



Heteroplasmy in the mitochondrial D-loop region in Turkish domestic pigeon breeds (*Columba livia domestica*) obfuscates their phylogeny

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ABSTRACT

Columba livia domestica, the domesticated version of the rock pigeon, has long accompanied people for various purposes or activities for thousands of years. Nowadays, pigeon breeding is mostly done as a hobby and it is estimated that domestic pigeons have more than 350 breeds throughout the world. Because of the strong artificial selection applied to them, these different breeds exhibit dramatically diverse phenotypic traits.

In this study, partial mitochondrial D-loop sequences from 96 different individuals, mainly from western Turkey, were used to uncover genetic diversity, the presence of heteroplasmy, and phylogenetic relationships within and among local and foreign breeds. It was discovered that the partial D-loop regions of domestic pigeon breeds vary in length, ranging from 714 to 1020 bp, due to heteroplasmy in the 61 bp long VNTR found at the end of the D-loop. The number of VNTR repeats changes from 2 to 7, with a T stretch between locations 382 and 391, containing 10 or 9 thymines. Although the D-loop is a promising marker for animal phylogenetic research due to its high polymorphism and high haplotype diversity, and despite the discovery of 30 different D-loop haplotypes, Bayesian phylogenetic analysis was not sufficient to separate different pigeon breeds in Turkey, except for the Modern Oriental Frill breed. In conclusion, mainly due to the heteroplasmy and possible introgression among distinct breeds, it is hard to use the D-loop as an informative marker for getting valid phylogenetic results and to distinguish morphologically distinct Turkish domestic pigeon breeds.

1. Introduction

The Rock Pigeon (*Columba livia*) is a bird species belonging to the Columbidae family. It can be found everywhere on Earth excluding Antarctica. (Gibbs et al., 2010; Shapiro and Domyan, 2013; Zhang and He, 2015). The rock pigeon is known to be the first domesticated bird species, probably domesticated several thousand years ago, with an important history with humans (Gilbert and Shapiro, 2014). Today, pigeon breeding is quite a common hobby for thousands of breeders all around the world (Jerolmack, 2007). It is estimated that there are approximately 350 different breeds of domestic pigeons subjected to great selective pressure by breeders to develop desired features, such as acrobatic skills, specific appearances, or flight styles. Due to this selective pressure, their characteristics differed large enough to get the attention of scientists to investigate their phylogenetic structure (Stringham et al., 2012; Shapiro and Domyan, 2013; Vickrey et al., 2015).

Pigeon breeding is a common hobby in Turkey with a long history. Unfortunately, not enough scientific studies have been done on the phylogenetic structure of domestic pigeon breeds in Turkey. This might cause these breeds to lose their unique genetic composition due to uneducated and haphazard breeding (Yılmaz and Boz, 2012).

The avian D-loop region has been utilized as a good marker in many studies and produced promising results for numerous different bird species (Fry and Zink, 1998; Guan et al., 2007; H. F. Li et al., 2010) and the *Columba* genus (Young and Allard, 1997; Seki, 2006; Oka et al., 2007; Butkauskas et al., 2008; Marrero et al., 2008). Analyses of the complete mitochondrial DNA D-loop of *Columba livia* revealed the presence of many polymorphic sequences, which are indels, SNPs, VNTRs (Variable Number of Tandem Repeats), and STRs (Short Tandem Repeats) (Tsai et al., 2009). Lee et al. (2010) found 131 randomly chosen birds of *Columba livia* to exhibit VNTR numbers varying between 2 and 8, and that the D-loop was heteroplasmic in 54 individuals. It has also

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been shown that the STR locus has different repeat numbers in different individuals. In a recent study by Stefaniuk-Szmukier et al. (2021), sequencing of a 748 bp region between the start of the D-loop and the VNTR and STR loci of 80 bird samples belonging to 18 breeds and six groups of fancy pigeons, racing pigeons and feral populations of pigeons revealed five new polymorphic sites. These results demonstrated that the D-loop might be a useful genetic marker for resolving maternal relationships (Lee et al., 2010; Stefaniuk-Szmukier et al., 2021). Furthermore, tandem repeats in mitochondrial genomes might lead to excessive variability of mitochondrial control regions, together with other processes such as regulation and recombination (He et al., 2013; Padhi, 2014; Termignoni-Garcia et al., 2023).

