Investigating the Genetic Diversity of Squab Pigeon Breeds using Mitochondrial DNA COI Region

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Abstract
Pigeon breeding is a long-established activity, with archaeological and written evidence dating back thousands of years. Hungarian pigeon breeding has been influenced from several directions in the past, as several trade routes crossed the historical Hungary. Therefore, the ancestors of today's breeds probably originate partly from the East and partly from the West. The Turkish conquest left a large number of diverse pigeon breeds in Hungary, and pigeons from Russia also arrived in the Carpathian Basin through Polish mediation. Pigeons were introduced from the West thanks to Danube sailors. Seven breeds were sampled (n=5/breed) via random sampling from different districts of Hungary to carry out this study. In this study, a 540-bp sequence of the COI region of mtDNA (mitochondrial DNA) was used for analysis. CLUSTALW was used for sequence alignment and MEGAX and Network for reconstruction of phylogenetic trees and with ARLEQUIN to detect diversity values, haplotype distributions, the number of polymorphisms, and nucleotide frequency values. A total of 35 haplotypes were identified in the populations studied. Nucleotide diversity (π) was 0.2889 (Hungarian breeds) and (π) 0,3364 (International breeds). Our results help to reveal the extent to which populations are genetically uniform, and to what extent they are separated from each other.

Keywords: Columba livia domestica, genetic diversity, squab pigeon, mtDNA, COI region,

1. Introduction

The long history of pigeon breeding is demonstrated by the fact that there is archaeological and written evidence from the Sumerian period, which suggests that the domestication of the pigeon probably started in the Middle East 4000-6000 BC [1]. Pigeon breeding in Hungary has been influenced from many directions in the past, as several trade routes crossed historical Hungary. Thus, the ancestors of our present breeds probably originated partly from the East and partly from the West. The Turkish conquest left a large number of diverse pigeon breeds in Hungary [2], and pigeons from Russia also arrived in the Carpathian Basin through Polish mediation [3]. From the West-Europe, pigeons were introduced into the country by Danube sailors [4,5]. These influences played a role in the development of our own breeds. The 19th and the first half of the 20th century was the period when Hungarian pigeon breeding was at its peak, as most of the Hungarian pigeon breeds were developed and adopted during this period. Evidence of this can be found in Prütz's book from 1866 [6]. Following the German pattern, the naming of the breeds was mostly linked to cities, which is important because most of Hungarian breeds were developed in a single city or in small rural areas. Thus, for example, the Hungarian larger-bodied 'giant pigeon' breeds are typically associated with the Great Hungarian Plain. The

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characteristics of the breeds are largely a faithful reflection of the tastes of the breeders of the region and the type of use. It has been suggested that these breeds may have developed from a common base, or even from a completely independent lineage which based on the Mediterranean giant pigeons of world-wide distribution. The Hungarian breeds developed from these breeds and lines also demonstrate that these genotypes are well adapted to environmental changes, which can be very useful against the negative effects of climate change. The success of these breeds is also shown by the fact that Hungarian squab pigeon breeding was very important in the 1970s and 1980s, with the country exporting 1,000 tonnes of squab pigeons (2 million pigeons) per year.

However, after the change of regime, there was a significant decline, mainly due to the loss of export markets and the subsequent collapse of the domestic buying and processing chain. The Hungarian squab pigeon flock has not developed over the last 30 years due to the sector's slump and is currently uncompetitive due to the backwardness. The Hungarian squab pigeon population was reduced to 249,000 squab pigeons in 2014 [7], and modern breeds imported from abroad are used for breeding in the country. Positive changes in consumer habits (alternative sources of protein, increasing demand for healthy food and premium products) could provide a perspective for the sector, so that today up to ten times the amount of pigeons kept in Hungary could be exported and sold on Western European markets.

