Research Article

Advances in Applied Biological Research

Year 2024, Volume-1, Issue-1 (January - June)

www.acspublisher.com

CO-1 gene-based DNA barcoding and phylogenetic analysis of genus Columba from Kasur, Pakistan

Faiqa Zulfiqar[1], Ayesha[1], Muhammad Imran[2], Mudasar Hussain[2], Moshin Masood[2], Naseem Akhtar[2], Hamad Alam[2], Sehresh Ghafoor[1], Ariba Masood[2], Waqas Ali[2]

[1]Institute of Molecular Biology and Biotechnology, University of Lahore, Lahore.
[2]Department of Wildlife and Ecology, University of Veterinary and Animal Sciences, Lahore.

ARTICLE INFO

Key Words: COI, Cytb, 16S rRNA, pigeon

doi: 10.48165/aabr.2024.1.04

ABSTRACT

The present study was conducted from July to December, 2021 to find out phylogenetic relationship among different species of pigeons captured from different sites of district Kasur. The live specimens were shifted to the Avian Conservation and Research Center (ACRC), Department of Wildlife and Ecology, UVAS for molecular analysis. All the specimens were morphologically identified via classical taxonomic keys. The blood samples were collected into EDTA tubes by puncturing the brachial vein. Salt extraction was used to isolate DNA from whole blood samples. An agarose gel electrophoresis was used to confirm the DNA's purity and quantification was done through Nano drop-One. All the DNA samples were Sanger sequences on 13730XL DNA Analyzer from Korea. After trimming bases, the obtained COI fragments were 675 bp. The obtained DNA sequences were submitted to Genbank and accession numbers were obtained (OL774678, OL778850, OL778945 and OL780846). The obtained DNA sequences have shown clear species identification of all the captured specimens. Closely related DNA sequences were downloaded in Blast searches from the NCBI and incorporated in the Neighbour-joining tree. Neighbour-joining tree was constructed based on p-distance using MEGA 10.0. The whole genetic divergence of *Columba livia* and *Columba rupestris* was 0.003±0.001 and 0.014±0.004 respectively. It was concluded that more studies related to DNA barcoding should be done to find any new or subspecies from study area.

Introduction

The universal biomarker based on DNA barcoding is the most promising strategy because there is more diversity between species than within species, allowing for species differentiation. Furthermore, the US FDA recommend DNA barcoding as a possible tool, particularly for seafood speciation (Khaksar *et al.,* 2015). The mitochondrial 16s rRNA gene, which has a large number of insertions and deletions, may be used to differentiate most species, but the CO1 gene, which has a higher interspecies variability than intraspecies variability, is commonly used for barcod-

*Corresponding author.
E-mail address: faiqa.zulfiqar6@gmail.com (Faiqa Zulfiqar)
Received 05.07.2024; Accepted 28.07.2024

ing (Sarri *et al.,* 2014; Sultana *et al.,* 2018).It is possible to verify and identify certain species using DNA barcoding, which employs a little DNA sequence (Hebert *et al.,* 2003). For the first time, researchers have used DNA sequences to identify a new species inside an established categorization system (a monophylum) rather than to investigate the evolutionary relationships between the species in question (Kress *et al.,* 2005). To correctly identify a taxon, a short DNA sequence from the genome may be employed in a DNA barcoding approach. A barcoding gene may be useful for taxa from the same major kingdom, such as mammals, plants, or fungi, since it can be repeated and compared among them (Yan *et al.,* 2013).

The mitochondrial CO1 gene is located in a region of around 650 base pairs (bp) in mammals and other eukaryotes (Hebert *et al.,* 2003). It was employed in the early stages of DNA barcoding to identify microbial communities. For the identification of species and the classification of unidentified sequences to higher taxa, such as orders and subphyla, researchers at the University of Guelph issued a study in 2003 by Paul DN Hebert and his colleagues (Hebert *et al.,* 2003). Folmer *et al.,* (1994) employed an outstanding approach for phylogenetic analyses at the species level to discriminate between metazoan invertebrates (Hebert *et al.,* 2003). It is common to use the CO1 gene's "Folmer region" to differentiate taxa. The Food and Agriculture Organization advocates for the preservation of genetic diversity around the world. As a result, native species conservation as a genetic resource is critical in order to meet future species demands (Tadano *et al.,* 2007).

Domestication of pigeons is said to have begun as early as 3000 B.C. Over 300 species of domestic pigeons exist today, all descended from a single wild parent, the rock dove (Ramadan *et al.,* 2011). There are two well-known bird families in the order Columbiformes: Raphididae and Columbidae (Pereira *et al.,* 2007). These birds are often light grey in colour with two black stripes on each wing. Pigeons have been tamed for millennia, and multiple new varieties have emerged from just one bird. Many domestic pigeons that have escaped or been released into the wild have given rise to feral pigeons. Many cities and towns across the world have feral pigeon colonies (Kan *et al.,* 2010). Racing pigeons are only one of many uses for pigeons in addition to their other responsibilities of being food providers and display birds, and pilots.

Rock Doves, including domestic pigeons, are a member of the *Columba livia domestica* family (also called the rock pigeon). Rock pigeons are without a doubt the earliest tamed birds. Archaeological finds in Mesopotamia and Egypt show that humans have been domesticating birds for at least 5,000 years (Blechman *et al.,* 2007). Pigeons may have been domesticated as early as 10,000 years ago, according to research (Blechman *et al.,* 2007).