Despite the extensive use of mitochondrial DNA in phylogenetic studies to reveal biogeographic events (Avise, 2009; Paijmans et al., 2013), the heteroplasmy aspect of the mtDNA in such studies has been largely overlooked. Mitochondrial heteroplasmy, defined as the genetic variation of mtDNA in an individual, can be attributed to two primary mechanisms: mutational processes or a paternal leak of mtDNA (Ingman and Gyllensten, 2006; Ye et al., 2022). Previously considered rare, the phenomenon is now readily discernible through the use of high-throughput sequencing (HTS) technology in several taxa (van den Ameele et al., 2020; Pereira et al., 2021; Termignoni-Garcia et al., 2023). Tikochinski et al. (2020) indicated that this is an important mechanism for maintaining genetic diversity during a sudden decline in population size in natural populations. In addition, Termignoni-Garcia et al. (2023) analysed whole mitogenomes of nine species of New World Jays using HTS and found that heteroplasmy was mostly found in the control region and indicated an interaction between heteroplasmy and tandem repeats and its importance in regulation, recombination and paternal leakage in the mitochondrial genome.

The purpose of this study is to design specific primers for the partial mitochondrial D-loop region, to sequence and analyse the D-loop region of several Turkish local breeds, to determine haplotype and genetic diversity and the level of heteroplasmy for local and foreign breeds, and to reveal phylogenetic relationships between local and foreign breeds. We also aimed to understand both the impact of heteroplasmy on the accuracy of phylogenetic results and to what extent the phylogenetic results coincide with the classification of pigeon breeds according to their morphological characters, acrobatic abilities, and flight styles.

2. Material and methods

Most of the samples used in this study were provided by local pigeon breeders who had been previously informed of the specifics regarding the collection and transmission of feather samples. Those included refraining from touching the feather calamus with bare hands, refraining from mixing feather samples from different individuals, and preserving feathers in paper envelopes. Active communication with the breeders during the sampling period (2018–2019) helped to ensure the validity of this sampling procedure. 96 samples were provided from Manisa (Aegean Region, 30 samples), Denizli (Aegean Region, 19 samples), Çanakkale (Marmara Region, 14 samples), Uşak (Aegean Region, 10 samples), İzmir (Aegean Region, 10 samples), Mersin (Mediterranean Region, 3 samples), Afyon (Aegean Region, 2 samples), Konya (Central Anatolia Region, 1 sample), Bursa (Marmara Region, 1 sample), and Adana (Mediterranean Region, 1 sample). Except for samples from Australia (5 samples), all breeds sampled were local. The Oriental Frill Pigeon, which is thought to have originated in Anatolia first (Lyll, 1887; Türkes and Gündüz, 2021), and other short-beaked breeds, which are thought to have their origins in the Middle East, were represented with more samples than the other breeds.

2.1. Genomic DNA extraction from feathers

The feathers were kept at +4 °C in paper envelopes until DNA extraction. Prior to extraction, they were rinsed with distilled water for

15 min. Using sterile forceps and scalpels, the calamus parts of the feathers were cut and placed in 2 ml eppendorf tubes. For DNA isolation, the spin column technique and the Qiagen DNeasy Blood and Tissue Kit (Qiagen GmbH, Hilden, Germany) were performed according to manufacturing procedure. The quantity and purity of extracted DNA were measured using a NanoDrop 2000 UV–Visible spectrophotometer. The dissolved DNA was stored at –20 °C before analysis.

