Characterization of the complete mitogenomic and phylogenetic of the *Columba livia* breed Şebap pigeon

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ABSTRACT

The initial phase of the studies focused on the conservation of gene resources involves the phenotypic and genetic characterization of existing breeds. By elucidating the differences at the mitochondrial DNA (mtDNA) level, a particularly suitable source for genetic analyses, it becomes feasible to calculate genetic distances between local breeds, identify potential domestication centers through evolutionary analyses, and construct phylogeny trees depicting kinship relationships between among breeds. Şanlıurfa holds significance in pigeon breeding due to its prolonged history of traditional pigeon breeding, establishing itself as one of the pigeon domestication centers. This study aims to genetically characterize Şebap pigeon breed which is specific to Şanlıurfa province in Türkiye. The mitogenome of the Şebap pigeon was analyzed by next generation sequencing and determined to be 17,232 bp in size, comprising 13 Protein-Coding Genes (PCGs), two Ribosomal RNAs (rRNAs), 22 Transfer RNAs (tRNAs). The overall mitogenome composition includes 29.6% for A, 24.5% for T, 31.2% for C, and 14.7% for G, with a notable A–T richness of 54.1%. Notably, ND6 is encoded on the L strand while the others are encoded on the H strand. Our study has provided a comprehensive examination of whole mitochondrial DNA. Using this information, we have constructed a phylogenetic tree, locating the Şebap pigeon within the rock pigeon lineage.

Introduction

Prior to the development of contemporary molecular genetic methodologies for detecting variants at DNA-level, breed differentiation was dependent exclusively on phenotypic traits; nowadays, DNA-level variances serve to enhance phenotypic distinctions. Analyzing genetic diversity enables the calculation of genetic distances between local breeds, which sheds light on vicariance biogeography (6), identifies potential domestication centers through evolutionary analysis, and constructs phylogenetic trees illustrating relationships among breeds (20). Mitochondrial DNA (mtDNA) is stands out as a valuable genetic resource in research, particularly in phylogenetic investigations, owing to its non-recombining and uniparental inheritance characteristics, which enable the elucidation of evolutionary relationships across animal species and identification of domestication origins (7, 20).

Although the mitochondrial genome size in pigeons varies across breeds, it generally consists of 2 ribosomal RNA (rRNA) genes, 13 protein-coding genes (PCG), 22 transfer RNA (tRNA) genes and a non-coding control region (D-loop) (8, 14, 17, 21). While comprehensive whole-genome mtDNA analyses remain notably absent for pigeon breeds in Türkiye, various studies conducted in different countries have shed light on the mtDNA characteristics of pigeon breeds (20**)**. Notably, the

mitochondrial genome size varies among pigeon breeds across the globe. For instance, in Egyptian rock pigeons (specifically, the *Columba livia* breed Egyptian swift), the mtDNA size is reported as 17,239 base pairs (bp) (15). Similarly, in king pigeon breed (*Columba livia* breed king) which is selectively developed for meat production over several years, the mtDNA size is documented as 17,221 bp (23). Additionally, Chinese pigeons, as studied by Kan et al. (10), exhibit a mtDNA size of 17,229 bp. These findings underscore the need for a comprehensive exploration of the mtDNA landscape in Turkish pigeons to contribute to the broader understanding of global pigeon genetics.

Pigeon breeding in Şanlıurfa province which resides in south-eastern part of Türkiye, holds a distinctive position, deeply rooted in its longstanding tradition and recognized role as a significant pigeon domestication center. Tracing back to the pigeons of Şanlıurfa the city's heritage intertwines with the genetic lineage of numerous pigeon breeds across Türkiye. Boasting a vibrant community of pigeon breeders, the city also hosts dedicated venues for pigeon shows and trading activities, establishing itself not only as a hub for enthusiasts but also as a thriving commercial venture in the region (12, 16). While Şanlıurfa's pivotal role in pigeon breeding and trade is undeniable, it's notable that the city currently lacks formal registration for its unique pigeon breeds. This gap is particularly intriguing given the historical and geographical significance of Anatolia, renowned for its rich genetic resources, including the pigeons, traditionally bred in these lands for centuries (5, 17). Türkiye, with its plethora of indigenous pigeon breeds, stands as a testament to the nation's diverse avian heritage (1). Çelik (4) reported the morphological characteristics of the Şebap pigeon, which are typically used to classify pigeon breeds based on various traits globally; however, Türkiye's classification system prioritizes flight display traits, underscoring the cultural and practical importance of pigeons within Turkish tradition and society. This study aims to elucidate the genetic composition of the Şebap pigeon, a distinct breed specific to the Şanlıurfa province, thereby contributing to the enhancement of our knowledge regarding Türkiye's avian genetic diversity.