Over 309 species of pigeons and doves in live status belong to the Columbidae subfamily. Pigeons are wild and street birds that come in grey, blue, brown, and largely white colours (Johnson and Clayton, 2001). Birds of the Columbidae family may be found across the world, except in Antarctica (Gibbs *et al.,* 2001). Taxonomic authorities disagree on how to classify the Columbidae. The categorization of the Columbidae is a matter of debate among taxonomic authorities that ITIS relies on to classify the five families (Columbidae, Didunculinae, Otidiphabinae, and Treroninnae), which only utilizes two families (Integrated Taxonomic Information System 2020). In China's Catalog of Animals, all green-pigeon (Treron) species are second-class national protected wildlife. Despite the fact that the majority of the genus' species are in decline, only a tiny number of Treron's genetic resources are still available (Claramunt and Cracraft, 2015). Green-Pigeon *Treron curvirostra* may be found in the tropical and subtropical forests of Southeast Asia and South Asia on a regular basis. This is based on a study by Gibbs et al. (2001). Seeds and fruits are the primary food sources for this species, which belongs to the Columbidae family (Korzun *et al.,* 2008). With their unique grey or green heads and necks, medium-sized birds with bright plumage may be recognized (Korzun *et al.,* 2008). Bright yellow wing patches contrast with the otherwise dark black wings, which are framed by yellow. There is one green patch in the midst of the tail's secondary end patches, which are grey with black patches (Nair, 2010). A common bird that descended from the rock pigeon is the domestic pigeon (*Columba livia domestica*). Their natural habitats are Europe, North Africa, the Middle East, and South Asia (Shapiro and Domyan, 2013). Columba livia is a well-known for a variety of research projects, such as ecology, genetics, physiology, behaviour, and anatomical variation (Holt, 2018). Pigeon breeds' geographic origins have not been thoroughly documented. The origins of major breed groupings of pigeons, however, have been proven to originate in the Middle East and North America (Shapiro *et al.,* 2013). Natural and artificial selects have had a significant impact on the genetic development of pigeons, just as they have in other domestic animals.

For the identification of species, a short sequence of DNA is compared with reference database in DNA barcoding technique (Hajibabaei et al. 2007). In DNA barcoding, the genomic regions that are mostly used are 12s rRNA, 16s rRNA, Cytb and COI consisting on 600-1200 bp (Hebert et al. 2003). The Cytochrome Oxidase I is reliable and efficient in most animal group as compared to other markers (Nagy et al. 2012). For taxonomical classification and phylogenetic relationship molecular markers and DNA sequencing are

included among the good markers. The laboratorial diagnostic protocols efficiency has been improved significantly by PCR technique as it allows the in vitro the amplification of huge amount of DNA by using a particular genomic region as template. Since in this technique only small amount of DNA is required this technique is particular used for the identification of forensic samples, museum specimens and also in archeological remains (Perl et al. 2014). Keeping in view above mentioned problem, this study was therefore planned to find out phylogenetic relationship among different species of pigeon captured from Kasur.

Materials and Methods

Sample Collection and Morphological Analysis

This research was conducted to determine the genetic diversity and phylogenetic relationship among different pigeon species including wild and domestic pigeon. The pigeon species belonging to the family Columbidae were captured from selected sites of district Kasur. The species was selected on the basis of phenotypic characteristics for the molecular analysis. The captured specimens of each species (n=5) was brought to Avian Conservation and Research Centre (ACRC), Department of Wildlife and Ecology, UVAS, Ravi Campus. The specimens of each species were kept in nest separately having dimensions of 8ft×4ft×12ft (length, width and height) for acclimatization.

DNA Extraction

The total genomic DNA was extracted using phenol chloroform and through salt extraction methods. The blood samples were collected in EDTA tubes from the brachial vein of specimens. After that blood samples were transfer into a 1.5 ml Eppendorf tube containing 600 µl of TNES buffer (5ml of 1M Tris, pH-8.0, 8 ml of 5M NaCl, 20ml of 0.5M ethylene Diamine Tetra Acetic Acid [EDTA], pH-8.0, 5 ml 10% Sodium Dodecyl Sulfate [SDS], 62 ml of distilled water) and Proteinase-K at a final concentration of (20 mg/ml). Then blood samples were incubated for lysis at 56 ºC for 4 hours. After the lysis step, 166.7 µLof 6 M NaCl was added and samples were vigorously vortexed to homogenize the lysate and centrifuged at 12000 rpm for 10 minutes. Subsequently, 50 ml of 2M NaCl and an same volume of cold 100% ethanol were used to precipitate the genomic DNA. The DNA pellet was washed in 70% ethanol (chilled at -20 °C for 24 hours) and re-suspended in 100–500 µLof TE buffer or Tris-EDTA (1 M Tris of 5 ml, pH-8.0, 0.5 M EDTA of 1 ml, pH-8.0) or sterile water.

Gel Electrophoresis

The quality of DNA was checked through gel electrophoresis. 1% agrose gel was prepared by adding 1g of agrose in 100 ml of 1X TBE buffer. The solution was heated on a hot plate and a magnetic stirrer was used to mix it. The solution was allowed to cool down and until the solution is clear and the agarose has entirely dissolved. Ethidium bromide was used to stain the gel. The comb was inserted into the gel casting tray and the mixture was poured into the tray to solidify. The comb was carefully removed when the gel was solidified and the casting tray was placed into the gel electrophoresis device or gel tank. The 2 µL of loading dye and 1.5 µL of genomic DNA were well mixed before being gently injected into the gel's wells. Electrophoresis were processed at 110 volts for 20-25 minutes. The 1kb ladder was loaded in one well as a standard to compare the DNA bands. After the completion of gel electrophoresis, the gel is seen under the UV trans-illuminator for visualization of DNA bands.