2.2. Primer design, PCR and sequencing

It was aimed to amplify the 850 bp mitochondrial D-loop fragments by the primers “DLF2: 5'-CTTGGCGCCTTTGGTTTT-3' and 5'-TTTCGCGGTTACTGTACG-3' DLR2” to demonstrate the phylogenetic relationships between local breeds and others. All 96 samples were amplified. Primer3version 4.1.0 (Untergasser et al., 2012) was used to design primers based on the full *Columba livia* mitochondrial genome (Accession no. NC 013978.1, Kan et al., 2010). The primers were supplied commercially by the Metabion firm (Planegg, Steinkirchen, Germany). The defined structure of *Columba livia*'s D-loop by Tsai et al. is given in Fig. 1 (Tsai et al., 2009)

PCRs were carried out in a final volume of 25 µl containing 1x HOT FIREPol Blend Master Mix (HOT FIREPol DNA polymerase, 5x blend master mix, 2.5 mM MgCl₂, 2 mM dNTP), BSA, blue and yellow dyes) (Solis BioDyne), 0.3 µM of each primer, 1 µl of genomic DNA. PCR conditions consisted of an initial incubation at 94 °C for 12 min, 38 cycles of 95 °C for 30 s, 61 °C for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 10 min. Negative controls were always included to the reactions to verify that no contamination had occurred. Since PCR results of partial D-loop region resulted in more than one band (Fig. S1) due to heteroplasmy within individuals, these bands were carefully cut out for all samples and extracted from the gel individually before sequencing reaction. For extraction of the DNA from agarose gel, the EURX GeneMATRIX Agarose-Out DNA Purification Kit was used according to manufacturing procedure (EURx Ltd., Gdansk, Poland)

After purification of the products was completed, PCR products were sequenced both forward and reverse to ensure accuracy and complete the targeted region of the D-loop. ABI Terminator 3.1 was used to conduct each sequencing process (Applied Biosystems Inc., Foster City, CA, USA). For the electrophoresis and detection of fluorescently tagged nucleotides, an automated DNA sequencer was used (ABI 3730x1 Genetic Analyzer, Applied Biosystems).

2.3. Data analysis

The Clustal W algorithm (Thompson et al., 1997) in MEGA X (Kumar et al., 2018) was used to align all 96 D-loop sequences. Alignments were controlled by eye and, if necessary, corrected manually. Unique haplotypes were identified using the Arlequin 3.5.2.2 software (Excoffier and Lischer, 2010). D-loop sequences, which were analysed in this study and represented different haplotypes, were deposited in GenBank with the accession numbers listed in Table S1. The DnaSP program version 6.x was used to determine haplotype diversity (*H_d*), nucleotide diversity (*π*) (Nei, 1987), and the number of segregating sites (*S*) or polymorphic sites (Rozas et al., 2017).

For phylogenetic analysis of the mtDNA D-loop region, 18 mtDNA D-loop sequences belonging to *C. livia* were downloaded from GenBank and combined with D-loop haplotypes detected in this study (Table S1), while *Columba rupestris* was used as the outgroup.

The best evolutionary sequence model was calculated according to the Bayesian information criterion (BIC) (Schwarz, 1978) using MEGA X (Kumar et al., 2018). The best-fit molecular evolution model for the mitochondrial D-loop region was the Hasegawa-Kishino-Yano (HKY) (Hasegawa et al., 1985) with uniform site rate variation (ln L = –1679.61, BIC = 3718.92)

Using the software MEGA X (Kumar et al., 2018), the molecular clock test was performed by comparing the maximum likelihood value for the

