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Hina Rafiq

Institute of Natural and Management Sciences (INAM), Rawalpindi, hinafaizan0@gmail.com

Fakhar -i-Abbas

Centre for Bioresource Research (CBR), Islamabad, Pakistan, syed.abbas@wright.edu

Fakhra Nazir

Institute of Natural and Management Sciences (INAM), Rawalpindi, Pakistan

Fida Muhammad Khan

Centre for Bioresource Research (CBR), Islamabad, Pakistan

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MOLECULAR TAXONOMY AND PHYLOGENETIC ANALYSIS OF DOVE AND PIGEON SPECIES (AVES: COLUMBIDAE) OF PAKISTAN, BASED ON COI REGION OF MITOCHONDRIAL DNA

HINA RAFIQ¹, FAKHAR-I-ABBAS², FAKHRA NAZIR^{1*} AND FIDA MUHAMMAD KHAN²

¹*Institute of Natural and Management Sciences (INAM), Rawalpindi, Pakistan*

²*Centre for Bioresource research (CBR), Islamabad, Pakistan*

*Corresponding author: fakhra.979.nazir@gmail.com

ABSTRACT

Doves and Pigeons are the members of living family Columbidae (Order: Columbiformes) having a wide range of taxonomic diversity and geographic distribution. Seven species with one sample each of family Columbidae were collected via random sampling from different districts of Pakistan to carry out this study. The targeted gene region was sequenced and identified by using BLAST tool at National Center for Biotechnology Information (NCBI). CLUSTALW was used for sequence alignment and MEGA6 for reconstruction of phylogenetic trees to predict the effective ancestry of different Columbidae species. The following phylogenetic trees were obtained i.e. Maximum Likelihood tree, Neighborhood joining tree, Maximum parsimony tree and UPGMA tree. In the current study, COI gene barcoding and phylogenetic analysis of family Columbidae gave results of multiple alignment which showed that *Columba livia livia* and *Columba eversmanni*, closely resembled as well as *Spilopelia senegalensis* and *Streptopelia decaocta*. While *Streptopelia tranquebarica* and *Spilopelia chinensis* have great affinity due to small clade difference and *Treron phoenicoptera* was distinctly related to other species due to large clade difference.

Key words: Aves, Columbidae, COI gene, molecular taxonomy, phylogenetic analysis

INTRODUCTION

Doves and Pigeons are the members of family Columbidae a living family of order Columbiformes (Farner and Ziswiler 1972). The Columbiformes are bisected into Columbidae subfamily and the Raphidae subfamily (Pereira et al., 2007). The subfamily Columbidae is entitled currently by over 309 species of pigeons and doves in living status (Robert, 1992). Dove is a peaceful and soft looking smart bird while a pigeon is a feral and street bird that can be found in gray, blue, brown and mostly white color. They have slight differences in their habitat preferences, feeding lifestyles as well as their songs and calls (Johnson and Clayton, 2001).

Pigeons and doves have a wide range of taxonomic diversity and geographic distribution (Crome, 1991). However, their population is declining due to anthropogenic activities. In Pakistan almost fifteen species of family Columbidae have been reported which are found in every kind of environment (Robert, 1992).

Traditional taxonomic identification based on morphological characters has many shortcomings. These include wrong identification of species as a result of genotype fluctuation, physical appearance in characters and dominating complications in sorting authentic character cryptic tax owing to extensive maturity duration etc. (Lazcono et al., 1988).

To reanimate traditional taxonomical studies and to decrease

taxonomic disasters, a substitute and complimentary access have been flourished together with molecular taxonomy (Jae-Heup et al., 2001), information technology and increased utilization of cyber tools (Abd-El salam, 2003). Among them, DNA barcoding has been particularly useful in the description and detection of new species from distinct groups and subgroups on the basis of authenticity, ease and affordable measures (Hebert et al., 2003a; Kerr et al., 2009; Tobe et al., 2010). The main goal of DNA barcoding to match the barcodes of identified or an unidentified specimen to establish and construct online libraries that can assist as a standard for well-known species of barcode arrangements (Rubinoff et al., 2006).