DNA Quantification

DNA quantification was done using Nanodrop One using the formula. The absorbance of the extracted DNA was measured at wavelengths of 260 nm and 280nm (A260DF50) /1000 = DNA concentration (g/L). The A260/A280 ratio was used to determine the DNA quality. The quality ratio of all genomic DNA extracted from three replicates for each sample ranges from 1.7 to 2.0.

PCR Amplification

The total genomic DNA was amplified using COI primer set. Primer pair mentioned in Table 1. Primer pair was ordered from Macrogen, Korea.

Table 1: The detail of primer pair used for amplification of COI region.

Primers	Primer sequences	Annealing temperature (°C)	Product size
BirdF1	TTCTCCAACCACAAAGACATTGGCAC	54-55	760bp
BirdR1	ACGTGGGAGATAATTCCAAATCCTGG		

Primer dilution

To make the primers dilute, 220µl of DNAase free water was added into the primer and vortexed. Then centrifuged for 10 minutes and stored at 4°C. After that 12µl of primer was taken from stock solution and diluted with 90µl of injection water as working solution. Primer was vortexed before PCR amplification.

Primer Optimization

By taking the average temperature of forward and reverse primer, the temperature of forward primer was 60°C and reverse primer was 59°C. The average temperature was 58°C and 2°C less was 56°C. The PCR was performed at 56°C to check whether it was the optimum temperature for amplification or not. There was no annealing of primers at 56°C. Then PCR was performed 2°C down at 54°C and optimum temperature was attained.

PCR Conditions

A BioRAD thermal cycler was used for PCR amplification. PCR amplification was performed in 25µL volume reaction with 3-5µl of DNA. The PCR amplification consist of 95 °C for 5 minutes; 35 cycles at 95 °C for 1 minute, 54 °C for 1 minute, 72 °C for 1 minute; and a final 10 minute at 72 °C. PCR products were checked through 1.2% agarose gel (Bio-Rad apparatus). Sanger sequencing was done of all the DNA samples on I3730XL DNA Analyzer from Korea according to standard protocols.

Data analysis

The newly produced DNA sequences were checked in BioEdit 7.5 and sequences were aligned in Clustal X. All the DNA sequences were exposed to BLAST searches and closely matched sequences from the GenBank were downloaded and incorporated in the NJ tree analysis. Neighbor-joining tree was constructed in MEGA 10.0 using 100 bootstrap replicates. Within and between species, genetic distances were calculated using MEGA 10 based on p-distance.

RESULTS

The present study was performed from July to December, 2021 at Postgraduate Laboratory, Department of Wildlife and Ecology, UVAS Ravi Campus, Pattoki.

Sampling and identification

The pigeons were captured from selected sites of district Kasur, Punjab (Figure 1). The description of captured specimens is as follows;

Family Columbidae (*Columba livia*)

Pigeons belong to the family Columbidae, a surviving family within the order Columbiformes. This order is divided into two subfamilies: Columbidae and Raphidae. The Columbidae subfamily currently includes over 309 species of pigeons and doves. Pigeons are commonly found as feral or street birds and appear in various colors, such as gray, blue, brown, and predominantly white. They exhibit slight differences in habitat preferences, feeding habits, and vocalizations. Pigeons have significant taxonomic diversity and are widely distributed geographically. However, their populations are decreasing due to human activities. In Pakistan, approximately fifteen species from the family Columbidae have been documented, inhabiting a variety of habitats.

Pigeons are kept and bred for many purposes, including meat production in the form of squabs, as well as for exhibition as fancy and ornamental birds, for flying competitions, and sports like racing. They are also used in laboratory experiments, particularly in cognitive science research. The domestic pigeon (Columba livia domestica), commonly found worldwide, is descended from the rock pigeon. These birds are native to South Asia, North Africa, Europe, and the Middle East. *Columba livia* is widely regarded as an excellent model organism for studies in ecology, genetics, physiology, behavior, and anatomical diversity.

DNA Extraction

The purity of DNA was tested through agarose gel electrophoresis (Figure 3 and 4) and quantification was done through NanoDrop-ONE (Table 1).

Amplification and sequencing

DNA of specimens were amplified using primer set cited in Table 1. 25µL reaction mixture was used in PCR amplification. 8µL DDW, I µL (25 mM) forward Primer and 1µL (25 mM) reserve primer, 14 µL PCR master mix and 1uL of DNA template was added. The following steps were performed for the amplification of COI gene, 3 minutes denaturing at 95°C for 2 minutes followed by 35cycles of for