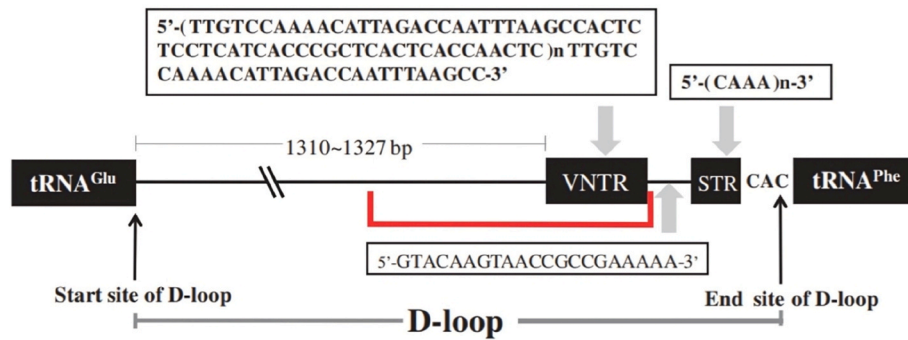


Fig. 1. D-loop structure of *Columba livia*, defined by Tsai et al. (2009). The region amplified in this study is emphasized in red.

given topology of D-loop data set with and without the molecular clock constraints under the HKY model (Hasegawa et al., 1985) with uniform site variation. The null hypothesis of equal evolutionary rate throughout the tree was rejected at a 5 % significance level ($P = 0.05$).

BEAST version 1.10 (Suchard et al., 2018) was used for conducting Bayesian phylogenetic analyses. The data set was prepared for analysis by BEAUTI version 1.7.5. The random starting tree using the Hasegawa-Kishino-Yano (HKY) mutation model (Hasegawa et al., 1985) using 4 discrete mutation classes selected before initializing the Bayesian analyses. A relaxed molecular clock (Drummond et al., 2006) was estimated using an uncorrelated lognormal prior and the Yule model as a tree prior. To determine posterior distribution parameters, the MCMC was run for 100,000,000 steps and sampled every 10000 steps. As burn-in, the first 1000 trees (10 % of the sampled trees) were discarded. The effective sample sizes for posterior probability were calculated using TRACER version 1.7 log files (Rambaut et al., 2018). The morphological classification of distinct breeds, as shown in Table 1, was used to colorize haplotypes in the phylogenetic tree.

3. Results and discussion

The partial mitochondrial D-loop of 96 individuals, including a 61 bp long VNTR at the end of the D-loop sequences, which was both predicted from the agarose gel electrophoresis (see Fig. S1) and validated by the sequencing data with clean peaks and all noisy parts removed, was amplified successfully. According to sequencing results, the length of the partial D-loop region is 713 bp in six individuals (two haplotypes), 774 bp in 73 individuals (10 haplotypes), 835 bp in 75 individuals (four haplotypes), 896 bp in 36 individuals (seven haplotypes), 957 bp in 18 individuals (five haplotypes), and 1020 bp in 7 individuals (two haplotypes) due to the different copy numbers of the repetitive region (VNTR) at the end of the D-loop. The number of VNTRs changes from two to seven and the most common haplotypes (Hap_13 and Hap_14) have four VNTRs.

In the partial D-loop region of Turkish domestic breeds, another length heteroplasmy was also discovered at the T stretch, which had 9 or 10 thymines and was located between the 382nd and the 391th positions. Additionally, heteroplasmic individuals did not only have different numbers of VNTR and T stretches but also SNP polymorphism throughout at different positions of the sequenced partial D-loop region. Haplotypes with various repeat counts are shown in Table S2; in addition, 17 novel SNPs were discovered and are shown in Table S3, combined with the SNPs discovered in previous studies.

While nucleotide diversity is low ($\pi = 0.00020$), haplotype diversity was found to be high ($h = 0.882$). The number of unique haplotypes detected in this study is 26, with the most frequent haplotype (Hap_13) carried by 39 individuals. Haplotypes numbered 21, 22, 23, and 26 were only carried by the Modern Oriental Frill individuals.

