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Genetic Characterization and Identification of Pakistani Avian Fauna through DNA Barcoding

Muhammad Zia ul Haq[1], Sehrish Firyal[1], Aftab Ahmad Anjum[2], Muhammad Wasim[1], Rana Manzoor Ahmad[3], Maryem Hussain[1], Asim Iqbal[1], Agha Wasif Ali Khan[1], Muhammad Tayyab[1] and Ali Raza Awan[1]*

[1]*Institute of Biochemistry and Biotechnology, University of Veterinary and Animal Science, Lahore, Pakistan*
[2]*Institute of Microbiology, University of Veterinary and Animal Science, Lahore, Pakistan*
[3]*Department of Zoology, Government College University, Lahore, Pakistan*

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Authors' Contribution
MZH conducted this research. MW and AAA helped in research work as supervisory committee. MH, AWAK and AI helped in sampling and basic DNA work. RMA, SF, MT guided for data analyses and supported in manuscript preparations. ARA supervised the complete research project.

Key words
Birds, DNA barcode, *CO1* gene marker, Phylogenetic analyses, Cytochrome oxidase, Avian fauna

ABSTRACT

Bird species that are difficult to identify taxonomically using morphological methods remain a challenge for ornithologists in accurate species identification. International migratory bird route number 4 (Indus Flyway) traverse through Pakistan. Current study present a DNA-based method that enables taxonomic identification of selected Pakistani avian fauna and is based on amplification and sequencing of *CO1* region (650 bp) of mitochondrial genome. For this purpose, a comprehensive avian barcode reference database was established from the Punjab province of Pakistan. A total of 60 mitochondrial *COI* barcode sequences inferred in this research from 20 different bird species (03 replica from each species). Further, phylogenetic analyses of these species revealed their phylogeny among species reported from different regions. As the bird species possess a barcode distinct from each other thus, *COI* sequencing can be used to identify avian fauna present in any particular region so this study will help in accurate identification of the most of the Pakistani bird species. In addition, the current study would be beneficial for further systematic genetic investigations of these bird species. The inferred DNA barcode will also provide valuable insight and foresight about evolutionary progression and genetic drift in Pakistani avian fauna.

INTRODUCTION

Birds are important as they are natural indicators of ecosystem. Pakistan is considered as a remarkable ecological region of the world because of dynamic geological history, broad altitudinal range and latitudinal spread. It is bestowed with diversified array of avian fauna due to variety of habitats (Grasteau and Minvielle, 2003). Indus flyway is one of the most important international migratory bird routes that traverse through Pakistan.

Pakistan avian fauna includes a diverse array of wild species. There are several indigenous and migratory bird species that exist in Pakistan. Wild avian fauna include species: pond heron (*Ardeola grayii)*, owl (*Athene brama)*, bulbul *(Pycnontus barbatus)*, ring necked pheasant (*Phasianus colchicus)*, crow (*Corvus splendens)*, egret (*Egretta garzetta)*, cuckoo (*Cuclus canorus*), koel (*Eudynamyss colopaceus)*, common quail (*Coturnix coturnix),* blue peacock *(Pavo cristatu),* green peacock *(Pavo muticus),*white peacock *(Pavo muticus leucistic),* red jungle fowl (*Gallus gallus),* aseel chicken (*Gallus gallus domestica),* desi chicken *(Gallus gallus domesticus),* common tragopan *(Tragopan melanocepals),* house sparrow *(Passer domesticus*), kalij pheasent (*Lophura leucomelanos),* black francolin *(Francolinus francolinus)*, and grey francolin *(Francolins pondicerianus).*

The process of identifying species gain a key significance for explaining relationship and interaction among natural biodiversity, humans and society. Additionally, there are certain few limitations too that have been faced by taxonomists during morphological identification process of species (Knowlton, 1993). Sometimes morphological variability in species may lead to incorrect taxon recognition. To avoid this kind of ambiguity, a new approach for species recognition is necessary and should be kept in consideration (Jarman and

* Corresponding author: arawan77@uvas.edu.pk
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Elliot, 2000).