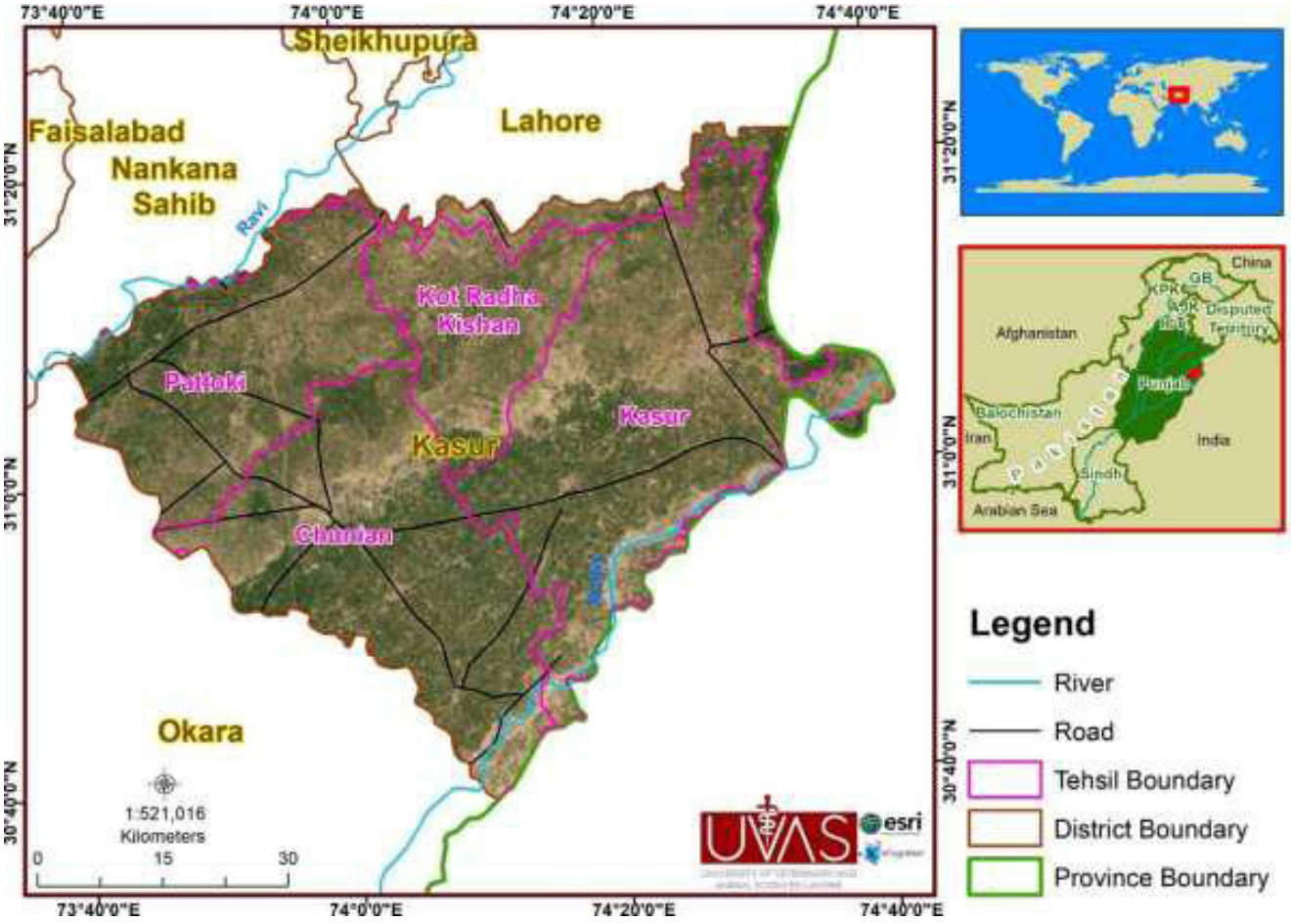

Fig. 1: GIS map of the study area.

30 sec at 95°C, primer annealing for 30 sec at 50°C and elongation for 1 minutes at 72°C, with final 10 minutes at 72°C and infinity at 4°C. PCR products were checked on 1.2% agarose gel and purification was done using Qiagen purification kit according to manufacturer's instruction. All the DNA samples were Sanger sequenced on 13730XL DNA Analyzer from Korea. The newly obtained DNA sequences were submitted to Genbank and accession numbers were received. The details of voucher specimens and accession numbers are given in Table 2. Clear species identification of all the captured specimens were shown in the obtained DNA sequences. The obtained COI fragments were 675 bp after trimming the ambiguous bases.

Figure 2: (a) Wild pigeon (b) Domestic pigeon.

Phylogenetic analysis

Recently few DNA barcoding studies of birds have been carried out and sequences for related species were available at NCBI. Closely aligned sequences of genus Columba were recovered from NCBI in blast searches and incorporated in N-J tree. Neighbour-joining tree of genus Columba was constructed based on p-distance using MEGA 10.0 (Figure 5).

Genetic diversity and variation

Table 4 summarizes the genetic divergence of *Columba livia* based on p-distance. The overall, genetic divergence of *Columba livia* was 0.003±0.001. Table 5 summarizes the genetic divergence of *Columba rupestris* based on p-distance. The overall, genetic divergence of *Columba rupestris* was 0.014±0.004. Table 6 summarizes the genetic divergence of *Columba palumbus* based on p-distance. The overall, genetic divergence of *Columba palumbus* was 0.043±0.006.