The Bayesian phylogenetic analysis demonstrated that the D-loop marker is not sufficient to separate different pigeon breeds in Turkey,

except for the Modern Oriental Frill breed (Fig. 2). The branch including haplotypes 26, 23, 21, and 22 exclusively belonging to the Modern Oriental Frill has the highest posterior probability of 1. However, since the Modern Oriental Frill samples carrying these haplotypes were all provided by the same breeder, it is not possible to ensure that the reason for this significant separate branching is whether these birds were developed abroad for a significant period of time before being reintroduced to Turkey, or they were sourced from the same farm. Similarly, for the other three significantly separated branches having high posterior probabilities (Haplotypes 19 and 20, 7 and 8, 15 and 16, 27 and 28), the haplotypes were carried by the same individuals, only the repeat numbers in the VNTR differs.

The remaining haplotypes were distributed randomly across the Bayesian phylogenetic tree (Fig. 2) rather than clustering to associate with specific breeds or breed groups. Since a large proportion of the pigeons were heteroplasmic and carried more than one type of mitochondrial molecule, such as different repeat numbers in the 61 bp-long VNTR region or varying numbers of T repeats, it is difficult to create a reliable phylogenetic tree that can classify pigeons based on their breed, geographic origin, or poultry type to which they belong. Therefore, the morphological grouping of the pigeon breeds based on the EFLP classification (European Association of Poultry Pigeon and Rabbit Breeders, 2018) was not concordant with the D-loop haplotype phylogeny of distinct breeds.

Although the D-loop region is shown to be variable, particularly in terms of length polymorphism, it was unable to produce a strong phylogenetic signal due to the high levels of heteroplasmy and likely introgression among various breeds. The observed heteroplasmy in the D-loop region is consistent with the previous findings (Tsai et al., 2009; Lee et al., 2010): Mainly, the number of VNTR repeats ranges from 2 to 7 and the most common haplotype has four VNTR repeats, consistent with the result of Lee et al. (2010), in which the number of VNTR repeats changes from two to eight and the most common haplotype has 4 VNTR repeats too. Additionally, the T stretch was also heteroplasmic in terms of the number of thymines in the stretch.

Multiple mitochondrial types are present in heteroplasmic individuals, which also means that these mitochondria harbour a variety of mutations, including SNPs, deletions, and various amounts of VNTRs (Huang et al., 2019; Paneto et al., 2007). According to the results of Lee et al. (2010), the number of SNPs in the D-loop region was low, and only 4 haplotypes could be detected in 131 individuals. Similarly, in Stefaniuk-Szmukier et al. (2021) study, D-loop regions of 80 birds from fancy, racing, and feral pigeon populations revealed only 11 distinct haplotypes with seven polymorphic sites, indicating low haplotype diversity. They discovered only five novel SNPs when compared to the reference genome and the sequences of the Lee et al. (2010) study. In the present study, the number of novel SNPs was 17, which was quite high when compared with the previous studies. 30 unique haplotypes were discovered in our study, while Lee et al. (2010) discovered 21 haplotypes when VNTR repeat numbers were considered alone and 38

Table 1

Grouping of the pigeon breeds based on the EFLP classification (European Association of Poultry Pigeon and Rabbit Breeders, 2018).

Group Name	Breeds
Utility Pigeons (1)	Romer (Runt)
	Adana Dewlap*
	Damascene
	Egyptian Swift
	King Pigeon
Colour Pigeons (2)	Gimpel (Archangel)
	Nuremberg Lark
	Ice Pigeon
Trumpeter Pigeons (3)	Demirgech Pigeon*
Structure Pigeons (4)	Chinese Owl
	Seldschuk (<i>Selçuklu in Turkish</i>)*
	Fantail (<i>Tavuskuyruk in Turkish</i>)
Owl Pigeons (5)	African Owl (Tunisian Owl)
	Modern Oriental Frill (<i>Modern Hünkari in Turkish</i>)
	Classical Oriental Frill* (<i>Hünkari in Turkish</i>)
	Manisa Azman*
	Polish Owl
	Mısiri Pigeons* (Misiri İzmir*, Misiri Manisa*, Enice*)
	Mısiri İstanbul*
	Bulgarian Bango*
	Baska Pigeons* (Classical Baska*, Akbash Bango/Akbash Baska*, İstanbul Bango)
	Denizli Azman*
	Macar*
	Tokur*
	Tumblers (6)
Rostov Tumbler/Volski	
Uzbekian Tumbler	
Botoschan Tumbler	
Malatya Tumbler*	
Mosul Tumbler	
Shebap	