It is now widely spread that genetic diversity studies is crucial for the persistence of wildlife and domesticated populations as low diversity results in both low individual fitness and reduced adaptability of populations in the face of environmental changes [8,9]. No molecular genetic methods have been used to study the Hungarian pigeon flocks, so we do not have an accurate picture of the genetic characteristics, values and potential of the species. For this reason, the genetic diversity, population structure and phylogenetic relationships of the Hungarian flocks of large-bodied domestic pigeons should be explored, with a special focus on potential breeds for use in the production of squab pigeon. Mitochondrial DNA markers can be reliable in determining the origin of species and phylogenetic relationships of species due to their simple structure, easy molecular weight and maternal inheritance [10,11]. DNA barcoding techniques use a short DNA sequence of the genome, which provides sufficient variance at the species level to unambiguously determine the taxon. Mitochondrial cytochrome oxidase I region is the standard marker for barcoding of birds [12]. Phylogenetic studies in case os Columbadie have been carried out mainly in wild pigeon populations. In 2020, Rafiq et al. [13] investigated the genetic distances and phylogenetic position of 7 species randomly collected from the family Columbidae in different areas of Pakistan. They found that the Eurasian collared dove (*Streptopelia decaocto*), the Red collared dove (*Streptopelia tranquebarica*) and the Laughing dove *(Spilopelia senegalensis*) are closely related while the Spotted dove (*Spilopelia chinensis*) shows a large genetic distance to this group [14,15]. Furthermore, the Yellow-eyed pigeon (*Columba eversmanni*) and the Yellow-footed green pigeon (*Treron phoenicoptera*) are phylogenetically closely related, while the Yellow-footed green pigeon (*Treron phoenicoptera*) is highly separated from these species. During the analyses, the species seemed to have descended from a common ancestor, but eventually 3 distinct clades emerged. In South Korea, Hill pigeons (*Columba rupestris*) were on the brink of extinction, and as part of an urgent conservation effort, Kim et al. (2022) [16] set out to breed and reintroduce Hill pigeons in captivity by studying the extent of hybridisation between mountain pigeon and domestic pigeon populations. Hill pigeon habitats are divided into three regions in South Korea, so these regions were investigated and genetic distance and diversity were analysed in three global areas (Mongolia, Russia, South Korea). Hill pigeons in South Korea were genetically indistinguishable from Mongolian and Russian populations and showed relatively low genetic diversity from other Columbidae endangered species. Two of the three regions have pure populations, and only one region is more hybridized. The endemic pigeon species on the Caribbean islands are highly sensitive to environmental degradation, extreme climatic events and changes resulting from interactions with exotic species. In 2021, Cambrone et al. [17] investigated gene flow,

genetic diversity and genetic structure among island Scaly-naped pigeon (Patagioenas squamosa) populations from Puerto Rico, Guadeloupe, Martinique and Barbados in the COI region. A significant genetic difference was found in the Barbadian population, where some individuals from a captive breeding population from a Bridgetown aviary were sampled. There was no differentiation between the populations of Puerto Rico and the French Antilles, so the species may largely consist of a single large, homogeneous population. These types of studies are also common in pigeons, Ramadan et al. in 2011 [18] studied 6 Egyptian pigeon breeds and Biała et al. in 2015 [19] studied Wroclaw meat pigeon breeds using the COI region. Also in domestic pigeon breeds, genetic diversity was investigated using two mtDNA regions (D-Loop, COI) by Biray et al. (2020) [12].

Population genetics studies are important for the management of endangered species, the determination of evolutionary and geographic limits of species, and the identification of subspecies and populations [20,21,22,23]. At the same time, there are also a number of advantages for agriculture, as knowledge of the genetic resources of a country can help to detect the development of potential breeds for production, and the results can help to develop new selection technologies in any species [24,25,26].