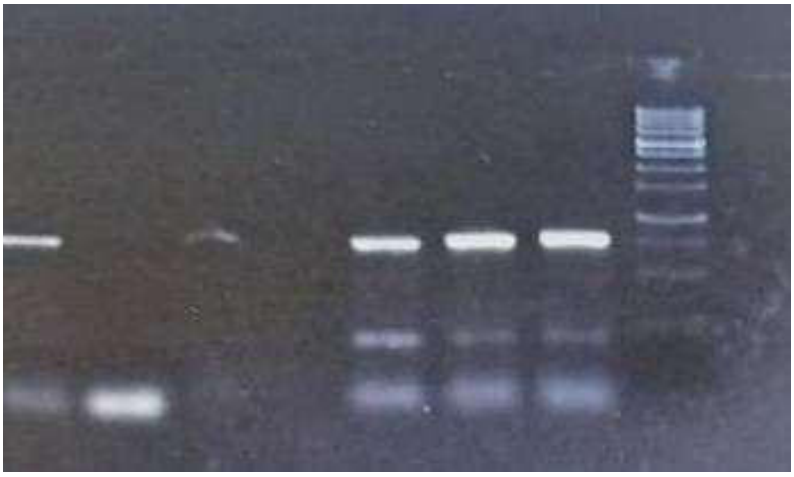

Fig. 3: Successfully amplified DNA samples of wild pigeon

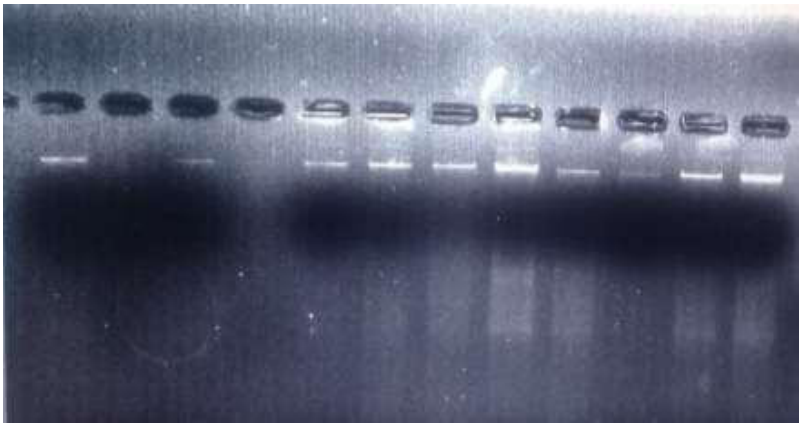

Fig. 4: Successfully amplified DNA samples of domestic pigeon.

Table 2: Quantification of extracted DNA samples using NanoDrop.

Spec ies	Nucleic Acid(ng/µL)	A260/A280	A260/A230	A260 60	A260 80	Nucleic Acid Fact or	Baseline Correction (nm)	Baseline Absorbance	Corrected (ng/µL)	Corrected %CV
Columba livia										
1	468.781	1.576	0.983	9.8 65	5.7 65	50	340	0.497	258.87	4.49
2	467.501	1.618	0.934	9.3 72	5.7 91	50	340	0.509	259.45	4.51
3	467.401	1.561	0.823	9.1 73	5.3 91	49	338	0.499	258.99	4.48
4	467.987	1.698	0.912	9.2 83	5.5 87	50	337	0.532	259.11	4.52

Table 3: The details of successful amplified DNA samples and Genbank accession numbers.

Family	Order	Genus	Scientific Name	Common Name	Voucher No.	Accession No.
Columbidae	Columbiformes	Columba	Columba livia	Rock pigeon	ACRCUVAS1	OL774678
					ACRCUVAS2	OL778850
					ACRCUVAS3	OL778945
					ACRCUVAS4	OL780846

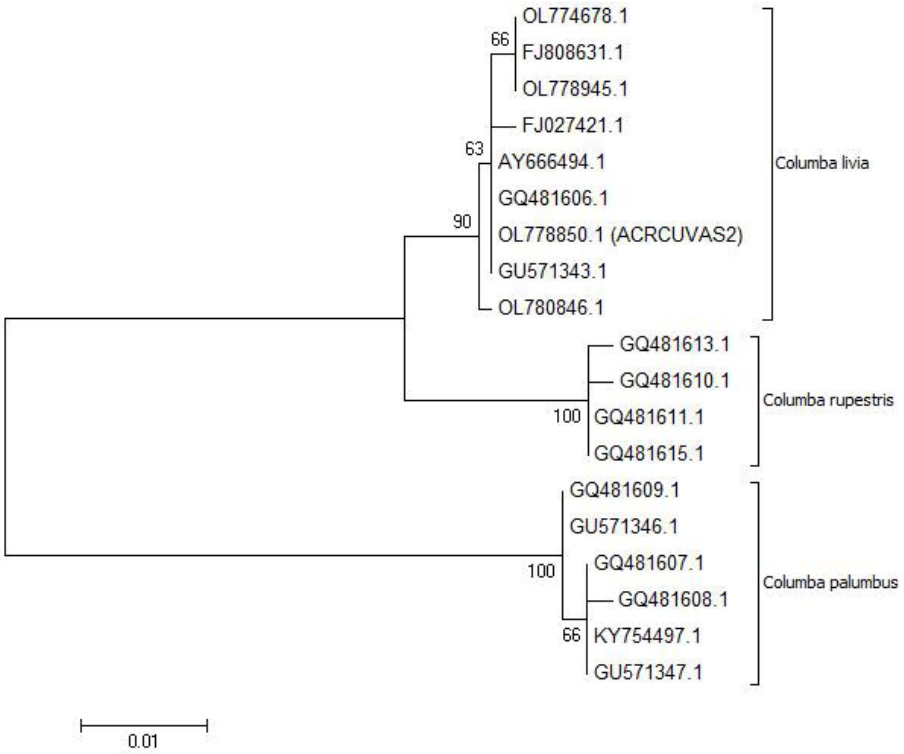

Figure 5: N-J tree of genus Columba based on p-distance.

