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RESEARCH ARTICLE

Mitochondrial DNA D-loop and 12S Regions Analysis of the Long-Crowing Local Breed Denizli Fowl from Turkey

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Summary

Aim of this study is to analyze the genetic structure of long-crowing Denizli chicken, a Turkish local fowl, using nucleotide sequences of the mitochondrial DNA 12S and D-loop regions. DNA isolation was carried out using feather samples of cocks of this local breed. D-loop and 12S regions were amplified using polymerase chain reaction technique and *ca* 1230 bp and 950 pb PCR products were obtained respectively. Native genotype Denizli fowl's D-loop and 12S regions were sequenced and sequencing data were analyzed and compared with the related published data. Nucleotide content of the 12S region showed a 32% A, 19% T, 19% G and 30% C, whilst AT and GC ratios were found as 59.88% and 40.12% respectively for D-loop region. D-loop sequence data analysis clearly identified the Denizli fowl within the clade of long-crowing cocks. The results give the first informative data about the mitochondrial DNA D-loop and 12S regions of this local breed from Turkey.

Keywords: *12S rDNA, D-loop, Denizli fowl, Local breed, mt-DNA*

Türkiye Uzun Ötüşlü Yerli Irkı Denizli Tavuğunun Mitokondriyal DNA D-loop ve 12S Bölgeleri Analizi

Özet

Çalışmanın amacını ülkemiz yerli tavuk ırklarından olan uzun ötüşlü Denizli tavuğunun mitokondriyal DNA 12S ve D-loop bölgelerinin gen dizi analizine göre genetik yapısının ortaya çıkarılması oluşturmaktadır. Bu amaçla DNA izolasyonu için Denizli horozlarından alınan tüy örnekleri kullanılmıştır. D-loop ve 12S bölgeleri polimeraz Zincir Reaksiyonu (PZR) ile amplifiye edilmiş ve sırasıyla 1230 bç ve 950 bç uzunluğunda PZR ürünleri elde edilmiştir. D-loop ve 12S bölgelerinin gen dizi analizleri çıkarılmış ve daha önce yayınlanmış ilgili gen bölgeleriyle analize tabi tutulmuştur. 12S bölgesinin gen dizisi incelendiğinde %32A, %19T, %19G ve %30C olarak bulunmuştur. D-loop bölgesi için AT ve GC oranları ise sırasıyla %59.88 ve %40.12 olarak gözlemlenmiştir. D-loop bölgesi gen dizi analizi sonuçları Denizli ırkının „uzun ötüşlü ırklar arasında yer aldığını açık olarak ortaya koymuştur. Sonuçlar bu lokal ırkın mitokondriyal DNA'sı hakkında ilk informatif verileri ortaya koymuştur.

Anahtar sözcükler: *12S rDNA, D-loop, Denizli tavuğu, Lokal ırk, mt-DNA*

INTRODUCTION

Although most animal species were used and domesticated as work animals or as a food source, historical and archaeological findings strongly suggest that chicken was first functioned in religious ceremonials, decorative art or as entertainment. Domesticated fowl species are now widely distributed around the world and are bred in various categories for different purposes such as food source as egg type and meat type, decorative art as ornaments, fighting or game instrument. With regard to any role, human culture has had a strong influence on the domestication of chickens. Throughout history, however, chickens have been bred for various other purposes. Long-crowing chickens and fighting cocks are typical examples of chickens that have been bred for purposes rather than as human food or decorative ornament or adornment material , and are therefore excellent species for studying the relationship between selective breeding and human culture [1,2]. Chickens have played an important role in the

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development of human culture, particularly in Asian countries such as China, Thailand and Japan.

Four species of Gallus have currently been reported as grey junglefowl *(Gallus sonnerati),* red junglefowl *(Gallus gallus),* ceylon junglefowl *(Gallus lafayettei)* and green junglefowl *(Gallus varius),* only *Gallus gallus* is suggested to be the main progenitor of all contemporary domesticated chicken breeds [3,4]. This view is consistent with the discussion that the domestication and breeding of chickens was initiated with junglefowls, which then only inhabited Southeast Asian regions such as Indochina and South China [3,4]. Exact location of domestication of *Gallus gallus,* however, is still remain controversial [5] since the findings of these researchers suggest that contemporary fowls have multiple origin in South Asia and Southeast Asia.