Materials and Methods

The animal material of the study consisted of 30 pigeons from Şanlıurfa (37° 9′ 30″ N; 38° 47′ 30″ E), sourced from the Şebap Pigeon Association and local breeders within the Şanlıurfa province. For each pigeon the roots of 5 feathers were collected, cut into 0.5-1 cm lengths, and transferred to a 1.5 ml centrifuge tube. The DNA isolation process utilized the phenol-chloroform extraction procedure as reported by Sambrook (19). In the tube 360 µl Lysis solution (10 mM TRIS-HCl, 1 mM EDTA, %0.1 Sodium Dodecyl Sulphate, pH:8.0), 50 µl DTT, 50 µl Proteinase K (10 mg/ml) were added followed by an overnight incubation in a thermo-shaker TS-100C (Kisker biotech, Germany) device at 56 °C. Subsequently, 400 µl of phenol and 400 µl chloroform was introduced to the lysate followed by gentle shaking and a 30-minute incubation at room temperature. After centrifugation at 10,000 rpm for 4 minutes at 4 °C the upper phase containing DNA was carefully transferred to a new tube. A second step involved the addition of 800 µl chloroform to remove the DNA from phenol residues. After vigorous shaking for 5 seconds, the mixture was left at room temperature for 10 minutes followed by centrifugation at 10,000 rpm for 4 minutes at 4 °C. The upper phase was transferred to a new tube and an equal volume of isopropanol was added and centrifuged at 14,500 rpm for 30 minutes for precipitation of the DNA. The DNA pellet was washed with %70 ethanol by centrifuging for 5 minutes at 14,500 rpm. After drying the DNA pellet, it was diluted in 50 µl ddH_2O (19). Concentrations of the DNA samples were set to 100 ng/µl.

Oligonucleotide design involved the creation of primers for polymerase chain reaction (PCR) utilizing the reference Pigeon mtDNA NC_013978.1 from National Center for Biotechnology Information (NCBI) database as a template. The design and selection of candidate primers were accomplished with Primer3Plus (22). Subsequently, the chosen candidate primers were evaluated for their capacity to create secondary structures using IDT OligoAnalyzer (10). The specificity of the primers was tested by "Primer-BLAST" tool within the relevant databases. Two sets of oligonucleotides were used for each sample (Table 1).

Table 1. Oligonucleotide sequences and amplicon lengths.

Name	Sequence	Amplicon lengths
RD_mtDNA_F1	ATAACCTCCCCGACGCATTC	10505bp
RD_mtDNA_R1	CAGTAACACCGGAAGCGAGT	
RD_mtDNA_F2	TCCTACTCGCCCTTCCATCA	9661bp
RD_mtDNA_R2	GTTTTGGACAATTGATGAGTGAAAA	

Oligonucleotide PCR optimization was conducted utilizing the Master Cycler epgradient (Eppendorf, Germany). The amplification of long DNA segments within mtDNA was achieved with the Phire II enzyme (Thermo, Germany). The $MgCl_2$ concentration in the buffer solutions provided with enzyme for long-region amplification was maintained as standard 2.5mM. Annealing at a variable temperature ranging from 58 to 60°C for a duration of 3 minutes were tried for optimization. The PCR amplification protocol began with denaturation at 98°C for 60 seconds, followed by a second denaturation step at the same temperature but for a shorter duration of 30 seconds. Subsequently, annealing temperature of 60°C for 30 seconds followed by the elongation phase took place at 72°C for 3 minutes, and a final elongation step is carried out at the same temperature but extended to 5 minutes. The PCR components included using Buffer containing $MgCl_2$ 5X at a 1X concentration, 200nM for dNTP, 1.25 units of Phire II, 50ng/µl for DNA, and 0.5µM for both oligonucleotides. The reaction mixture is completed with ddH_2O to 25µl. Adhering to these specified conditions ensures the successful execution of the PCR process, leading to the desired replication of the target DNA sequence.

Bioinformatics analyses were carried out on mtDNA data obtained through next-generation sequencing in the “fastq” file format. The Geneious Prime Bioinformatics Software v2023.2.1 package program (https://www.geneious.com) was utilized for various analysis. Trimming and quality filtering operations, including the removal of adapters, low-quality bases (quality score less than 30) at the ends, and short reads (less than 50 bp) from the raw reads, were performed with the BBDuk trimming tool within the same program. Duplicated readings were removed using the "Remove Duplicate Reads" tool and erroneous readings were addressed with the "Error Correct & Normalize Reads" tool.