Table 4: Interspecific genetic identities of *Columba livia* calculated based on p-distance.

	Species	*Columba livia*					*Columba livia* voucher ACRCUVAS1-4			
		FJ027421.1	GU571343.1	GQ481606.1	AY666494.1	GQ481605.1	OL774678.1	OL780846.1	OL778850.1	OL778945.1
Columba livia	FJ027421.1	ID								
	GU571343.1	0.985	ID							
	GQ481606.1	0.997	0.988	ID						
	AY666494.1	0.997	0.988	1	ID					
	GQ481605.1	0.994	0.985	0.997	0.997	ID				
Columba livia	OL774678.1	0.973	0.988	0.976	0.976	0.973	ID			
	OL780846.1	0.983	0.998	0.986	0.986	0.983	0.986	ID		
	OL778850.1	0.755	0.768	0.757	0.757	0.754	0.775	0.767	ID	
	OL778945.1	0.754	0.762	0.756	0.756	0.753	0.772	0.761	0.992	ID

Table 5: Interspecific genetic identities of *Columba rupestris* and *Columba livia* calculated based on p-distance.

	Species	*Columba rupestris*				*Columba livia*			
		GQ481613.1	GQ481610.1	GQ481611.1	GQ481615.1	OL778850.1	OL778945.1	OL774678.1	OL780846.1
Columba rupestris	GQ481613.1	ID							
	GQ481610.1	0.996	ID						
	GQ481611.1	0.998	0.998	ID					
livia	GQ481615.1	0.996	0.996	0.998	ID				
	OL778850.1	0.755	0.755	0.757	0.757	ID			
	OL778945.1	0.752	0.752	0.754	0.754	0.996	ID		
Columba	OL774678.1	0.975	0.975	0.977	0.975	figire	0.772	ID	
	OL780846.1	0.978	0.978	0.98	0.978	0.772	0.769	0.996	

Table 6: Interspecific genetic identities of *Columba palumbus* and *Columba livia* calculated based on p-distance.

		Columba palumbus											
Species		GQ481607.1	GQ481608.1	MN122869.1	KY754497.1	GQ481609.1	GU571347.1	GU571346.1	EF012603.1	OL778850.1	OL778945.1	OL774678.1	OL780846.1
Columba palumbus	GQ481607.1	ID											
	GQ481608.1	0.998	ID										
	MN122869.1	1	0.998	ID									
	KY754497.1	1	0.998	1	ID								
	GQ481609.1	0.998	0.996	0.998	0.998	ID							
	GU571347.1	1	0.998	1	1	0.998	ID						
	GU571346.1	0.998	0.996	0.998	0.998	1	0.998	ID					
	EF012603.1	0.925	0.924	0.925	0.925	0.927	0.925	0.927	ID				
livia Columba	OL778850.1	0.708	0.707	0.708	0.708	0.71	0.708	0.71	0.765	ID			
	OL778945.1	0.705	0.704	0.705	0.705	0.707	0.705	0.707	0.762	0.996	ID		
	OL774678.1	0.913	0.911	0.913	0.913	0.915	0.913	0.915	0.849	0.772	0.772	ID	
	OL780846.1	0.913	0.911	0.913	0.913	0.915	0.913	0.915	0.849	0.772	0.769	0.996	ID

DISCUSSION

The study's results highlighted the effectiveness of COI barcodes in identifying species, as each species was clearly differentiated into distinct clades on the phylogenetic tree. The structure of the data seems to align with the theory of complete or significant isolation between breeds. Nevertheless, Columba livia, being a widespread species across various countries and commonly bred for both meat and recreational purposes, is unlikely to exhibit such total breed isolation as commonly thought. Although there have been only a few recent DNA barcoding studies focused on birds, related species sequences were available in the NCBI database. Sequences closely related to Columba were recovered from NCBI through BLAST searches and incorporated into the Neighbor-Joining (N-J) tree. The N-J tree for the Columba genus was generated based on p-distance using MEGA 10.0 software. Further phylogenetic studies and molecular dating at the species level may offer more insights into the historical biogeography of Columbiformes and the impact of key paleoevents on their diversification.

Commonly used mtDNA genes for barcoding includes COI, Cytb, 12S rRNA, and 16S rRNA. Among these, Cytochrome C Oxidase I has been identified as a distinct genetic marker for animals. However, due to difficulties in amplifying and evaluating COI sequences in birds, researchers have increasingly turned to the 16S ribosomal RNA gene as a preferred option for DNA barcoding. By DNA barcoding analysis we can genetically identified a species by comparing short mitochondrial fragments and for taxonomical studies it is one of the reliable technique (Hebert et al. 2003). Although DNA barcoding is a powerful tool but one should be cautious before using it because if sequence were generated from misidentified specimens it can lead to misinterpretations. For detailed taxonomical studies molecular genetics provide large amount of information. Until 2010 in global DNA barcoding initiative the amphibians and reptiles COI sequence were not produced but in phylogenetic analysis COI was used as a genetic marker. For phylogenetic analysis in genbank COI sequences were obtained by using ND1 and ND2 partial or full genes , intervening tRNAs and COI gene 5¢ terminus short section of (100–200 bp).