Denizli chicken (*Gallus gallus domesticus*) is a local breed of Turkey and vast majority of them are habituated at Western Anatolia. Because of poor commercial performance they provide an excellent example of seriously threatened populations with immediate risk of extinctions, and therefore, Turkey has undertaken a conservation program operated by the Lalahan Livestock Central Research Institute and supported by Turkish Ministry of Agricultural and Rural Affairs since 1997. The females of this breed are considered as layer type chicken breed although their egg production is about 110-120 eggs for 29 week laying period, whilst their cocks are famous for long-crowing [6]. They can crow up to 20 seconds for 1-year-old and up to 24 seconds for 3-year-old mature cocks, and in some cases they could become unconscious following a long crowing session. There is no report (to our best knowledge) that Denizli chicken exists in nature, but semi-selectively bred by local people for their relatively robust body and long-crowing characters. One recent report by Kaya and Yildiz [7] about the estimation of genetic diversity by microsatellite markers seems the only study aiming the exploration of genetic structure of this local breed. Therefore, the origin, domestication story and pyhlogenetic relationship with other fowls in particular with long-crowing cocks of this local chicken remain unknown.

This study aims to examine the mitochondrial DNA regions, D-loop and 12S, sequences of long-crowing Denizli chicken from the viewpoint of molecular phylogeny. The sequence data of the current study was also analyzed after combination with the earlier published data representing game cocks and long crowing cocks located in GenBank web site (http://www.ncbi.nlm.nih.gov/).

MATERIAL and METHODS

Samples

Feather samples of two pure bred cocks were obtained from the Denizli Provisional Directorate of Agriculture in where this bred is under conservation scheme. The feather samples were washed twice using 70 % EtOH and kept under aseptic conditions at -20°C until required for DNA extraction.

DNA Extraction

Feather samples were cut by a sterile scissors into small pieces and treated using liquid nitrogen to disrupt the cells prior to DNA extraction. Phenol-chloroform based DNA isolation was performed according to Villalta *et al*.[8] (originally devised to extract fish tissue DNA) with the slight modification of the incubation time which was elongated to 48 h in extraction buffer at 55°C. Extracted DNAs were either used freshly or kept in 100 ml of TE buffer [10 mM Tris-HCl (pH=8.0), 1mM EDTA] [9] at -20 for further studies.

Primer Design for D-loop and 12S Regions

Designing of forward and reverse primers for both D-loop and 12S mitochondrial regions were conducted using published mitochondrial DNA sequence data of *Gallus gallus* derived from the web server of National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/). For amplification of the D-loop region forward 5′-TTA ACC TAA CTC CCC TAC TAA GTG TA-3′ and reverse 5′-TCT TCC GTA AAA CAC AAA CC-3′primers were used, whilst forward 5′-GGT TTT TGC TAG ACA TAT ACA TGC-3′ and reverse 5′-CAT CAG ATT CAC GTG GAA GGC -3′ primers were designed for the amplification of 12S region. The designed primers for both mitochondrial D-loop and 12S regions were synthesized by a commercial company (Iontek, Istanbul-Turkey).

PCR Amplification

PCR amplification of the extracted DNAs was performed for 50 ml in size. Amplification primers were synthesized by a commercial company (Iontek, Istanbul), dNTPs and Hi-Fi Taq DNA polymerase were supplied by Favorgen (Favorgen Biotech Corp., Taiwan). The optimized denaturation, annealing and extension characteristics of PCR amplification steps for both regions are shown in *Table 1*. Following the amplification, all PCR products were run in 1% agarose gel and post stained using ethidium bromide solution 0.1% (w/v) for 15min, and finally photographed with the aid of a digital camera (Canon Powershot S3 IS, Japan) under 312nm wavelength ultra violet (UV) trans-limunator light (UVP, UK). The PCR products were purified using PCR clean up kit (Favorgen Biotech Corp., Taiwan) according to manufacture's instruction prior to sequencing process.