In DNA barcoding, COI gene may be utilized to identify animal species. Mitochondrial COI gene sequence is considered suitable for this purpose, by using rapid mutation rate to differentiate closely related species and due to conserved sequences in conspecifics (Roe and Sperling, 2007). COI has a special characteristic that it evolves much more slowly than other mtDNA genes (Verboom et al., 2009). This evolution rate variation has an important practical application to design universal primers, that may effectively be used as the basis for a comprehensive DNA description system for animal kingdom (Hebert et al., 2003b).

For genetic analysis different bioinformatic tools (FASTA, BLAST, ClustalW and MEGA6) are used. The phylogenetic study is possible with the help of these tools which are being used since the last two decades. For genomic analysis, like for the search of sequence similarity, BLAST and FASTA are used. ClustalW is used for multiple sequence alignment and phylogenetic analysis, while MEGA6 software is used for the sequence alignments to determine the evolutionary history and for

constructing phylogenetic trees (Altschul, 1997; Kim et al., 1999; Tamura et al., 2013).

The current study was designed to fulfill the objectives of finding genetic distance among the members of family Columbidae, compare their phylogenetic position based on ranking and determine major phases of diversification among different species of family Columbidae collected from different regions of Pakistan. It is the first study about molecular taxonomy and phylogenetic analysis of family Columbidae from Pakistan up to our knowledge.

MATERIALS AND METHODS

Sample Collection

The samples of seven species of family Columbidae were collected via random sampling from five districts of Punjab, one each from Islamabad and Nowshera (KPK) to carry out this study. Each sample was labeled properly along with sample number, locations and GPS coordinates by using GPS meter, Garmin eTrex 30x, Kansas, US (Table 2). Samples were collected in summer and spring season (2017). For detailed comparative analysis the samples were taken from keel tissues and preserved in plastic bottles containing 70% ethanol solution at room temperature (25-28°C). All samples were ethically collected under the set guidelines by the Centre for Bioresource Research (CBR), Islamabad. The current research study was accomplished at Molecular Ecology Laboratory of Centre for Bioresource Research (CBR), Islamabad.

Morphological Identification

Each specimen was morphologically identified via classical taxonomy by observing the features such as spindle shaped body and its size (Robert, 1992). The head, neck, alignment, colors of feathers and size

of tail are used as keys for identification (Andrew, 2007).

DNA Extraction

The seven samples were processed to extract DNA for subsequent analysis for getting the gene sequences. The chloroform/phenol protocol defined by Sambrook and David (2001) with some modifications was used for DNA extraction.

DNA Quantification

DNA quantification was carried out through spectrophotometer (584A Diod Array Spectrophotometer, Hewlett-Packard, USA). The extracted DNA solution absorbance was recorded at the value having a wavelength of 260nm and 280 nm using following formula (Rossella et al., 2009).

$$\text{DNA concentration } (\mu\text{g}/\mu\text{L}) = (\text{A}_{260} \times \text{DF} \times 50) / 1000$$

The DNA quality was assessed by measuring A260/A280 ratio. For genomic DNA, the quality ratio ranges from 1.7 to 2.0 of all DNA extracted from three replicates for each sample. Purified DNA was quantified by measuring the wavelength ratio of A260. For genomic DNA the quantity ratio ranged from 500 to 3000 ng / μL of extracted genomic DNA samples.

Polymerase Chain Reaction (PCR)

After confirmation of DNA, the samples were subjected to amplification of COI region of mitochondrial genome of Columbidae species by using PCR technique. A pair of primers (BirdF1/BirdR1) was selected for the amplification of COI region of DNA (Ivanova et al., 2006). Primer pair was supplied by Oligo, MACRO GEN (Seoul, Republic of Korea). The product size along with annealing temperature of primer is listed in Table 1.

Table 1: Primer pair used for amplification of COI region

Sr. No.	Primers	Primer sequence (5' to 3')	Annealing temperature (°C)	Product size
1	BirdF1	TTCTCCAACCACAAAGACATTGGCAC	60	756bp
2	BirdR1	ACGTGGGAGATAATTCCAAATCCTGG	59.6	

DNA Sequencing and Analysis

After confirmation of amplification, PCR products were sequenced by using Sanger's method (Sanger et al., 1977). The sequence analysis was done by Bio Edit software Version 7.0.2 (Taraan, 1997), to convert them into FASTA format. The sequences with fine peaks were selected for further analysis while sequences with noise in peaks were sequenced again. After proofreading, the peaks were subjected to BLAST at National Centre for Biotechnology Information (NCBI) Gene Bank portal to

identify the samples of these sequences under study.