At the moment, DNA barcoding is considered a recent tool for identification of species and for discovering new species by using molecular methods. The 650 bp mitochondrial cytochrome c oxidase subunit I *(COI)* serve as fast and accurate marker for species identification due to its high interspecies variation using universal primers (Hebert *et al*., 2004a; Ward *et al*., 2005; Johnsen *et al*., 2010; Stoeckle and Herbert, 2008; Savolainen *et al*., 2005). So, the present study aimed for identification of local avian species by making use of *CO1* gene polymorphism (DNA barcoding) as a molecular marker.

MATERIALS AND METHODS

Selection of birds and their identification

To develop DNA barcode and to investigate the phylogenetic classification, total 60 birds of 20 different species (03 birds from each species) were taken. Bird samples were collected from 2012 to 2016 with collaboration of legal hunters, taxidermist, Punjab Wildlife and Parks Department and Pet Centre of University of Veterinary and Animal Sciences (UVAS), Lahore. All bird species were identified on basis of their phenotypic diagnostic characteristics. This study was approved from departmental/faculty and advanced board of studies of University of Veterinary and Animal, Sciences, Lahore.

Blood samples collection and isolation of DNA

Blood samples (50 µL) of above mentioned birds were collected in a vial with 40 µl EDTA (0.5M, pH 8), drawn from brachial wing vein from different regions of Province Punjab, Pakistan. Genomic DNA was extracted from each blood sample using standard phenol-chloroform extraction method (Sambrook and Russel, 2001). Besides that concentration of DNA was measured with help of Nano drop.

Amplification of CO1 Gene and DNA sequencing

Extracted DNA samples were run on 0.8% agarose gel electrophoresis following standard protocol. For amplification of *CO1* gene, reported primers BirdF1 (TTCTCCAACCACAAAGACATTGGCAC) and BirdR1 (ACGTGGGAGATAATTCCAAATCCTG) were used and designed with help of Primer3 (https://primer3.ut.ee/) (Hebert *et al*., 2004).

The conditions of PCR concentration were optimized by changing the concentration of magnesium chloride ($MgCl_2$), deoxy ribonucleotide triphosphates (dNTPs), and Taq polymerase. The appropriate annealing temperature (52°C) for time period of 30 sec was also modified to achieve amplification of *COI* gene. The PCR products were sequenced on Applied Bimolecular system instrument ABI 3100 Genetic Analyzer in CAMB, University of the Punjab, Lahore.

BioEdit was used for analysis of sequences. Sequences were then copied in FASTA format using Bio Edit and pasted in BLAST for similarity. Clustal W2 was used for homology and pairwise alignment.

Phylogenetic analysis

Phylogenetic trees for each bird species were constructed using fast minimum evolution method in which (*COI*) gene sequences of Pakistani birds were compared with all available sequences of birds at NCBI to investigate the taxonomy and phylogeny of Pakistani birds (Desper and Gascuel, 2004; Danish *et al*., 2008). Further, phylogenetic analysis was done with help of MEGA 6.0 software.

RESULTS AND DISCUSSION

In this study, the sequencing of cytochrome c oxidase subunit I *(COI)* gene of Pakistani avian species were conducted. The gene sequences were then conformed to NCBI and prepared for various analysis of *COI* gene sequences. The analysis include homology analysis in which the reference sequence and *COI* gene sequences of twenty avian species namely *Ardeola grayii, Athene brama, Pycnontus barbatus, Phasianus colchicus*, *Corvus splendens*, *Egretta garzetta*, *Cuclus canorus*, *Eudynamyss colopaceus, Coturnix coturnix, Pavo cristatu, Pavo muticus, Pavo muticus leucistic, Gallus gallus, Gallus gallus domestica, Gallus gallus domesticus, Tragopan melanocepals, Passer domesticus*, *Lophura leucomelanos, Francolinus francolinus and Francolins pondicerianus* showed SNPs in *COI* gene sequences at various sites (Supplementary Figs. 1 to 20).