Sequencing Procedure and Data Analysis

Sequencing process was performed by a commercial company (Iontek, Istanbul-Turkey) as direct sequencing from purified PCR products. Sequencing process for D-loop and 12S region were conducted in both forward

and reverse directions and all sequence data were edited using Bioedit 7.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and FinchTV Version 1.4.0 (http://www.geospiza.com/finchtv) following naked eye checking. The sequence data produced were aligned to earlier reported *Gallus gallus* sequence data using CLUSTAL X program [10] with default parameters. The bootstrap analysis of 1000 replications was performed to assess the statistical confidence of the phylogenetic trees which were constructed by the neighbor joining method. Conversion of the data format and DNA polymophism analysis were carried out with the aid of DNaSP 4.02 [11].

RESULTS

PCR Amplification and Nucleotide Ratios of D-loop and 12S Regions

A partial PCR amplification of the D-loop region of the Denizli fowl yielded a *ca* 1210bp PCR product *(Fig. 1)* and 1094 bp of that fragment was successfully sequenced and analyzed. The sequence data of D-loop region of Denizli cock was submitted to the GenBank (http://www.ncbi.nlm.nih.gov) and located there with the accession number of EU194446. Nucleotide ratios of the D-loop region were calculated as 26% A, 34% T, 14% G and 26% C. Relatively higher AT ratio compare to GC content for this region was observed, and ratios for these data were found as 59.8% and 40.2% respectively.

For 12S region, newly designed primers amplified a *ca* 950bp PCR fragment *(Fig 1b)* and 872bp length of this product was successfully sequenced and analyzed. The sequence data of 12S region of Denizli fowl is deposited on the GenBank web site with the accession number of FJ610338 (http://www.ncbi.nlm.nih.gov). The nucleotide content of the sequenced data was calculated as 32% A, 19% T, 19% G and 30% C. Balanced AT and GC ratios were observed for 12S region since these values were found as 51.8% and 48.2% respectively.

Table 1. *The optimized polymerase chain reaction amplification characteristics of mitochondrial D-loop and 12S regions*
Tablo 1. *Mitokondriyal DNA D-loop ve 12S bölgeleri için Polimeraz Zincir Reaksiyonu optimizasyonu*

PCR Steps	**D-loop**		**12S**	
Initial Denaturation	**94°C 4 min**			
Denaturation	94°C 1 min	35 cycle	94°C 1 min	35 cycle
Annealing	55°C 30 sec		55°C 30 sec	
	63°C 30 sec		58°C 30 sec	
Extension	72°C 2 min		72°C 2 min	