Molecular Taxonomy

The sequence alignment was carried out with sequences already available on NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to identify specimens on genetic basis. BLAST result of DNA barcode sequence of seven species of family Columbidae showed the sequence that matches with the isolate cytochrome oxidase subunit I (COI) gene.

The results having sequence ID, accession numbers, range, score, identities, gaps and lengths are listed in Table 3.

Phylogenetic Analysis

MEGA6 (Tamura et al., 2013) and CLUSTAL W (Thompson et al., 1994) softwares were used for sequence alignment, and reconstruction of phylogenetic trees.

i. Sequence Alignment

The sequence alignment of COI genes for seven specimens of family Columbidae were aligned by using CLUSTAL W bioinformatics tool (Li, 2003). Results are given in Figure 3.

ii. Reconstruction of Phylogenetic Tree

MEGA6 was used to draw the phylogenetic tree for the available nucleotide

sequences of family Columbidae to exploit the resolution power of each gene for predicting the effective ancestry of different Columbidae species. The different aligned sequences were entered in the phylogeny option of the MEGA6 and phylogenetic trees were obtained i.e. Maximum likelihood tree, neighborhood joining tree, maximum parsimony tree and UPGMA tree based on different methods which helped to analyze the phylogenetic relations among different Columbidae species.

RESULTS

In the current research, out of fifteen reported species of family Columbidae in Pakistan, seven species were collected from various locations and were studied on both morphological and molecular basis (Table 2).

Table 2: GPS co-ordinates of samples along with their area of collection

Sample No.	Location	District	Province	GPS Coordinates		
				Latitude (N)	Longitude (E)	Altitude
S1	Kund Park	Nowshehra	KPK	33°55'43.32"	72°14'5.28"	277m
S2	Wah Cantt	Attock	Punjab	33°45'6.48"	72°39'43.56"	478m
S3	Narri	Khushab	Punjab	32°29'3.88"	72°23'43.37"	201m
S4	Nakha	Chakwal	Punjab	33°45'6.48"	72°39'43.56"	422m
S5	Balkassar	Chakwal	Punjab	32°56'34.80"	72°39'42.48"	528m
S6	Nilore	Islamabad	Federal area	33°38'37.32"	73°14'56.76"	549m
S7	Bagga	Jehlum	Punjab	32°54'26.28"	73°41'47.76"	226m

Table 3: BLAST analysis of sequences under study

Sample ID	Accession Number	Score (Bits)	Expect	Identities	Gaps	Strand	Length
S1	GU571344.1	1216(658)	0.0	682/694 (98%)	0/694 (0%)	Plus/Plus	722
S2	KU722397.1	922(499)	0.0	500/501 (99%)	0/501 (0%)	Plus/Plus	657
S3	KU946864.1	1280(693)	0.0	693/693 (100%)	0/693 (0%)	Plus/Plus	693
S4	KC439337.1	1131(612)	0.0	614/615 (99%)	0/615 (0%)	Plus/Plus	612
S5	KU946864.1	1280(693)	0.0	693/693 (100%)	0/693 (0%)	Plus/Plus	693
S6	JQ176292.1	1168(632)	0.0	636/638 (99%)	0/6638 (0%)	Plus/Plus	632
S7	KF446965.1	667 (361)	0.0	383/394 (97%)	0/394 (0%)	Plus/Plus	648