2. Materials and methods

Sampling

Seven domestic pigeon breeds – Hungarian Giant House Pigeon (n=5), Hungarian Cropper (n=5), Buga Pigeon (n=5), Giant Salonta (n=5), King (n=5), Mondain (n=5) and Runt Pigeon (n=5) – were studied in Hungarian populations. These breeds have evolved originally in three major geographical regions: the Carpathian Basin, the Mediterranean and North America. The study was sampled in 2018 and 2019 from flocks of pigeon breeders living in different municipalities in Hungary. Samples were collected from the wing vein of the individuals and individually marked. Blood samples were stored at -20 C˚ until use. The tests were performed at the Centre for Agricultural Genomics and Biotechnology, University of Debrecen.

Double stranded DNA was amplified in a total reaction volume of 30 µl containing 7.2 µl dH2O, 0.6 µl of DreamTaq polymerase (Thermofisher), 6 µl of (DreamTaq Flexi Buffer) reaction buffer, 6 µl MgCl2, 3 µl of dNTP, 0.6 µl (1 pmol/µl) of each primer and approximately 6 µl 50–100 ng of DNA.

Thermocycling conditions were 94°C for 5 min, followed by 35 cycles of 56 °C 1 min, 72 °C 1 min, 72 °C 10 min. This was followed by a final extension step for 10 min at 72 °C and 10 °C ∞. After PCR, the success of amplification was verified by agarose gel electrophoresis. The PCR products were sent to Macrogen Europe BV, Amsterdam for commercial sequencing.

DNA isolation

Genomic DNA was isolated from the samples according to the protocol of [27].

PCR

Based on literature data, 2 primer pairs in the COI region of mtDNA were selected for preliminary analysis, of which AWAN showed better performance in optimization [28] and was used for further analysis. The COI region of mtDNA, a 540 bp region, was used for the analyses. Amplification of the primer pair defined sequence was performed at the Centre for Agricultural Genomics and Biotechnology of University of Debrecen using the Biometra Tone PCR machine.

Data analysis

The sequences of the samples were obtained electronically, and the nucleotides were checked manually for correctness. After manual verification, the sequences were aligned using ClustalW [29]. To cut the sequences and draw the dendogram, we used MEGA11 [30] software. Then, DnaSP 6 (DNA Sequence Polymorphism Analysis of Large Data Sets) software [31] was used to measure the number and distribution of haplotypes, the number of polymorphisms and the nucleotide frequency values. Arlequin 3.5.2.2 software [32] was used to perform fixation index (Fst), genetic distance and AMOVA tests. The Network 5.0.1.1 [31] software package, a phylogenetic tree was constructed using Neighbor-Joining analysis to illustrate evolutionary relationships.

3. Results and discussion

In assessing our results, we created two groups. The first group is the Hungarian breeds (Buga Pigeon, Hungarian Cropper, Hungarian Giant Pigeon, Salonta Pigeon) and the second group is the international breeds (King, Mondain, Runt Pigeon). The genetic diversity indices of the groups are summarized in Table 1.

