0.005	0.005	0.005	0.007	0.005	0.006	0.007	0.006	0.004	0.006	0.004
2	0.022		0.006	0.002	0.003	0.007	0.008	0.007	0.007	0.008	0.003	0.008	0.007	0.007	0.006	0.006	0.007	0.006	0.006	0.006	0.002	0.005	0.007	0.007	0.007	0.007	0.007	0.007
3	0.018	0.020		0.007	0.007	0.005	0.006	0.006	0.007	0.006	0.007	0.007	0.006	0.007	0.000	0.004	0.006	0.006	0.004	0.006	0.007	0.004	0.006	0.006	0.006	0.006	0.006	0.007
4	0.026	0.004	0.024		0.004	0.006	0.008	0.007	0.007	0.008	0.003	0.008	0.007	0.007	0.007	0.006	0.007	0.006	0.006	0.006	0.000	0.006	0.007	0.007	0.007	0.007	0.007	0.007
5	0.026	0.004	0.024	0.008		0.007	0.008	0.008	0.008	0.008	0.004	0.008	0.007	0.008	0.007	0.007	0.008	0.007	0.007	0.007	0.004	0.006	0.007	0.007	0.007	0.008	0.008	0.008
6	0.022	0.024	0.016	0.024	0.028		0.007	0.006	0.007	0.007	0.007	0.007	0.006	0.007	0.005	0.003	0.007	0.003	0.004	0.003	0.006	0.005	0.006	0.007	0.006	0.006	0.007	0.007
7	0.022	0.028	0.016	0.028	**0.032**	0.023		0.007	0.007	0.000	0.008	0.008	0.006	0.007	0.006	0.006	0.002	0.006	0.006	0.006	0.008	0.006	0.006	0.007	0.006	0.007	0.005	0.007
8	0.008	0.026	0.022	0.026	0.030	0.022	0.026		0.002	0.007	0.007	0.003	0.006	0.002	0.006	0.006	0.007	0.005	0.006	0.005	0.007	0.006	0.006	0.007	0.006	0.003	0.006	0.002
9	0.010	0.028	0.024	0.028	**0.032**	0.024	0.028	0.002		0.007	0.007	0.002	0.006	0.000	0.007	0.007	0.007	0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.006	0.003	0.007	0.000
10	0.022	0.028	0.016	0.028	**0.032**	0.023	0.000	0.026	0.028		0.008	0.008	0.006	0.007	0.006	0.006	0.002	0.006	0.006	0.006	0.008	0.006	0.006	0.007	0.006	0.007	0.005	0.007
11	0.026	0.004	0.024	0.004	0.008	0.024	0.032	0.026	0.028	**0.032**		0.008	0.007	0.007	0.007	0.007	0.008	0.006	0.006	0.006	0.003	0.006	0.007	0.007	0.007	0.007	0.007	0.007
12	0.012	0.030	0.026	0.030	0.030	0.026	0.030	0.004	0.002	0.030	0.030		0.007	0.002	0.007	0.007	0.007	0.006	0.007	0.006	0.008	0.006	0.007	0.008	0.007	0.004	0.007	0.002
13	0.016	0.022	0.018	0.026	0.026	0.018	0.021	0.020	0.022	0.021	0.022	0.024		0.006	0.006	0.005	0.006	0.005	0.004	0.005	0.007	0.003	0.000	0.006	0.000	0.006	0.006	0.006
14	0.010	0.028	0.024	0.028	**0.032**	0.024	0.028	0.002	0.000	0.028	0.028	0.002	0.022		0.007	0.007	0.007	0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.006	0.003	0.007	0.000