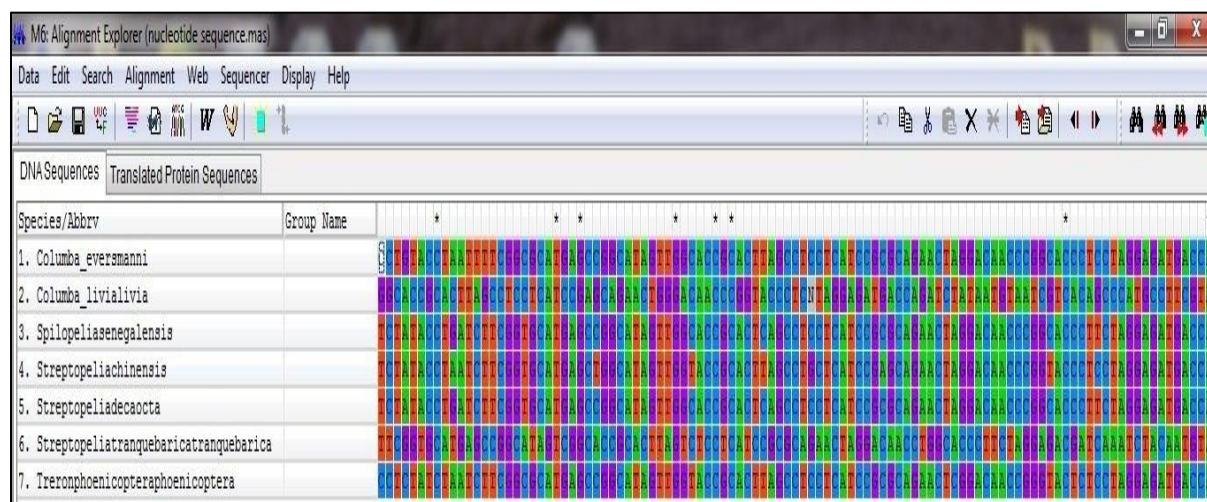


Figure 1: Aligned sequences of COI gene of family Columbidae (seven specimens) by ClustalW.

Phylogenetic Analysis

Phylogenetic relationship was determined using MEGA6 software. The analysis involved seven nucleotide sequences of Columbidae species. Codon positions

included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were, a total of 500 positions in the final dataset. The following phylogenetic trees (Figure 4-7) were constructed by using MEGA6 software.