In the Hungarian and International groups, the number of polymorphisms was evenly balanced, although the Hungarian breed group had a higher number of elements. The haplotype diversity values were high in all cases, while the nucleotide diversity values were generally considered low. For nucleotide diversity, the International group shows higher values (π=0.3364 ± 0.0793) compared to the Hungarian group (π=0.2887 ± 0.0699) (Table 1). Prakas et al (2021) [33] examined 258 Turtle dove (*Streptopelia turtur*) samples in the D-loop region of mtDNA. Eighty haplotypes were identified. The average frequency of haplotypes was 3.2, which is higher than in our case (1.0) and the nucleotide diversity was also higher in their case in the group with multiple haplotypes (π= 0.00722 ± 0.00032), which is considered low compared to our values (Hungarian breeds: 0,2887 ± 0,0699, International breeds: 0,3364 ± 0,0793). In the Turtle dove study, probably due to the high haplotype number, the values were higher than in the groups we studied, but comparing the nucleotide diversity values, [33] obtained lower values in the wild pigeon flock compared to the flocks of domestic pigeons we studied, which may also indicate a good segregation of the pigeon breeds. The Hungarian Giant Pigeon showed the lowest nucleotide diversity (0.1305 ± 0.0187) and the King showed the highest nucleotide diversity (0.3785 ± 0.1382). The differences in nucleotide diversity between the breeds could be due to the breeding background of the breeds and differences in utilization factors. The King breed was bred in the early 1900s from Runt, Maltese, Racing Pigeon and Duches breeds, and the crosses resulted a white variety and later a silver variety [34], which is still one of the national breeds of the USA. In comparison, the Hungarian Giant Pigeon was established and spread within Hungary, mainly in the villages of the Great Plain. Our result can be explained by the large difference in size between these geographical regions, the King being a more widespread breed with more individuals, and the breed being more developed. In a previous study [35] (Nyiri, 2020), the genetic diversity of the King, Hungarian Giant, Salonta Pigeon and Runt Pigeon breeds was investigated, where King and Hungarian Giant Pigeon were also the most distant from each other when compared on the basis of Fst value per pair.

Table 1. Diversity indices of the studied groups

Indices / Groups	Number of elements (n)	Number of polymorphisms	Number of haplotypes	Haplotype diversity (H_d) ± SD	Nucleotide diversity (π) ± SD
Hungarian breeds	20	504	20	1..000 ± 0.016	0.2887 ± 0.0699
Buga Pigeon	5	396	5	1.000 ± 0.126	0.3472 ± 0.1305
Hungarian Cropper	5	411	5	1.000 ± 0.126	0.3526 ± 0.1485
Hungarian Giant Pigeon	5	113	5	1.000 ± 0.126	0.1305 ± 0.0187
Salonta Pigeon	5	408	5	1.000 ± 0.126	0.3545 ± 0.1438
International breeds	15	505	15	1.000 ± 0.024	0.3364 ± 0.0793
King	5	420	5	1.000 ± 0.126	0.3785 ± 0.1382
Mondain	5	411	5	1.000 ± 0.126	0.3707 ± 0.1334
Runt Pigeon	5	354	5	1.000 ± 0.126	0.3120 ± 0.1175

In table 2., the nucleotide composition of the two groups was analyzed to provide insights into their genetic background. The results show that both groups are slightly different in the percentages of each nucleotide, with cytosine (C) being the most abundant nucleotide, followed by adenine (A) and thymine (T), and guanine (G) being the least abundant.

Table 2. Nucleotide composition of the groups

Groups / Nucleotide composition (%)	Hungarian breeds	International breeds
Cytozine (C)	31.84%	31.89%
Tyhime (T)	25.35%	25.60%
Adenine (A)	25.98%	26.43%
Guanine (G)	16.82%	16.07%

Table 3. shows pairwise comparisons between the two groups based on Fst values. The extremely low Fst value of -0.02008 indicates that there is no detectable genetic difference between Hungarian breeds and International breeds.

Table 3. Pairwise comparison of groups by Fst values

Groups	Hungarian breeds	International breeds
Hungarian breeds	0.000	0.000
Squab breeds	-0.02008	0.000

The significance level of the Fst value ($p<0.05$).

Table 4. shows a pairwise comparison of the Fst values of the pigeon breeds tested. Overall, the values obtained are very low, which may indicate that there are not that many differences between the populations. However, it can be clearly seen that the Hungarian Cropper is quite distinct from the Salonta pigeon (-0.14801), the Mondain (-0.12267), and the Runt pigeon (-0.12240). It can also be seen that the Salonta pigeon is distinct from the Mondain (-0.12033) and the Runt pigeon (-0.13916), which is used as an outgroup. In this comparison, it is clear to see that besides King, Mondain is also relatively distinct from the native Hungarian breeds. The value of the Salonta Pigeon can be explained by the fact that the breed was initially spread in the town of Salonta in the Partium region and the surrounding villages, and even today it still mainly covers the area between Oradea and Arad but has also spread to other parts of Romania and Hungary. However, the number of elements found in Hungary is very low, and 10 years ago the population in Hungary was considered to be at critical status based on the threat level [36].