The same process was independently applied to each sample and consensus results were obtained for 20 clean samples. These consensus results were then aligned in Geneious Prime using the MAFFT v.7 (11) procedure based on the multiple alignment algorithm found in “Allign/Assemble”. The clean and high-quality reads aligned to the latest pigeon mitochondrial genome (NC_013978.1), were obtained using the "Map to Reference" algorithm in "Align/Assemble" with parameters set to "Sensitivity: Highest sensitivity/Medium, Fine Tuning: Iterate up to 25 times." This process enabled the acquisition of the entire mtDNA genome sequence of Şebap pigeons, followed by gene annotation.

We used 19 reference nucleotide sequences to construct the phylogenetic tree. *Didunculus strigirostris* was used as the outgroup. The neighbor-joining tree (18) was constructed with the Tamura-Nei Model using the "Tree" algorithm in the Geneious Prime® v2023.2.1 package program.

Results

In this study, we present the first-ever sequence of the mitochondrial genome of the Şebap pigeon (*Columba livia* breed Şebap). The complete mitochondrial genome spans 17,232 bp and comprises 37 genes encoding 13 proteins, 2 rRNAs, 22 tRNAs, and a control region. The mitogenome has an overall composition of 29.6% A, 24.5% T, 31.2% C, and 14.7% G, with a significant A–T richness at 54.1%.

The protein-coding genes (PCG) are distributed across both strands, with ND6 encoded on the light (L) strand and the remaining protein-coding genes on the heavy (H) strand. Of the 13 protein-coding genes, ten genes initiate with the common start codon ATG, while COX1 uses GTG, ND5 employs GGG, and ND3 utilizes AGT as initiation codons (Table 2). The lengths of the 12s rRNA and 16s rRNA genes were determined as 973 base pairs (bp) and 1,586 bp, respectively. Besides these genes are positioned between the tRNAPhe and tRNALeu genes and separated by the tRNAVal gene.

The control region (D-Loop) of the Şebap pigeon’s mtDNA spans 1,662 bp long and is located between the tRNAPhe and the tRNAGlu genes. L-stranded genes included ND6 gene and eight tRNAs (tRNA-Ala, tRNA-Cys, tRNA-Glu, tRNA Asn, tRNA-Gln, tRNA-Pro, tRNA-Ser and tRNA-Tyr) while all other mitochondrial genes are H-stranded (heavy in the helix) (Figure 1 and Table 3).

In the mitochondrial genome of Şebap pigeons, a total of twenty-two tRNA genes were identified. These tRNA genes are distributed across the genome, exhibiting sizes that vary from 65 nucleotides for tRNAHis to 74 nucleotides for tRNALeu (L2) (Table 3, Figure 2 and 3). Notably, tRNAPro, tRNAArg, and tRNAGly, undetected by tRNAscan-SE, were successfully identified through comparative analysis with their respective counterparts. Importantly, all tRNA gene sequences demonstrated the potential to form characteristic cloverleaf secondary structures, as illustrated in Figure 4. The DHU and TΨC arms of these tRNA structures exhibit variability, ranging from two to five nucleotide pairs. The neighbor-joining tree the Şebap pigeon located within the *Columba livia* branch as expected (Figure 5).

Table 2. Annotation of the complete mitochondrial genome of Şebap pigeon.