Birds like pigeons are among of nature's most sophisticated and intellectual species. Pigeons (Columbiformes) may be seen in the almost every city and town on the earth (Marques *et al.,* 2007). In addition, being a fantastic place to lounge about and reproduce, it is also a major food source. Humans utilize them for food, recreation, and even scientific research. This clever bird species has only been utilized for sport, as a food source, and as a messenger by human beings (Sari *et al.,* 2008). As a consequence of human overexploitation, the Passenger Pigeon (*Ectopistes migratorius*) was extinct in North America within a few decades. This precipitous decrease is likely one of the most well-known and recorded extinctions in the history of any species, and it serves as a sobering warning of the influence humans may have on the environment. At 1914, according to Schorger (1955) and Blockstein (2002), the last Passenger Pigeon was killed in a Cincinnati Zoo, where Schorger and Blockstein documented the bird's end. The Passenger Pigeon's natural history has been carefully recorded (Blockstein, 2002).

From the reviewed papers, it was evident that the COI gene were extensively applied in the bird's molecular identification, with studies ranging from resolving complex taxonomic phylogenetic relationships to identifying roadkill remains and fertile hybrids within wild populations. The primers most commonly used for this purpose are

those developed by the group that introduced and popularized DNA barcoding as a species identification method. These primers are likely favored because they were among the first designed to amplify and explore COI gene diversity across a broad range of bird orders next to the term "DNA barcode" was first introduced. When searching for barcoding primers, older and classic publications often provide the preferred options, which were also the case in my study (Hebert *et al.,* 2004).

While looking for barcoding primers, older or classic publications tend to be more commonly chosen, as was the case in my study. Maximum of these primers target the first 800 bp of the COI gene that corresponds to the widely recognized barcode region. This region contains sufficient nucleotide variation to be effectively used for molecular identification (Hebert *et al.,* 2003a, b).

Although the barcode region of the COI gene is commonly used for molecular identification of birds, it has demonstrated greater intraspecific diversity compared to interspecific diversity in groups with recent divergence, such as the genus Sporophila within the family Thraupidae. In such cases, other genetic barcode regions should be considered. The barcode region amplified by the primers discussed in this study, particularly the optimal subsets, can be further assessed for their effectiveness in taxonomic resolution across various levels of molecular identification (Hebert *et al.,* 2004; Tavares *et al.,* 2011).

Previous studies worldwide have shown that DNA extraction from tissue samples is more cost-effective and provides higher quality DNA compared to non-invasive methods. Animals living in the in close proximity to humans, may serve as major reservoirs for human pathogens. We observed pigeons (Columba livia) captured in urban areas of Madrid to assess the occurrence of three pathogens that are known to cause human diseases. The Neighbor-Joining (NJ) tree, constructed using mitochondrial COI gene analysis, indicated a close genetic similarity between *Spilopelia senegalensis* and *Streptopelia decaocta,* a relationship also noted by Wayne (1992). Additionally, the tree showed a strong phylogenetic connection between *Columba livia livia* and *Columba eversmanni*, suggesting a shared ancestral lineage. Teresa and Zbigniew (2009) similarly found a phylogenetic relationship between Streptopelia chinensis and Columba eversmanni.

Depending upon the percentage divergence of sequence within and among species using a DNA barcode enables to group individual to its conspecific. DNA barcoding helps in species identification and facilitates measure of biodiversity by using genomic sequences, mostly from the mitochondrial genome. COI based DNA barcoding is easiest and authentic tool for identifying most amphibians and non-avian reptiles. In DNA barcoding a short segment of a gene is taken from mitochondrial DNA region and is then used to identify species and their parts. DNA regions that show no or little intra- species variations and considerable interspecies variations are used in barcoding. DNA barcoding is authentic tool for quick and accurate verification of species and can also be used for screening of species. The 5' region of mitochondrial DNA (mtDNA) gene fragment is proved to be a universal marker for this purpose. Identification of species with high success rate can be performed using threshold method of analysis, BLOG techniques and tree-based analysis. In studying biodiversity measurements and ecological assessments including the identification of organisms at large scale, this technique has significant potential for discovering undescribed cryptic species.

DNA barcoding's effectiveness as a species identification tool can only be truly evaluated in groups with well-established taxonomies. While this holds for vertebrates, it is less consistent for many invertebrate groups. However, achieving over 90% species resolution using a single genetic marker is impressive and provides a cheap approach for biodiversity screening. The species resolution was higher (94%) when using the uniqueness of Neighbor-Joining (NJ) clusters, or monophyly, as a delimitation criterion, compared to the 89% threshold genetic distance suggested by Hebert et al. (2004). These results are consistent with recent research on DNA barcoding in closely related bird species.

REFERENCES

Aliabadian, M., Kaboli, M., Nijman, V., and Vences, M. (2009). Molecular identification of birds: performance of distance-based DNA barcoding in three genes to delimit parapatric species. *PLoS One,* 4(1): 4119-4127

Blechman, A. D. (2007). Pigeons: The Fascinating Saga of the World's Most

Claramunt, S., and Cracraft, J. (2015). A new time tree reveals Earth history's imprint on the evolution of modern birds. Science Advances, 1(11): 1-13.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R. (1994). "DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates". Molecular Marine Biology and Biotechnology, 3(5): 294–299.

Gibbs, D., Barnes, E., and Cox, J. (2001). Pigeons and doves: a guide to the pigeons and doves of the World. The Auk, 118(4): 1117-1118.

Gilbert, M. T. P., and Shapiro, M. D. (2014). Pigeons: domestication. Encyclopedia of global archaeology, 1382-1384. DOI 10.1007/978-1-4419-0465-2

Hebert, P. D. N., Stoeckle, M. Y., Zemlak, T. S., and Francis, C. M. (2004).