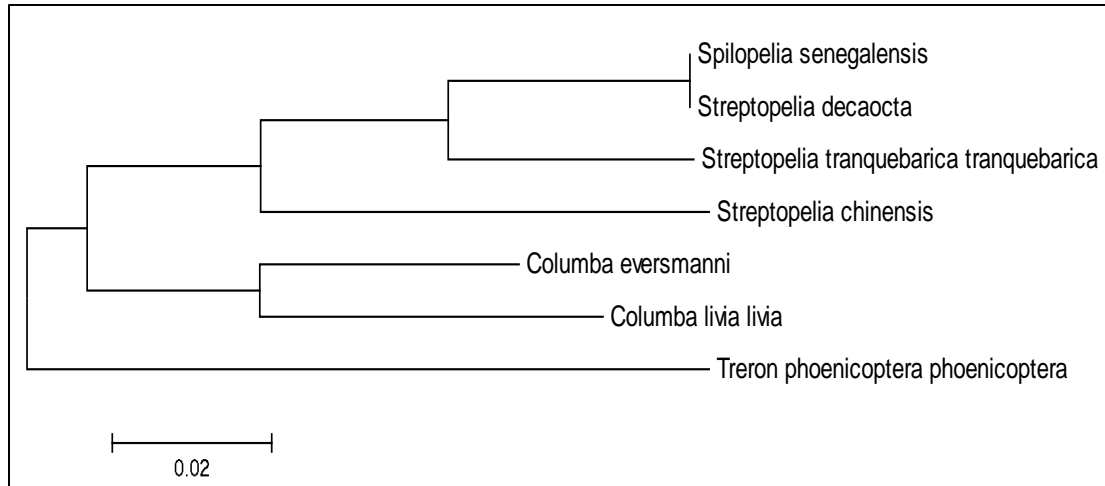


Figure 4: Molecular Phylogenetic analysis by using Maximum Likelihood Method

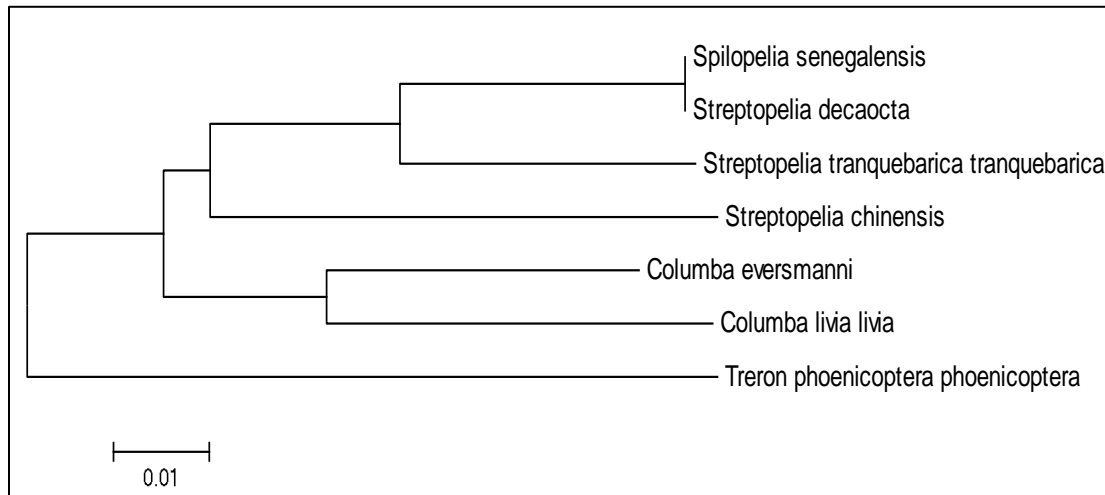


Figure 5: Molecular Phylogenetic analysis by using Neighborhood joining method

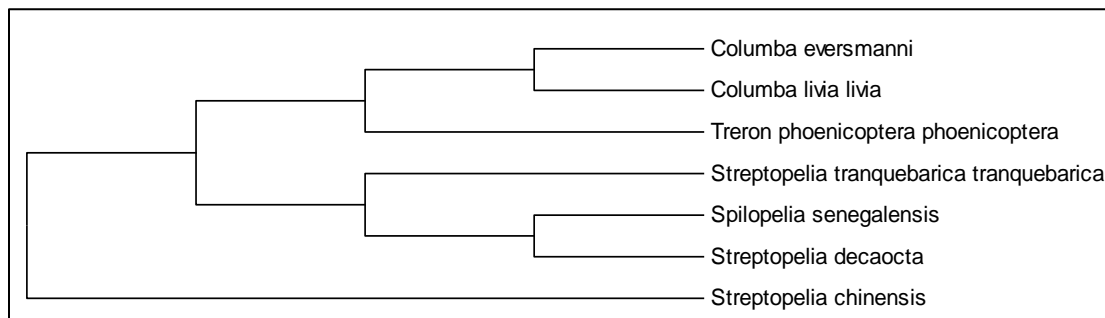


Figure 6: Molecular Phylogenetic analysis by using Maximum Parsimony method

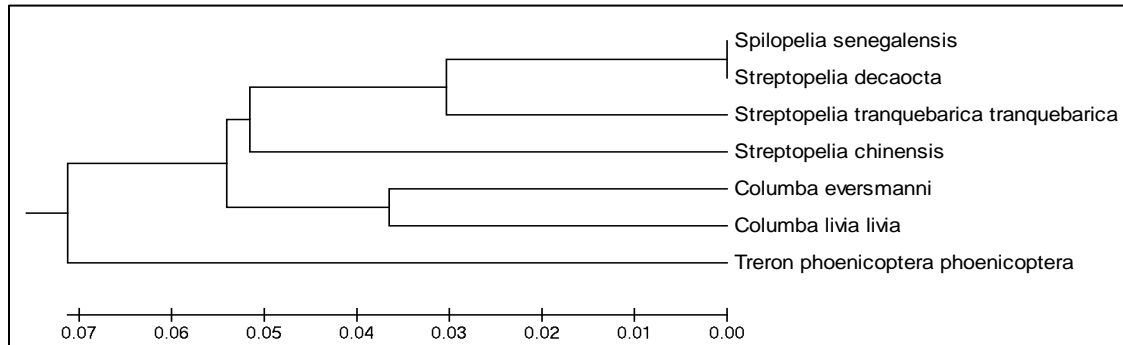


Figure 7: Molecular Phylogenetic analysis by using UPGMA method

DISCUSSION

In the current research study invasive samples (keel tissue) of seven species of family Columbidae were used for DNA extraction and sequencing in order to study the molecular taxonomy and phylogenetic relationships among these species. According to previous researches conducted worldwide, DNA extraction via tissue samples is cheaper and contains good quality of DNA as compared to non-invasive samples (Balasubramanian et al., 2016; Taberlet and Luikart, 1999).