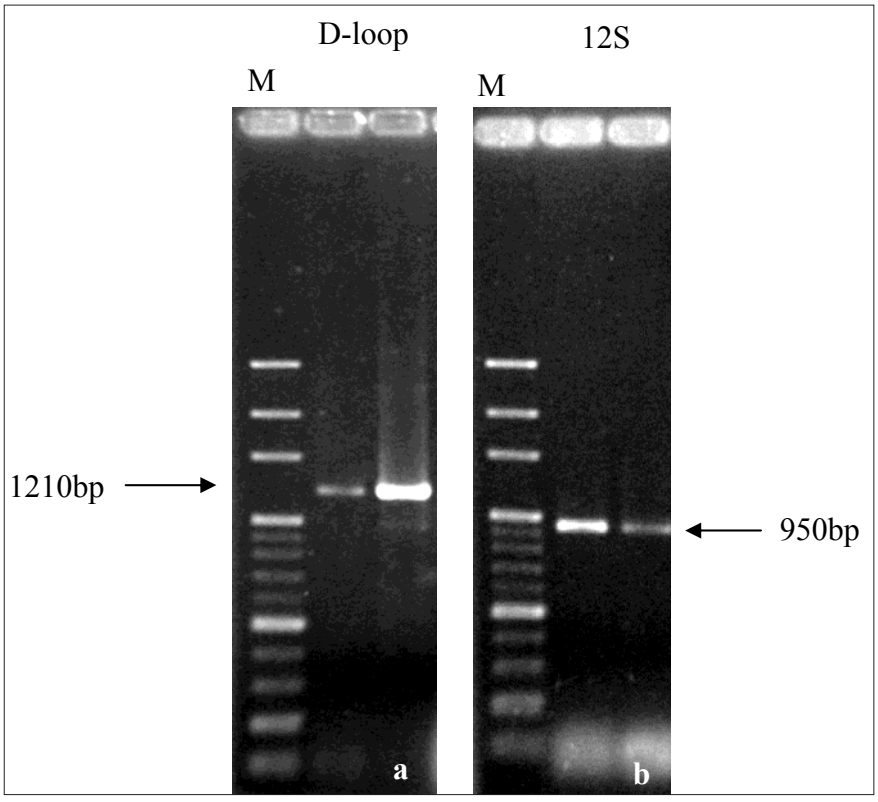

Fig 1. Polymerase chain reaction amplification of the D-loop **(a)** and 12S **(b)** regions yielded a *ca*1210bp and 950bp PCR products respectively. M: 1kb ladder from Favorgen Biotech. corp. (Taiwan)

Şekil 1. Polimeraz zincir reaksiyonu (PZR) amplifikasyonu D-loop (a) için ca 1210bç 12S bölgesi (b) için ca950 bç PZR ürünü ortaya koymuştur. M: 1kb merdiven, Favorgen Biotech. corp. (Taiwan)

Phylogenetic Trees According to Sequence Data of D-loop and 12S Regions

An unrooted tree was generated using the nucleotide sequence of D-loop region of mitochondrial DNA obtained from 30 samples of fighting, long-crowing and Denizli cocks. The unrooted Upgma tree of 30 samples revealed three divergent clades designated as A, B and C *(Fig. 2)*.

The haplotypes located in clades A and B represent the game-cocks, while clade C composed of long-crowing cocks and Denizli cock. Although Denizli cock shared the same clade with long-crowing cocks, a finer analysis showed that the sequence data of this Turkish local breed was differed from the remaining part of the clade by at least 7 single nucleotide mutations (see FJ610338 - Denizli fowls 12S region accession number).

The sequencing data of mitochondrial 12S region was analyzed for phylogenetic purposes too *(Fig. 3)*. To obtain a better alignment with the earlier reported data in GenBank, first 6 bases (GGAGGA) were excluded from the submitted 12S sequence of the Denizli fowl (Accession no: FJ610338). A rooted tree, for 12S region, was generated using the sequence data of local breed Denizli chicken and 18 published data belong to *Gallus gallus* derived from GenBank web site.

As seen in *Fig. 3,* 12S region of the Denizli chicken is quite similar to those various subspecies of *Gallus gallus*. Less than 1% differences was observed among the studied 12S regions of 18 fowls. Further, genetic structure of 12S region of Denizli fowl was identical to the White Leghorn *(Gallus gallus)*.