Highflyers (7)	Usak Highflyer*
	Iran Highflyer
	Budapest Highflyer
	Bulgarian (<i>Pazarcik</i>) Highflyer
	Denizli Katal*
	Szegedin Highflyer
Spinners and Rollers (8)	Thrace Roller*
	Bursa Roller*
	Saya* (<i>Kelebek in Turkish</i>)
	Odemiş (<i>Ödemiş Kelebek in Turkish</i>)*
	Aydin*
	Donek/Dunek (<i>Selanik and Rumeli Dönek in Turkish</i>)*
	Denizli Dolapci*
	Macedonian Dunek
Homing Pigeons (9)	Posta
Feral / No breed information (10)	Rock Pigeon/Feral Pigeon

Every breed group was colored differently. Local Turkish breeds were shown with *. These colours are used in the Bayesian phylogenetic tree of the D-loop haplotypes.

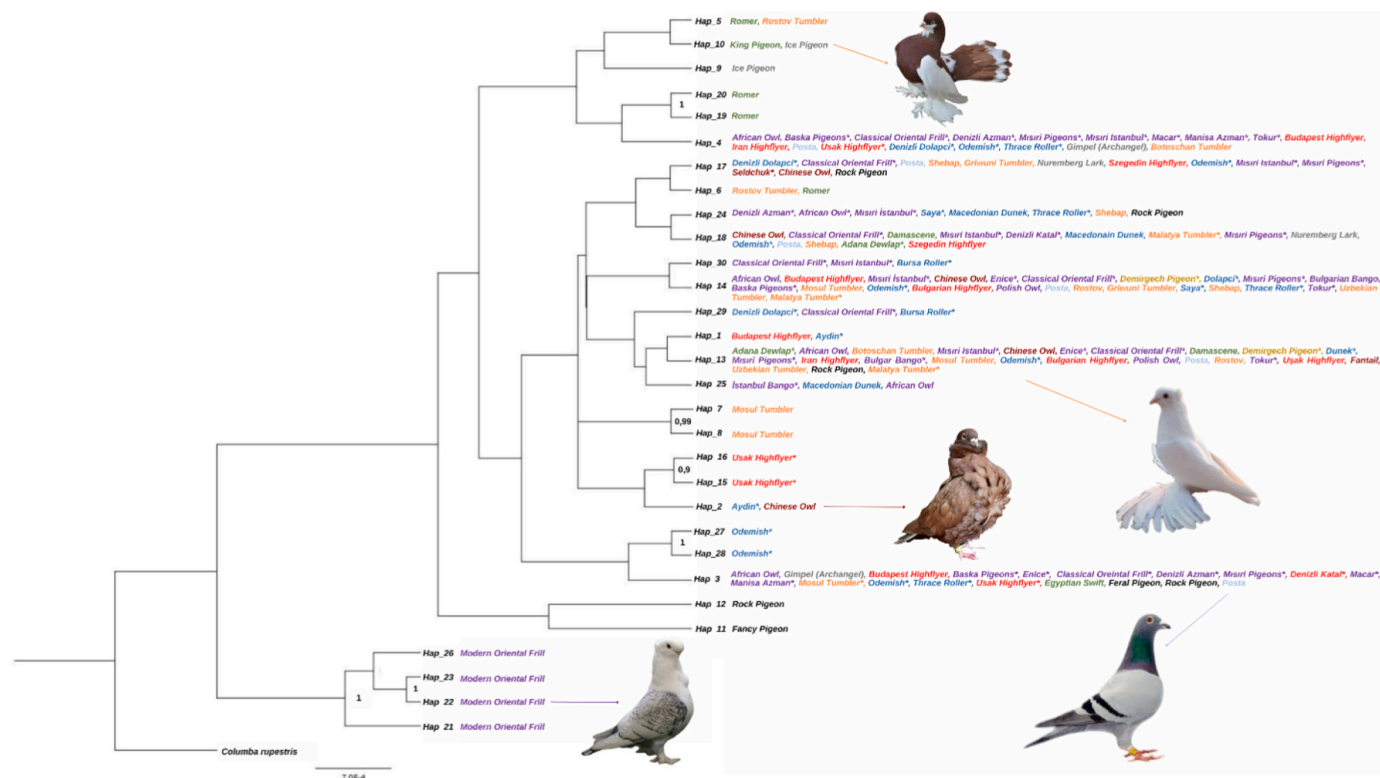


Fig. 2. Bayesian Phylogenetic Tree of D-loop Haplotypes. Numbers on nodes indicate the posterior probabilities of the branching structures. Colours indicate different pigeon groups based on the classification in Table 1.

haplotypes when SNPs were added to them. At this stage, it may be useful to emphasize again that the SNPs were detected by clean readings without any noise and the possibility of contamination was eliminated by negative controls in the beginning of the PCR reactions. Despite the low nucleotide diversity ($\pi = 0.00020$) found, the reason of high number of novel SNPs and high haplotype diversity ($h = 0.882$) in this study are probably the result of Anatolia being the centre of domestication for several breeds for a longer time, which is also supported by numerous historical artefacts and documents indicating the cultural and symbolic importance of pigeons for the region. (Shapiro and Domnyan, 2013; Yılmaz and Boz, 2012). Moreover, the increased popularity of pigeon breeding in Turkey, which often leads to indiscriminate interbreeding among local and non-local breeds, can also influence the high haplotype diversity discovered (M. Türkeş, personal communication).