Hebert, P. D. N., Cywinska, A., Ball, S. L., and De Waard, J. R. (2003a). Biological identifications through DNA barcodes. Proceedings of the Royal Society: Biological Sciences, 270(1512): 313–321.

Hebert, P. D. N., Ratnasingham, S., Jeremy, D. and Waard, D. (2003b). Barcoding animal life cytochrome oxidase subunit 1 divergences among closely related species. Proceedings of Royal Society of London: Biological Sciences, 270

Hebert, P. D., Cywinska, A., Ball, S. L., and DeWaard, J. R. (2003). Biological identifications through DNA barcodes. Proceedings of Biological Sciences, 270(1512): 313–321.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., and Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. Proceedings of the National Academy of Sciences, 101(41): 14812-14817..

Holt, C., Campbell, M., Keays, D. A., Edelman, N., Kapusta, A., Maclary, E., and Shapiro, M. D. (2018). Improved genome assembly and annotation for the rock pigeon (Columba livia). G3: Genes, Genomes, Genetics, 8(5): 1391-1398.

Johnson, K. P., and Clayton, D. H. (2001). Nuclear and mitochondrial genes contain similar phylogenetic. Signal for pigeons and doves (Aves: Columbiformes: Molecular Phylogenetics and Evolution, 14 (1): 141–151.

Kan, X. Z., Li, X. F., Zhang, L. Q., Chen, L., Qian, C. J., Zhang, X. W., and Wang, L. (2010). Characterization of the complete mitochondrial genome of the Rock pigeon, Columba livia (Columbiformes: Columbidae). Genetics and Molecular Research, 9(2): 1234-1249.

Kerr, K. C., Stoeckle, M. Y., Dove, C. J., Weigt, L. A., Francis, C. M., and Hebert, P. D. (2007). Comprehensive DNA barcode coverage of North American birds. Molecular ecology notes, 7(4): 535-543.

Khaksar, R., Carlson, T., Schaffner, D. W., Ghorashi, M., Best, D., Jandhyala, S., and Amini, S. (2015). Unmasking seafood mislabeling in US markets: DNA barcoding as a unique technology for food authentication and quality control. Food Control, (56): 71-76.

Korzun, L. P., Erard, C., Gasc, J. P., and Dzerzhinsky, F. J. (2008) Bill and hyoid apparatus of pigeons (Columbidae) and sandgrouse (family: Pteroclididae): a

Kress, W. J., Wurdack, K. J., Zimmer, E. A., Weigt, L. A., and Janzen, D. H. (2005). Use of DNA barcodes to identify flowering plants. Proceedings of the National Academy of Sciences, 102(23): 8369-8374.

Levi, W. (1977). The Pigeon Sumter, S.C. Levi Publishing Company: Sumter, S.C.

Meier, R., Shiyang, K., Vaidya, G., and Ng, P. K. (2006). DNA barcoding and taxonomy in Diptera: A tale of high intraspecific variability and low identification success. Systematic Biology, 55(5): 715–728.

Nair, M. V. (2010). Thick-billed Green-Pigeon Treron curvirostra in Similipal Hills, Orissa: an addition to the avifauna of peninsular India. Indian Birds, 6(1): 19– 20.

Pereira, K., Javier, C. G., and Leonard, A. M. (2007). Classification of Columbiformes. Journal of Science, 378 (1269): 1768-1774.

Ramadan, S., Abe, H., Hayano, A., Yamaura, J., Onoda, T., Miyake, T., and Inoue-Murayama, M. (2011). Analysis of genetic diversity of Egyptian pigeon breeds. Journal of Poultry and Science, 48(2): 79-84.

Sarri, C., Stamatis, C., Sarafidou, T., Galara, I., Godosopoulos, V., Kolovos, M., Liakou, C., Tastsoglou, S., and Mamuris, Z. (2014). A food additives and contaminants: part A 505 new set of 16S rRNA universal primers for identification of animal species. Food Control, 43: 35–41.

Shapiro, M. D., Kronenberg, Z., Li, C., Domyan, E. T., Pan, H., Campbell, M., and Wang, J. (2013). Genomic diversity and evolution of the head crest in the rock pigeon. Journal of Advancement of Science, 339(6123): 1063-1067.

Sultana, S., Ali, M. E., Hossain, M. A. M., Naquiah, N., and Zaidul, I. S. M. (2018a). Universal mini-COI barcode for the identification of fish species in processed products. Food Research International, 105: 19–28.

Tadano, R., Sekino, M., Nishibori, M., and Tsudzuki, M. (2007). Microsatellite marker analysis for the genetic relationships among Japanese long-tailed chicken breeds. The Journal of Poultry Science, 86(3): 460-469.

Tavares, E. S., Goncalves, P. F. M., Miyaki, C. Y., and Baker, A. J. (2011). DNA barcode detects high genetic structure within neotropical bird species. PLoS One, 6(12): 28543-28549.

Teresa, T., and Zbigniew, M.B., (2009). A Key for the Identification of Domestic Bird Bones in Europe: Galliformes and Columbiformes. 6th Ed, Institute of Systematics and Evolution of Animals, Polish Academy of Science: 58-61.

Ward, R. D., Zemlak, T. S., Innes, B. H., Last, P. R., and Hebert, P. D. (2005). DNA barcoding Australia's fish species. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1462): 1847-1857.

Wayne RK (1992). On the use of morphologic and molecular genetic characters to investigate species status. Conservation Biology, 6 (4): 590-592.