	Position			Nucleotide composition %				
Gene	**Start**	**End**	**Size**	**A**	**C**	**G**	**T**	**Strand**
tRNA-Phe	1	69	69	29.0	34.8	23.2	13.0	H
12S-rRNA	70	1042	973	31.2	27.5	20.1	21.1	H
tRNA-Val	1043	1114	72	31.9	27.8	19.4	20.8	H
16S-rRNA	1115	2700	1586	33.8	25.4	18.8	22.0	H
tRNA-Leu	2701	2774	74	24.3	25.7	24.3	25.7	H
ND1 CDS	2786	3751	966	26.0	34.2	12.9	26.9	H
tRNA-Ile	3769	3839	71	32.4	22.5	19.7	25.4	H
tRNA-GIn	3845	3915	71	38.0	22.5	9.9	29.6	L
tRNA-Met	3915	3983	69	29.0	30.4	18.8	21.7	H
ND2 CDS	3984	5023	1040	32.0	34.0	10.0	23.9	H
tRNA-Trp	5024	5094	71	35.2	25.4	16.9	22.5	H
tRNA-Ala	5096	5164	69	31.9	26.1	15.9	26.1	L
tRNA-Asn	5167	5239	73	30.1	31.5	17.8	20.5	L
tRNA-Cys	5242	5308	67	31.3	28.4	16.4	23.9	L
tRNA-Tyr	5308	5379	72	36.1	26.4	15.3	22.2	L
C0X1 CDS	5381	6931	1551	26.2	32.0	16.1	25.7	H
tRNA-Ser	6923	6996	74	29.7	28.4	17.6	24.3	L
tRNA-Asp	6999	7067	69	34.8	21.7	15.9	27.5	H
C0X2 CDS	7070	7753	684	30.4	30.8	14.6	24.1	H
tRNA-Lys	7555	7825	71	29.6	23.9	21.1	25.4	H
ATP8 CDS	7827	7994	168	32.1	38.7	5.4	23.8	H
ATP6 CDS	7985	8668	684	28.4	35.1	10.1	26.5	H
COX3 CDS	8668	9451	784	26.7	33.0	15.3	25.0	H
tRNA-GIy	9452	9520	69	27.5	23.2	14.5	34.8	H
ND3 CDS	9521	9871	350	23.1	30.0	14.9	32.0	H
tRNA-Arg	9873	9941	69	26.1	26.1	17.4	30.4	H
ND4LCDS	9943	10239	297	25.6	33.3	14.5	26.6	H
ND4CDS	10233	11609	1377	29.7	33.0	13.1	24.1	H
tRNA-His	11610	11678	69	31.9	23.2	15.9	29.0	H
tRNA-Ser	11679	11744	66	30.3	27.3	21.2	21.2	H
tRNA-Leu	11744	11815	72	34.7	18.1	18.1	29.2	H
ND5CDS	11816	13628	1813	29.7	32.8	13.6	23.9	H
CYTB CDS	13640	14784	1145	25.9	34.4	14.8	24.8	H
tRNA-Thr	14785	14853	69	34.8	21.7	17.4	26.1	H
tRNA-Pro	14860	14929	70	25.7	22.9	22.9	28.6	L
ND6CDS	14975	15496	522	38.7	35.8	12.5	13.0	L
tRNA-Glu	15500	15570	71	21.1	32.4	22.5	23.9	L
Control region	15571	17232	1662	30.6	28.6	13.3	27.5	H

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Table 3. Codon number and relative synonymous codon usage (RSCU) in Şebap pigeon mitochondrial PCGs.

Codon	AA	Count	RSCU	Codon	AA	Count	RSCU	Codon	AA	Count	RSCU
GCA	A	64	1.08	AGC	S	65	1.09	CTA	L	182	1.96
GCC	A	111	1.87	AGT	S	24	0.40	CTC	L	145	1.56
GCG	A	12	0.20	TCA	S	87	1.45	CTG	L	51	0.55
GCT	A	50	0.84	TCC	S	108	1.81	CTT	L	88	0.95
TGC	C	34	1.33	TCG	S	15	0.25	TTA	L	62	0.67
TGT	C	17	0.67	TCT	S	60	1.00	TTG	L	30	0.32
GAC	D	46	1.31	ACA	T	114	1.47	ATA	M	85	1.88
GAT	D	24	0.69	ACC	T	110	1.41	ATG	M	50	1.10
GAA	E	71	1.51	ACG	T	5	0.06	GTG	M	1	0.02
GAG	E	23	0.49	ACT	T	82	1.05	AAC	N	98	1.26
TTC	F	133	1.41	GTA	V	42	1.13	AAT	N	57	0.74
TTT	F	56	0.59	GTC	V	47	1.26	CCA	P	113	1.42
GGA	G	52	1.19	GTG	V	25	0.67	CCC	P	92	1.16
GGC	G	56	1.28	GTT	V	35	0.94	CCG	P	12	0.15
GGG	G	39	0.89	TGA	W	81	1.69	CCT	P	101	1.27
GGT	G	28	0.64	TGG	W	15	0.31	CAA	Q	81	1.38
CAC	H	88	1.11	TAC	Y	75	1.36	CAG	Q	36	0.62
CAT	H	70	0.89	TAT	Y	35	0.64	CGA	R	29	1.38
ATC	I	151	1.32	AGA	*	24	1.05	CGC	R	30	1.43
ATT	I	78	0.68	AGG	*	24	1.05	CGG	R	12	0.57
AAA	K	85	1.63	TAA	*	25	1.10	CGT	R	13	0.62
AAG	K	19	0.37	TAG	*	18	0.79				