The potential reasons for the observed heteroplasmy in the D-loop region of the domestic pigeons in Turkey can be listed as nuclear DNA of mitochondrial origin (NUMTs), somatic mutations, maternal inheritance, hair or feather color, age of the sampled individual, type of the sampled tissues, possible errors in DNA isolation or PCR, and paternal leakage of mitochondrial DNA. (Calloway et al., 2000; Paneto et al., 2007; de Camargo et al., 2011; Spicer et al., 2014; Ladoukakis and Zouros, 2017; Gandolfi et al., 2017; Latorre-Pellicer et al., 2019; Huang et al., 2019; Päckert et al., 2019).

Among those, only the NUMTs contamination could be eliminated, as NUMT tend to be shorter in length than the amplified gene (Gandolfi et al., 2017), and there were no significantly shorter bands observed on the gel alongside the desired bands in our study (see Fig. S1). In addition, heteroplasmic bands were carefully and distinctly separated from the agarose gel before sequencing, and back-to-back primers were used to prevent contamination from genomic DNA, as suggested by Huang et al. (2019). Other possible explanations for the observed heteroplasmy remain valid as the inheritance of the heteroplasmy of the D-loop region was not tested; no evaluations were made on the potential relationship between heteroplasmy and feather color or age of the pigeons, the effect of the different feathers could not be assessed as multiple feathers for each individual had to be used due to the insufficient DNA yield of a single feather, and it was not possible to repeat every PCR due to the limited tissue resources.

A founder effect might also possibly explain the observed results but as no pedigrees are kept by the breeders, this is difficult to explore. However, as exchange of birds between breeders is frequent, widespread and without any obvious directionality, a hierarchical descent of the observed genetic condition (i.e. a founder effect) is highly unlikely.