According to the maximum likelihood tree of mitochondrial COI gene, *Streptopelia decaocta*, *Streptopelia tranquebarica tranquebarica* and *Spilopelia senegalensis* were closely related while *Spilopelia chinensis* was distantly related due to large clade distance (Gibbs et al., 2001). However, *Streptopelia decaocta*, *Streptopelia tranquebarica tranquebarica* and *Spilopelia senegalensis* are considered as phylogenetically associated (Andrew, 2007). Similarly, *Columba livia livia* and *Columba eversmanni* have close phylogenetic association due to high affinity, while *Treron phoenicoptera phoenicoptera* showed slightly different position in phylogenetic

tree (Robert, 1992; Paul, 2009; Khan and Arif, 2013).

The neighborhood joining tree of COI genes revealed close association between *Streptopelia decaocta* and *Spilopelia senegalensis*, hence they have same ancestral lineage as compared to *Streptopelia chinensis* and *Streptopelia tranquebarica tranquebarica* as they are far apart due to wider genetic distance. Similarly, *Columba livia livia*, *Columba eversmanni* and *Treron phoenicoptera phoenicoptera* are far apart from the rest of the species due to high genetic distance (Saitou and Nei, 1987).

The maximum parsimony tree constructed in the current study according to mitochondrial COI analysis showed that *Columba livia livia*, *Columba eversmanni* and *Treron phoenicoptera phoenicoptera* were closely related due to least genetic distance. While *Streptopelia decaocta*, *Streptopelia tranquebarica tranquebarica* and *Spilopelia senegalensis* were not closely related so they have least phylogenetic association (Yuri et al., 2013). The phylogenetic tree also revealed the phylogenetic association between *Streptopelia decaocta* and *Streptopelia chinensis* as illustrated by Miller and Hareley (2002).

The UPGMA tree constructed based on mitochondrial COI gene analysis revealed close similarity between *Spilopelia senegalensis* and *Streptopelia decaocta*. Wayne (1992) also determined the close phylogenetic relation between *Spilopelia senegalensis* and *Streptopelia decaocta*. This tree also revealed the close phylogenetic association between *Columba livia livia* and *Columba eversmanni* hence, they have same ancestral line. Teresa and Zbigniew (2009) determined phylogenetic similarity between *Streptopelia chinensis* and *Columba eversmanni*. The UPGMA tree in this experiment also demonstrated some morphological convergence between *Streptopelia chinensis* and *Columba eversmanni*.

One thing was common among all the trees during construction that *Spilopelia senegalensis* and *Treron phoenicoptera* arise from a common ancestor and three clades diverge out in which *Spilopelia senegalensis* and *decaocta* are closely associated. Hence, they are placed in the same clade. Similarly, *Streptopelia tranquebarica tranquebarica* is genetically closer to *Streptopelia chinensis*, so they are placed in a similar clade. The *Columba livia livia* and *Columba eversmanni* have great affinity so they are placed in a common clade. *Treron phoenicoptera phoenicoptera* is least associated and resembled with the rest of the six species because of greater genetic distance and least affinity.

CONCLUSION

Phylogenetic analysis of family Columbidae showed that *Columba livia livia* and *Columba eversmanni*, as well as *Spilopelia senegalensis* and *Streptopelia decaocta* have a close resemblance. While *Streptopelia tranquebarica* and *Spilopelia chinensis* showed great affinity due to small clade differences whereas, *Treron phoenicoptera* showed large clade difference

and was distantly related to other species. To flourish greater authentication related to phylogeny of species these mitochondrial genes should be supplemented along with nuclear barcode. This would diminish the complication of dependency on single character and assist in identifying cases where mitochondrial DNA acts distinctive to nuclear genome.

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