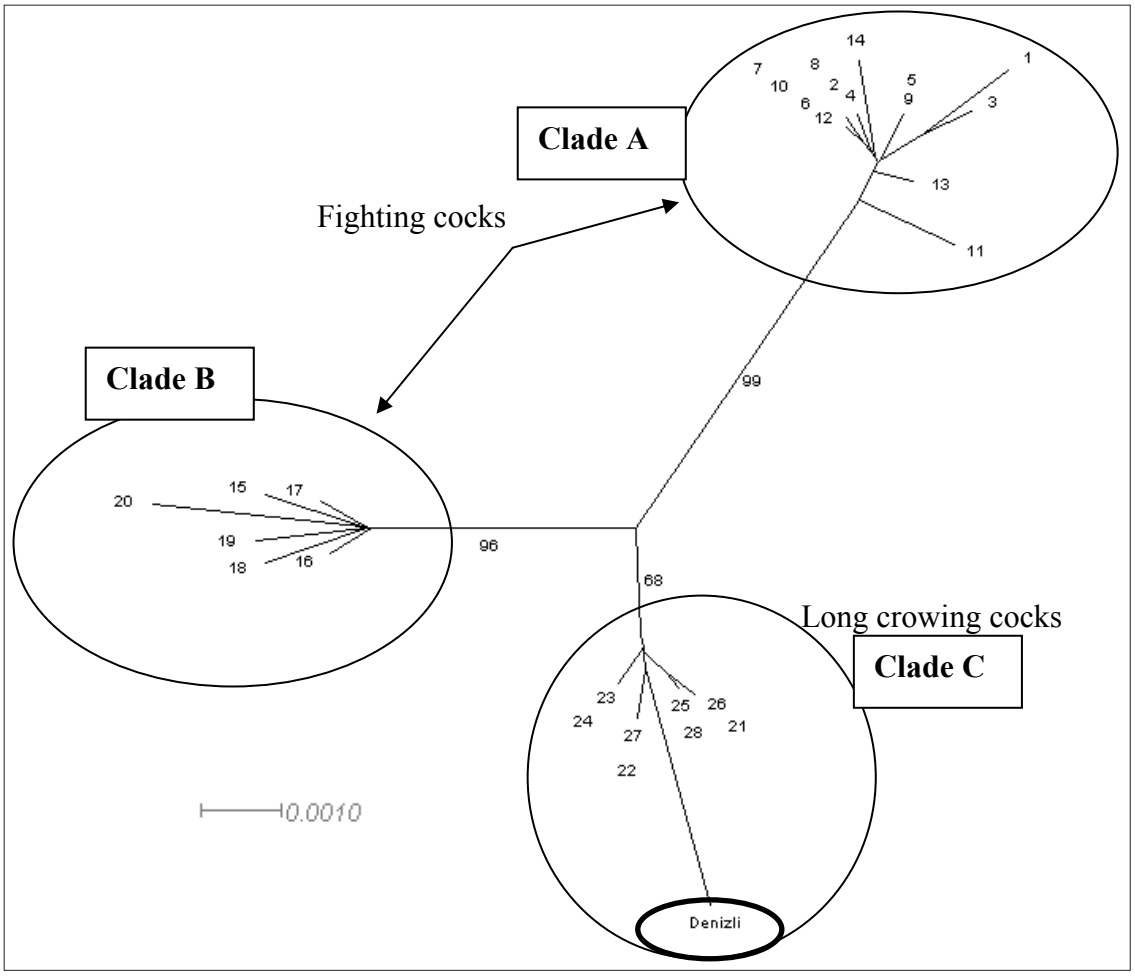

Fig 2. Unrooted phylogenetic tree of fighting and long-crowing cocks including Denizli local bred derived from mitochondrial D-loop region. Numbers of the samples and representing GenBank accession numbers are as follow; 1 (AB098699); 2 (AB098698); 3 (AB098697); 4 (AB098695); 5 (AB098694); 6 (AB098693); 7 (AB098692); 8 (AB098686); 9 (AB098660); 10 (AB098651); 11 (AB098649); 12 (AB098647); 13 (AB098644); 14 (AB098637); 15 (AB098662); 16 (AB098654); 17 (AB098653); 18 (AB098646); 19 (AB098639); 20 (AB098638); 21 (AB114065); 22 (AB114064); 23 (AB114063); 24 (AB114062); 25 (AB114059); 26 (AB114058); 27 (AB114066); 28 (AB114060); Denizli (EU194446)

Şekil 2. Dövüşcü ve Denizli ırkınında içerisinde yer aldığı uzun ötüşlü ırkların mitokondriyal DNA D-loop bölgelerine göre oluşturulmuş köksüz filogenetik yapı. İlgili GenBank ulaşım numaraları ise şu şekildedir; 1 (AB098699); 2 (AB098698); 3 (AB098697); 4 (AB098695); 5 (AB098694); 6 (AB098693); 7 (AB098692); 8 (AB098686); 9 (AB098660); 10 (AB098651); 11 (AB098649); 12 (AB098647); 13 (AB098644); 14 (AB098637); 15 (AB098662); 16 (AB098654); 17 (AB098653); 18 (AB098646); 19 (AB098639); 20 (AB098638); 21 (AB114065); 22 (AB114064); 23 (AB114063); 24 (AB114062); 25 (AB114059); 26 (AB114058); 27 (AB114066); 28 (AB114060); Denizli (EU194446)