In the case of paternal leakage, which was detected in NADH dehydrogenase subunit 2 of *Passer italiae*, a hybrid passerine species, the authors emphasized that heteroplasmy is more common in interspecific crosses than previously thought because the mitochondrial genomes from different species are more distinct, making it easier to see the mixed signals (Päckert et al., 2019). In our case, all birds are members of the same species, but when it is considered that the development of new breeds often involves hybridization, they might be the hybrids of different breeds. There are not many studies investigating whether heteroplasmy resulting from paternal leakage observed in interspecific hybrids also occurs in interbreed hybrids. However, it is known that heteroplasmy caused by paternal leakage typically is hard to detect and usually does not manifest in the form of copy number variants, which, in contrast, is the main form of heteroplasmy observed in this study (Päckert et al., 2019). Nevertheless, considering that more SNPs were detected in this study compared to other studies, it is worth further investigating whether interbreed hybridization has any effect on heteroplasmy.

Consequently, although the exact cause of the heteroplasmy observed in the D-loop region of domestic pigeons in Turkey remains unclear, heteroplasmy, which has become more easily detected with the recent advances technology (HTS), specifically in pigeons and generally in birds and other vertebrates, emerges as an interesting phenomenon to

study further.

The Bayesian phylogenetic analysis of the D-loop region could not distinguish domestic pigeon breeds in Turkey except for the branch exclusively including haplotypes representing the Modern Oriental Frill breed. The Modern Oriental Frill is thought to originate from the Classical Oriental Frill, which was first developed in Turkey (Lyell, 1887; Türkeş and Gündüz, 2021). Historical records have documented that the introduction of the Classical Oriental Frill breed from Smyrna to Europe in the 1880s might have led to the development of the Modern Oriental Frill breed (Naether, 1939). In a recent study, (Türkeş and Gündüz, 2021), it was also suggested that the Modern Oriental Frill is most likely to be descended from Turkish birds imported into the UK in the mid-nineteenth century. Our findings potentially support this historical background, although it is important to emphasize that both samples of the Modern Oriental Frill were provided by the same breeder. Further analyses with more samples are needed to confirm this conclusion.

Other haplotypes belonging to the distinct breeds were dispersed randomly across the phylogenetic tree rather than clustering to indicate specific breeds or breed groups. The most possible explanation is the heteroplasmy in the data. A large proportion of the pigeons carried more than one type of haplotype which had either a different VNTR repeat or a different number of T repeats in the T stretch. Due to this heterogeneity, it has been difficult to create a reliable phylogenetic tree that can truly reveal the phylogenetic relationships of pigeons based on their breeds, geographic origins, or the poultry to which they belong. As a result, the existence of heteroplasmy within the dataset has hindered the phylogenetic signals. Moreover, the geographical proximity of the sampled sites may also make the distinction difficult. Breeders that live in these close-by areas frequently stay in touch and are more inclined to trade birds among themselves. Genetic distinctions can be obscured because of this frequent gene flow between breeds. Collecting more samples from each breed and including samples from distant geographic areas would have probably produce results that are more informative.

4. Conclusion

In conclusion, as reported in many previous studies, this study once again confirmed that D-loop is a promising marker for animal phylogenetic studies due to its high level of polymorphism compared to less variable coding genes in avian mtDNA. However, mainly due to the heteroplasmy and suspected high levels of introgression, it is difficult to use it to distinguish morphologically different Turkish domestic pigeon breeds. As a result, presence of several distinct mitochondrial haplotypes in individuals, and the common outbreeding and crossbreeding between breeds may prevent the evolution of mitochondrial lineages unique to each distinct breed. However, the application of HTS to such groups of birds showing evidence of heteroplasmy might surely help to better understand the molecular and evolutionary mechanisms behind heteroplasmy and their role in genome regulation and evolution.

Authors contribution

B. B.: Sample collection, experiments, and writing, editing, and submission of the manuscript. Ç. A. P.: Experiments and writing, editing, and submission of the manuscript. C. C.: Sample collection, writing and editing of the manuscript.

Ethics approval

The study does not require an ethics committee's approval as DNA samples used in this study were provided by non-invasive sampling.

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Declaration of competing interest

All authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.eups.2025.100006>.

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