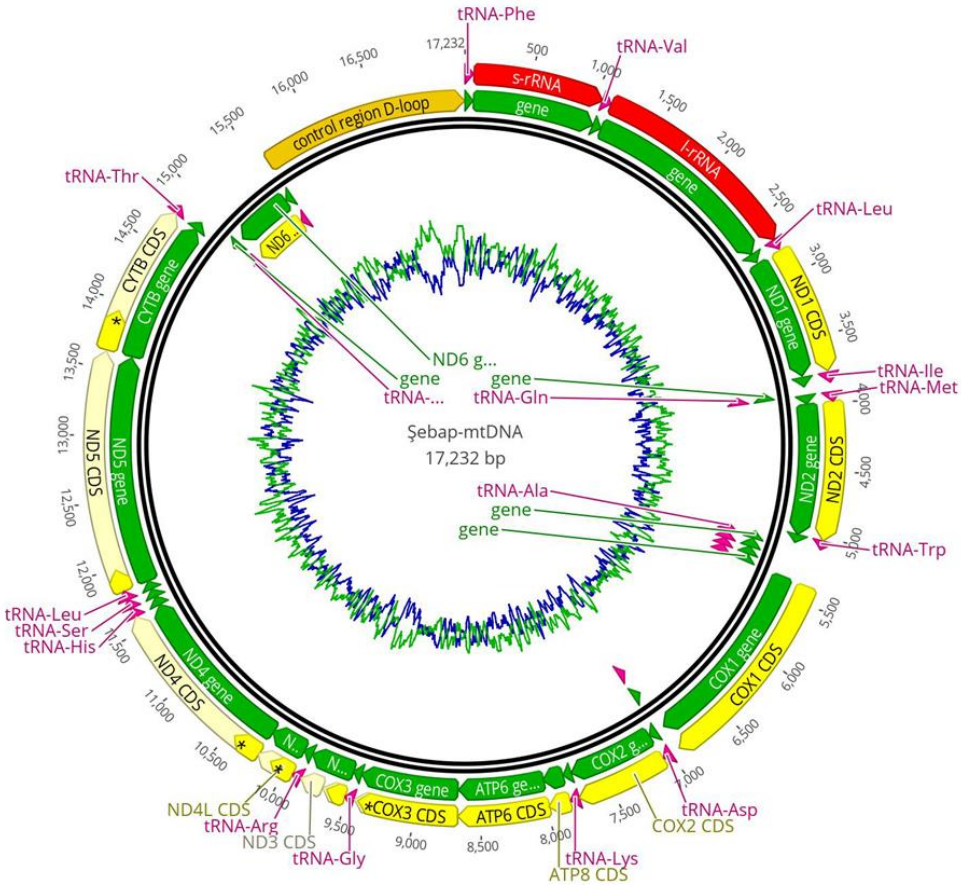

Figure 1. Gene map of the mitochondrial genome of Şebap pigeons (Columba livia breed Şebap).

Genes encoded in heavy or light strands are shown outside and inside the circular gene map, respectively. Twenty-two tRNA genes are indicated along with their amino acid codes. This graph was obtained using the Geneious Prime® v2023.2.1 package program.