15	0.018	0.020	**0.000**	0.024	0.024	0.016	0.016	0.022	0.024	0.016	0.024	0.026	0.018	0.024		0.004	0.006	0.006	0.004	0.006	0.007	0.004	0.006	0.006	0.006	0.006	0.006	0.007
16	0.018	0.020	0.012	0.024	0.024	0.004	0.020	0.022	0.024	0.020	0.024	0.026	0.014	0.024	0.012		0.006	0.003	0.003	0.003	0.006	0.004	0.005	0.006	0.005	0.006	0.006	0.007
17	0.020	0.026	0.018	0.026	0.030	0.025	0.002	0.024	0.026	0.002	0.030	0.028	0.020	0.026	0.018	0.021		0.006	0.006	0.006	0.007	0.006	0.006	0.007	0.006	0.007	0.005	0.007
18	0.016	0.022	0.018	0.022	0.026	0.006	0.021	0.016	0.018	0.021	0.022	0.020	0.012	0.018	0.018	0.006	0.019		0.005	0.000	0.006	0.004	0.005	0.006	0.005	0.005	0.006	0.006
19	0.016	0.018	0.010	0.022	0.022	0.010	0.018	0.020	0.022	0.018	0.022	0.024	0.008	0.022	0.010	0.006	0.020	0.012		0.005	0.006	0.002	0.004	0.005	0.004	0.006	0.006	0.006
20	0.016	0.022	0.018	0.022	0.026	0.006	0.021	0.016	0.018	0.021	0.022	0.020	0.012	0.018	0.018	0.006	0.019	**0.000**	0.012		0.006	0.004	0.005	0.006	0.005	0.005	0.006	0.006
21	0.026	0.004	0.024	**0.000**	0.008	0.024	0.028	0.026	0.028	0.028	0.004	0.030	0.026	0.028	0.024	0.024	0.026	0.022	0.022	0.022		0.006	0.007	0.007	0.007	0.007	0.007	0.007
22	0.014	0.016	0.012	0.020	0.020	0.012	0.020	0.018	0.020	0.020	0.020	0.022	0.006	0.020	0.012	0.008	0.018	0.010	0.002	0.010	0.020		0.003	0.005	0.003	0.006	0.006	0.006
23	0.016	0.022	0.018	0.026	0.026	0.018	0.021	0.020	0.022	0.021	0.022	0.024	0.000	0.022	0.018	0.014	0.020	0.012	0.008	0.012	0.026	0.006		0.006	0.000	0.006	0.006	0.006
24	0.022	0.024	0.020	0.028	0.028	0.024	0.028	0.026	0.028	0.028	0.028	0.030	0.018	0.028	0.020	0.020	0.026	0.022	0.014	0.022	0.028	0.012	0.018		0.006	0.007	0.007	0.007
25	0.016	0.022	0.018	0.026	0.026	0.018	0.021	0.020	0.022	0.021	0.022	0.024	**0.000**	0.022	0.018	0.014	0.020	0.012	0.008	0.012	0.026	0.006	**0.000**	0.018		0.006	0.006	0.006
26	0.008	0.026	0.022	0.026	0.030	0.022	0.026	0.004	0.006	0.026	0.026	0.008	0.020	0.006	0.022	0.022	0.024	0.016	0.020	0.016	0.026	0.018	0.020	0.026	0.020		0.006	0.003
27	0.020	0.026	0.022	0.026	0.030	0.026	0.014	0.024	0.026	0.014	0.030	0.028	0.020	0.026	0.022	0.022	0.012	0.020	0.020	0.020	0.026	0.018	0.020	0.026	0.020	0.024		0.007
28	0.010	0.028	0.024	0.028	**0.032**	0.024	0.028	0.002	**0.000**	0.028	0.028	0.002	0.022	**0.000**	0.024	0.024	0.026	0.018	0.022	0.018	0.028	0.020	0.022	0.028	0.022	0.006	0.026	