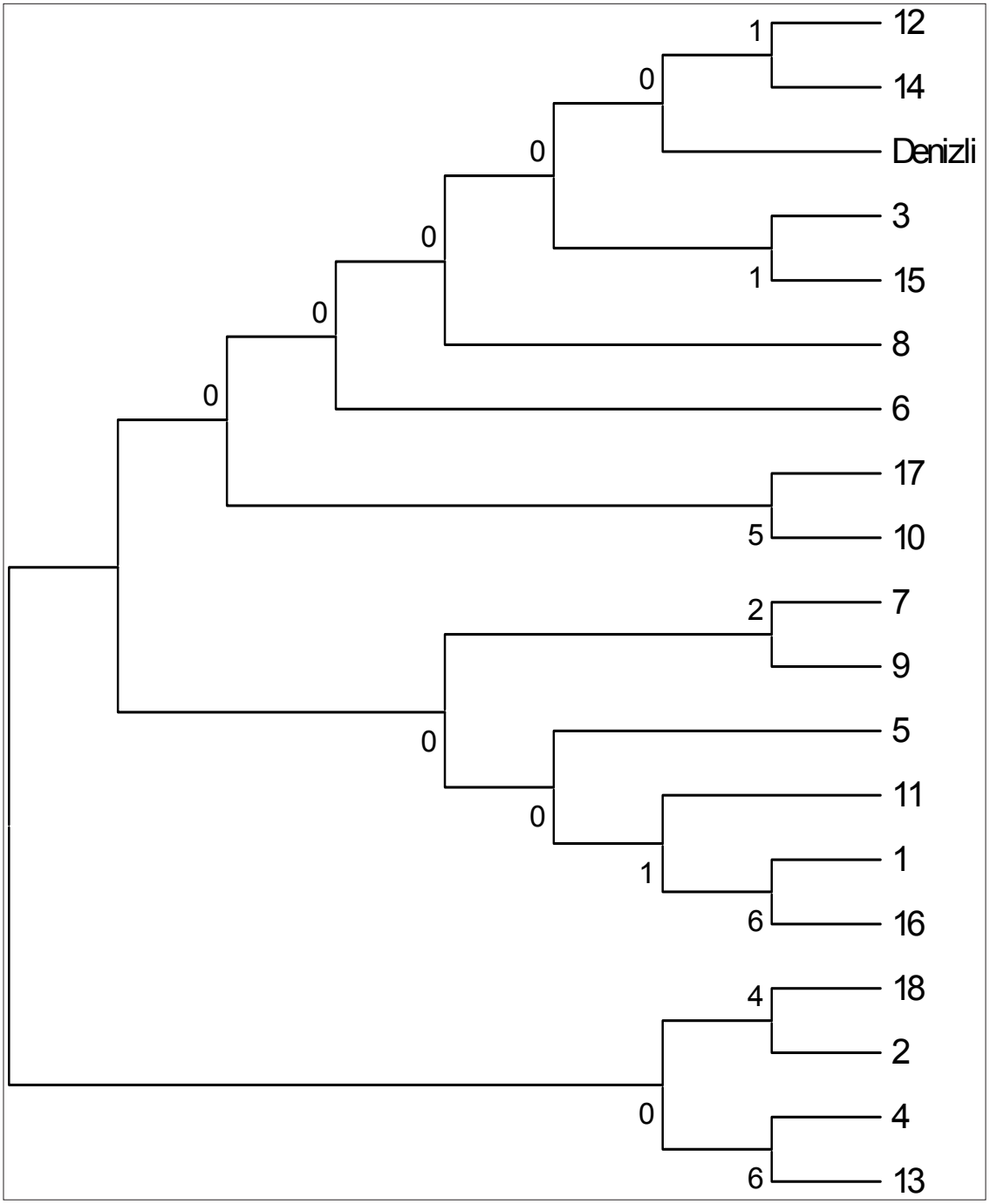

Fig 3. A rooted phylogenetic tree is generated using Denizli fowl and 18 *Gallus gallus* samples derived from the 12S rDNA sequence data reported in GenBank web server. The sample numbers representing GenBank accession numbers used to obtain the phylogenetic tree were as follow; 1 (DQ648776), 2 (AP003323), 3 (AP003322), 4 (AP003319), 5 (AP003318), 6 (AP003580), 7 (AY235571), 8 (AP003317), 9 (X52392), 10 (AB086102), 11 (AY235570), 12 (AP003321), 13 (EF373905), 14 (EF373873), 15 (DQ885561), 16 (AJ490505), 17 (AJ583547), 18 (AY309497), Denizli (FJ610338)

Şekil 3. GenBank'tan alınmış 18 *Gallus gallus* örneği ve Denizli ırkına ait 12S rDNA bölgesi gen dizisine gore oluşturulmuş köklü filogenetik yapı. İlgili GenBank ulaşım numaraları ise şu şekildedir; 1 (DQ648776), 2 (AP003323), 3 (AP003322), 4 (AP003319), 5 (AP003318), 6 (AP003580), 7 (AY235571), 8 (AP003317), 9 (X52392), 10 (AB086102), 11 (AY235570), 12 (AP003321), 13 (EF373905), 14 (EF373873), 15 (DQ885561), 16 (AJ490505), 17 (AJ583547), 18 (AY309497), Denizli (FJ610338)

DISCUSSION

Domesticated fowls are widely distributed throughout the world and recent archaeological studies strongly suggest that their domestication has originated in China nearly 8.000 years ago [12]. Domesticated chickens are currently bred for various purposes, not only because of their usefulness for human nutrition but also for their long crowing, outstanding characteristic songs, colorful appearance (as religious symbols), entertainment and

ceremonial purposes [13].

Turkey has two native chickens, Denizli and Gerze fowls, which are seriously threatened with extinction and they are under a governmental conservation program since 1997. The former one, Denizli chicken, is bred mainly because of its long crowing characteristics as hobby pet, rather than egg or meat production. Although several studies were carried out about their egg- and meat production characteristics [14,15], quite few works has focused on their genetic structures [7,16]. The current study is the first attempt which points out the mitochondrial DNA D-loop and 12S regions of this local breed of Turkey.