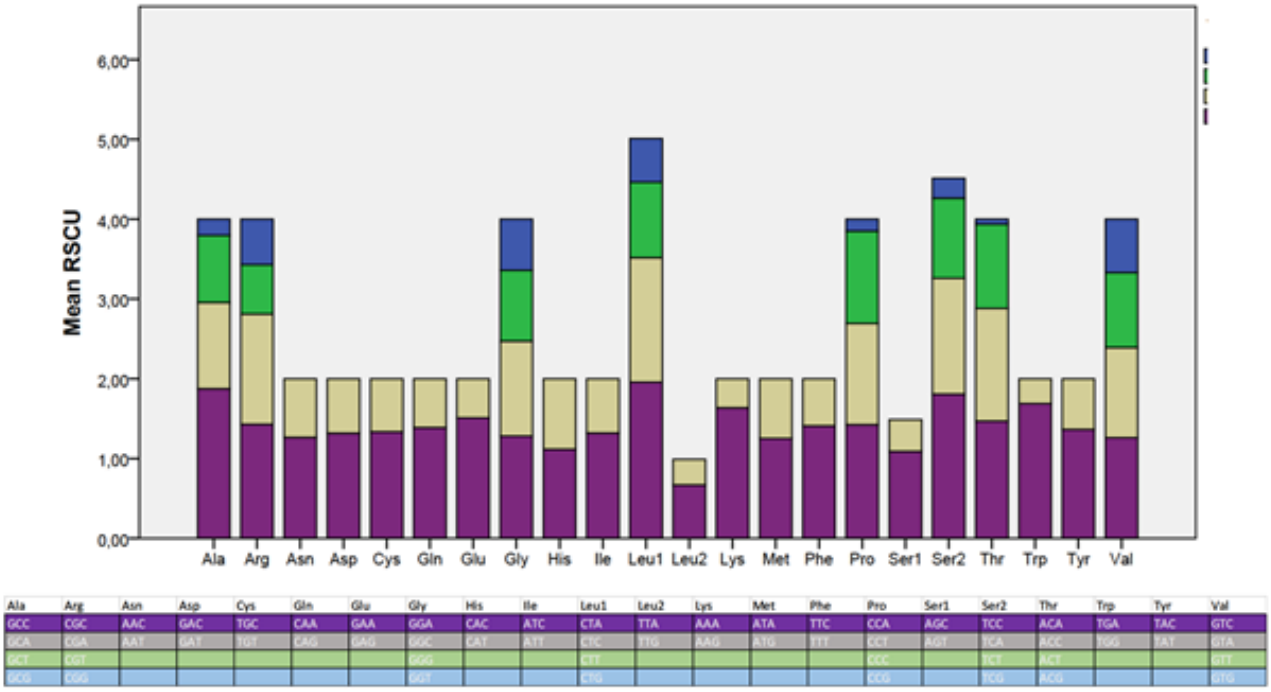

Figure 2. Codon distribution in the Şebap pigeon mitogenome.

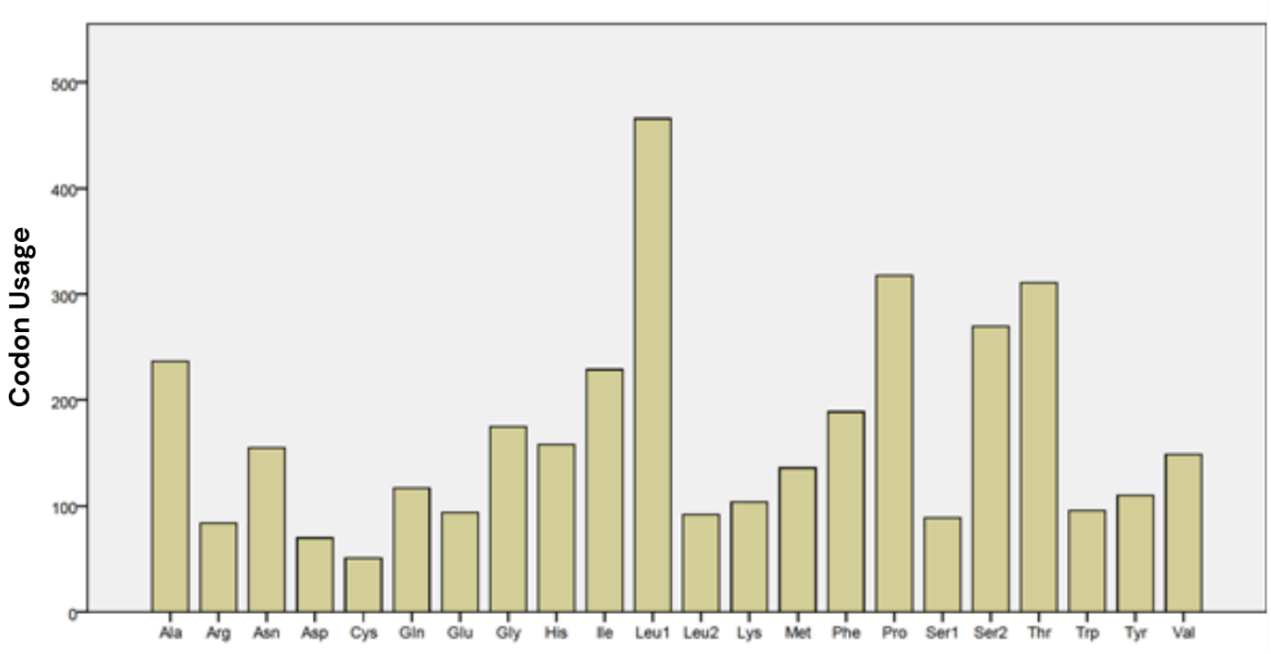

Figure 3. The relative synonymous codon usage (RSCU) in the Şebap mitogenome.