Table 4. *Results of Tajima's Neutrality Test*

N	S	P_s	Θ	π	D
28	42	0.080153	0.020597	0.018882	-0.311316

n = Total number of samples; S = Total number of polymorphic regions; p_s = Ratio of bases that show difference; Θ = Rate of mutation in population; π = nucleotide variety; D = Tajima test statistic

East [8]. In the study of mountain horse breeds of Bulgaria, it was reported that mainly Eastern and Western Eurasia and Middle East haplotypes were found [35]. In this study, it was observed that three Coloured horses in D and E haplogroups were found in haplogroups of Northern Europe horses and the horses in other haplogroups were found in haplogroups of Central Asia and the Middle East horses.

There are studies showing that F haplogroup is a haplogroup specific to *E. przewalskii* wild horses [8]. Studies have shown that Arabian horses in the Middle East (Khanshour and Chothran, 2013), Bulgarian horse breeds [35] and native horse breeds in Asia and Caucasia [32,37] do not have F haplogroup. Similar to other studies, this study showed that F haplogroup was not present in Coloured horses in Turkey close to above mention regions. Since horses included in this study are in the haplogroup of horses of Central Asia and the Middle East, similar results were obtained with those horse breeds in these countries.

It was reported that in Kabardey horse breed of North Caucasus, the highest haplogroup rate was observed to be 19% in G haplogroup, and this was followed by L, Q and B haplogroups, approximately 12% [32]. In this study, 24 out of 28 horse samples were collected from Erzurum, Kars and Ardahan provinces under the influence of Caucasus region and there was no G and B haplogroup detected; while L (5 horses, 17.86%) and Q (1 horse, 3.57%) haplogroups were detected. It was considered that because the Coloured horse population in Turkey has the haplogroups of horses of Central Asia and in the Middle East, Coloured horses population may be originated from the Eurasia region centre of domestication.

Phylogenetic trees indicate that populations with similar nucleotide sequences have more recent common ancestry than populations with different nucleotide sequences. As a result of the studies carried out on Asian, European, Middle East and American horse populations, it was detected that while all samples from these countries are divided into 2 groups in phylogenetic tree, they were in mixture with each other [8]. In other studies, it was reported that except one horse breed, the other horse breeds studied were in mixture with each other [25,28,36].

In a study conducted in 5 native horse breeds of Turkey, it was detected that there was no breed specific pylogenetic group and they were in mixture with each other [17]. In this study, although there is only one horse population, the samples were collected from different regions. It was determined that these horses were in mixture with each other and did not show a similar clustering as a single population. It was considered that the native horse breeds in many countries do not form their own groups as in this study, since the domestication regions of horses are different, and that they may have different ancestral origins and uncontrolled mating programs.

When neutrality tests and phylogenetic trees are interpreted, more detailed information can be obtained about the history of populations. In order to determine whether populations have undergone mutation and natural selection, Tajima's neutrality test was used [38,39]. When nucleotide diversity (1.89%) was compared with the number of polymorphic regions (42) in the Coloured horse population used in this study, it was detected that Tajima D has a negative value (-0.31), and that there was a population expansion, albeit small, in the investigated population.

In the studies in which D-loop region of mtDNA was investigated, the nucleotide diversity was found between 1.52 and 2.8% [26,28,31,35,36] and 2.1% in 5 native horse breeds of Turkey [17]. In this study, nucleotide diversity was found to be 1.89%, similar to those of these studies.

In the study, it was found that Korean horse breed was located in the same cluster as the Mongol horse breed, which was reported to be its ancestor [40] and there has been a close genetic relationship between Chinese Mongolian horses and other Mongolian horses [37]. Likewise, it is necessary to carry out additional studies that evaluate these horses and other horse breeds that are likely to be their ancestors and found in the same habitats with the Coloured horse breed.

As a result, according to the study findings, it was understood that the genetic structures of the horses examined are different from each other. Horses originate from different mothers, except a small number of horses. In addition, in this study where the horse population was compared with horse populations from different countries; it was detected that Coloured horses were located at different phylogenetic cluster compared with other horses breeds. This suggests that Coloured horses have had their own genotype characteristics over time. It is necessary to determine whether this horse population is separated from the other native horse breeds of Turkey and horse breeds of nearby countries, and to register them as a different breed.

Competing Interests

The authors declare that they have no conflict of interest.

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