The analysis of mitochondrial DNA D-loop region sequence data has clearly demonstrated that Denizli chicken is more closely related to long crowing fowls rather than fighting cocks. Finer analysis of D-loop sequence data suggested that within the long crowing clade, Denizli cocks could be separated into subclade. The lack of sequence data for mitochondrial DNA 12S region of long crowing cocks on web site of GenBank hampered the opportunity to discus the phlylogenetic position of Denizli chicken with other long crowing cocks using that particular informative region. 12S sequence data located in GenBank (for other *Gallus gallus*) were compared with the same region of Denizli fowl. The results of this comparison showed that 12S region of Denizli fowl were almost identical to the 12S nucleotide structure of 18 *Gallus gallus* samples.

Long crowing fowls are in special interest for human all over the world and, in particular, for the people living in Asia. Komiyama *et al*.[17] conjectured that the culture of cockfighting was closely related with that of long crowing and therefore long crowing culture was derived from that of cock fighting. There is no scientific report or cultural knowledge, however, that Denizli fowl is used for cock fighting although they are famed for their long crowing characters and people would like to have them as hobby animals because of their colorful appearance and exclusive songs.

Further studies are required to explore the genetic structure of this local breed which is under severe threaten in extinction. Complete mitochondrial DNA sequence analysis of this local breed will provide us more information and give as an opportunity to conjecture its possible origin and relationship with other chicken breeds in particular with long crowing cocks more accurately.

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