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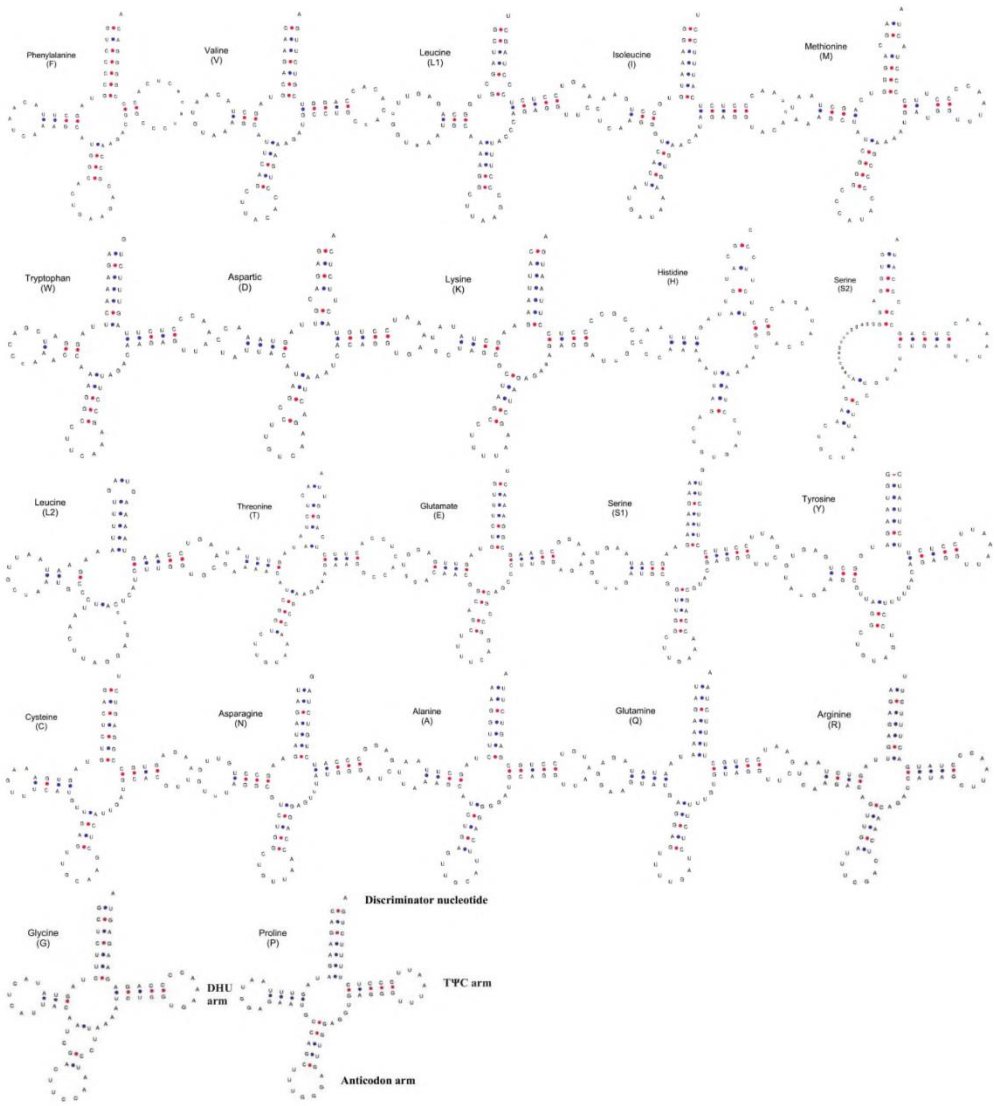

Figure 4. These are the secondary structures of the 22 tRNAs in the *Columba livia* breed Şebap mitochondrial genome.

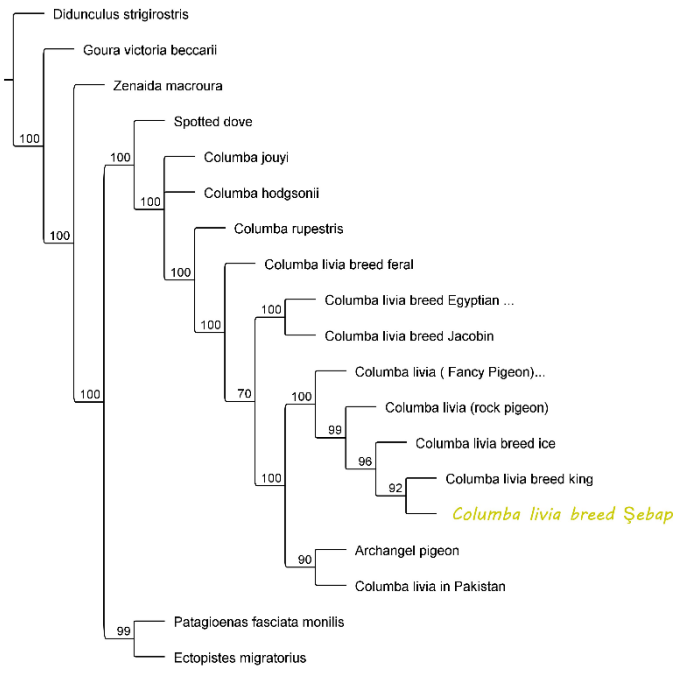

Figure 5. Nucleotide-based phylogenetic tree of 19 species and breeds belonging to the Columbidae family, with the bird *Didunculus strigirostris* as the outg.