Table 4. Pairwise comparison based on Fst values for all breeds

Breeds	Buga Pigeon	Hungarian Cropper	Hungarian Giant Pigeon	Salonta Pigeon	King	Mondain	Runt Pigeon
Buga Pigeon		-0.06128	0.02450	-0.06592	-0.05440	-0.06534	-0.04142
Hungarian Cropper			0.02794	-0.14801	-0.06980	-0.12267	-0.12240
Hungarian Giant Pigeon				0.07040	0.03150	-0.01189	0.09332
Salonta Pigeon					-0.06276	-0.12033	-0.13916
King						-0.07424	-0.04959
Mondain							-0.09942
Runt Pigeon							

Table 5. shows the results of the molecular analysis of variance (AMOVA). In this case, we also examined the values between species and between the two groups. In both clustering criteria, we obtained extremely high values within populations (breeds: 105.58; grouping by origin type: 102.01) but not between populations, which means that genetic differentiation is typical between breeds. In both cases, negative values were obtained between

populations, suggesting that there is no genetic structure between the populations. One of the reasons for this may be the low number of elements. Midot et al. (2019) [37] studied *Ganoderma boninense* populations, and also obtained extremely low Fst values, which are often associated with negative AMOVA values. For negative values obtained with a higher number of elements, a Mantel test is usually performed, as this test can be used to assess the degree of isolation of populations [38]. Since there is no significant geographic distance between the breeds we studied and the number of elements is low, it is not relevant to perform the Mantel test. Bigi et al. (2016) [39] also performed variance analysis according to different groupings between domestic pigeon breeds, showing in 21.34% of the variance between breeds and 74.69% of the variance within individuals. It resulted in 9.53% of the total variance between groups and 73.29% of the total variance within individuals for the pedigree-based grouping of breeds. In Figure 1., the relationship between the haplotypes of the tested breeds is shown in a dendogram constructed using the Neighbor-Joining method. The figure shows that 2 individuals are not directly related to the others, but indirectly, namely: MOND 114, and KING 42. No explicit groups are distinguished, but several blocks of mixed groups can be observed, which means that there are no haplotypes or haplotype groups that can be linked to a specific locality or breed.

Table 5. The result of the AMOVA test for groupings based on different criteria

	Source of variation	df	Sum of squares	Variance components	Percentage of variation (%)
Breeds	**Among populations**	6	382.486	-4.5747	-5.58
	Within populations	28	2425.400	86.6214	105.58
Grouping by origin	**Among populations**	1	55.269	-1.6417	-2.01
	Within populations	33	2752.617	83.4126	102.01

Figure 1. Neighbor-Joining dendogram of haplotype relationships (MEGA 11)

4. Conclusions

In the study, we assessed the genetic diversity and genetic structure of Hungarian populations of different squab pigeon breeds, and the extent to which international breeds influence the genetic stock of Hungarian breeds. Overall, the small number of elements used in the study may explain the low values obtained and the very high number of haplotypes (35 haplotypes for 35 samples). Although there is no significant difference between Hungarian and foreign breeds based on the source of variance. In case of King, Mondain and Hungarian Cropper showed a greater distance from the other breeds based on the FST value per pair and Nei genetic distances. In conclusion, our mtDNA COI region can be effectively used to assess the genetic diversity and genetic structure of Hungarian pigeon breeds and other domestic pigeon populations. The information from this thesis will provide useful input for mapping the genetic background of large-bodied pigeon breeds in Hungary and may also serve as a starting point for the genetic improvement of domestic pigeon breeds and the development of conservation/breeding programmes for native breeds. Genetic characterisation should be carried out with a larger number of elements in the future.

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