Discussion and Conclusion

The Şebap pigeon, a domesticated breed of the rock pigeon (*Columba livia*), represents a lineage within the Columbidae bird family, encompassing domestic pigeon, feral pigeon, and wild rock pigeon. This species has undergone several millennia of domestication, resulting in the emergence of diverse domestic breeds across various geographical locations (20, 24). Domestic pigeon descendants of wild rock pigeons have successfully adapted to urban habitats. This comprehensive analysis provides valuable insights into mitogenome diversity and features genes within the mitochondrial genome of Şebap pigeons.

The Protein coding genes (PCG) of the pigeon consist encoding *Cytochrome C Oxidases* (COXI, COXII and COXIII), two ATPases (ATP6 and ATP8), seven NADH dehydrogenases (ND1-6 and ND4L), and Cytochrome *b*. In the study of Kan et. al (6) on the rock pigeon (*Columba livia*), they found that the total length of 13 protein-coding genes in *C. livia* mtDNA was 11,388 bp, corresponding to 66.1% of the entire mitochondrial genome. Kan et al. (10) observed that the length of 13 protein-coding genes found in other bird species ranged from 11,379 bp for *Rhynochetos jubatus* to 11,412 bp for *Branta canadensis*. Additionally, the length of the 13 protein-coding genes found in *C. livia* mtDNA is similar to most other bird species. The longest protein-coding gene of *C. livia* mtDNA was determined to be the NAD5 gene (1,815 bp), and the shortest was ATP8 (168 bp). In a similar study on *Columba hodgsonii*, Liu et al. (16) determined that the total length of 13 protein-coding genes in mtDNA was 11,385 bp, corresponding to 65.1% of the entire mitochondrial genome. In our study the total length of 13 protein-coding genes on the mitochondrial genome of Şebap pigeons was 11,411 bp and corresponded to 66.2% of the entire mitochondrial genome. The general features, including mitochondrial genome organization and gene regulation pattern, align with those observed in other bird species (7, 8, 10, 15, 16) (Figure 1).

Biray et al. (3) and Khan et. al (13) who focused on *COI* and D-loop regions in *Columba livia* mtDNA emphasized the challenges establishing the phylogeny of domestic pigeon lineages using mtDNA. Our study, leveraging whole-genome next generation sequencing, provides a more comprehensive analysis of all mitochondrial genes revealing the Şebap pigeon's domesticated status within the rock pigeon lineage.

Balog et al. (2) studied the partial sequencing of COI region of mtDNA in 7 Hungarian and 35 international pigeon breeds and detected no significant difference between Hungarian and foreign breeds.

In conclusion, our study not only contributes to the understanding of the mitochondrial genome of the Şebap pigeon but also highlights the importance of comprehensive genomic analyses for a more accurate portrayal of genetic relationships within and between pigeon breeds. It is significant as it reveals the entire mitogenomic sequence of the Şebap pigeon breed of *C. livia* for the first time. The circular mitogenome, spanning 17,232 bp, exhibits the canonical configuration of 37 genes, including 13 protein-coding genes (PCGs), two rRNAs, and 22 tRNAs—features typical of Columbidae species. The nucleotide composition of the mitogenome of Şebap pigeon showed a distribution of 29.6% for A, 24.5% for T, 31.2% for C, and 14.7% for G, emphasizing a pronounced A–T richness at 54.1%. Notably, the encoding of ND6 on the L strand, in contrast to the H strand encoding of other genes, adds a layer of complexity to the genomic landscape. This comprehensive characterization significantly enhances our understanding of the mitochondrial dynamics in *C. livia* breed Şebap, laying a robust foundation for future investigations into its genetic and evolutionary intricacies.

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Ethical Statement

This research was approved by the Harran University Animal Experiments Local Ethics Committee (Approval no: 2021.005.04).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

RÇ, NB, and ŞG conceived the presented idea. FB developed the theoretical framework and conducted the computations. NB and RÇ designed the study. RÇ and AY were involved in data collection. FK and MYA contributed to the laboratory experiments. NB performed the data analysis. AY, FB, and NB contributed to the interpretation of the results. All authors discussed the findings and contributed to the preparation of the final manuscript.

Data Availability Statement

The datasets analyzed during the current study are available from the corresponding author on a reasonable request.

Animal Welfare

The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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