By using 650bp nucleotide segment of *COI* gene, the phylogenetic tree of all 20 species of birds were constructed in which comparison of all twenty studied birds were done with already available sequences of birds. It has been elucidated from analysis of DNA sequencing of *CO1* gene and phylogenetics that pond heron (*Ardeolla grayii)* formed a single cluster with *Nycticorax nycticorax* and *Ardeolla grayii.* Both are Indian which showed that *Nycticorax nycticorax* and *A. grayii* have a closer genetic relationship (Supplementary Fig. 1). Owl (*Athene brama)* formed a single clade with Indian *A. brama* as India is geographically in closed vicinity to Pakistan that showed a close genetic association between these two species (Supplementary Fig. 2). Bulbul *(Pycnontus barbatus)* formed a solitary cluster with American red velvet bulbul (*Pycnonotus cafer)* which is Hawaii (USA)

origined showing that Pakistani bulbul is closely related to bulbul from North America (Supplementary Fig. 3). The ring necked pheasant (*Phasianus colchicus)* created a monophyletic cluster with Chinese *P. colchicus* and *P. versicolor* showing that *P. versicolor* and *P. colchicus* are closest in evolutionary history (Supplementary Fig. 4). The phylogenetic tree of crow (*Corvus splendis)* represented a single clade which emerged directly from its descendent (Supplementary Fig. 5).

In evolutionary tree of egret (*Egretta garzetta*), it is seen that it formed a single clade with North American *E. garzetta* and western reef heron (*E. greta gularis),* although egret migrates from Russia but their sister species are present in North America (Supplementary Fig. 6). The cuckoo *(Cuculus canorus)* in its phylogenetic tree shows no variation in sequences of study samples. However, it formed a single clade representing *Cuculus micropterus* as its closest relative in the tree (Supplementary Fig. 7). Further, koel (*Eudynamys scolopaceus)* formed a single clade with American tree swallow (*Tachycineta cyaneovirides)* and shows similarity with it (Supplementary Fig. 8). Common quail (*Coturnix coturnix)* formed a cluster with helmeted guinea fowl (*Numida meleagris)* which is found in West Indies, North America and Australia. Wild quail (*Coturnix coturnix*) and Japanese quail (*Coturnix japanica*) species from Pakistan using 450 bp of *CO1* gene marker were also reported by Wajiha (2013) (Supplementary Fig. 9).

In addition, green peacock (*Pavo muticus)* showed a single cluster with blue peacock (*Pavo cristatus)* and white peacock *(P. muticus leucistic*) (Supplementary Figs. 10, 11, 12). Similar study was reported by Hussain (2014), in which she barcoded *Pavo cristatus, Pavo muticus* and *Pavo muticus leucistic* using 450 bp gene fragment of *COI* gene marker. Phylogenetic tree of red jungle fowl (*Gallus gallus),* aseel chicken **(***Gallus gallus domestica***)** and desi chicken (*Gallus gallus domesticus*) represented a cluster of the genus *Gallus*. The branch of one study sample in the tree radiated separately making it the out group which shows that it may have an evolutionary change (Supplementary Figs. 13, 14, 15). Local tragopan *(Tragopan temminckii)* was found as closer relative to western tragopan *(Tragopan melanocephalus)* in the evolutionary tree. *T. melanocephalus* belongs to the *Tragopan* genus of *Phasianidae* family (Supplementary Fig. 16).

House sparrow (*Passer domesticus)* shows single clade with Russet sparrow (*Passer rutilans)* and is found to be the most distant relative of that species (Supplementary Fig. 17). Kalij pheasant (*Lophura leucomelanos) also* show variations in their evolutionary tree (Supplementary Fig. 18). Black francolin **(***Francolinus francolinus)* formed a single cluster with its other sister species in the life tree. The genus *Francolinus* formed a single cluster indicated that *F. francolinus* and its sister species are sharing a common ancestor. Outer group of *Francolinus francolinus* and grey francolin (*Francolinus pondicerianus) are* Chinese *Arborophila brunneopectus* (KC352730.2). The bar-backed partridge showed that they had evolved from Chineese bar baked partridge. Similar studies including bird species such as *Francolinus (Francolinus francolinus* and *Francolinus pondicerianus)* were also studied using shorter *COI* gene fragment (450-500 bp) (Iqbal, 2016) (Supplementary Figs. 19, 20).

A study conducted by Hye-Sook *et al*. (2006) tested the effectiveness of COI barcode for discrimination of 92 Korean bird species. They indicated only one misidentified specimen out of 239 specimens from genetic resource bank. These results confirmed the accuracy of species identification through DNA barcoding. Kevin *et al*. (2007) studied widespread DNA barcode investigation for North American birds including 643 species demonstrating 93% of the breeding and pelagic avian fauna of USA and Canada. 94% species own different barcode clusters, with average neighbor-joining bootstrap support of 98%. In the remaining 6%, barcode clusters correspond to small sets of closely related species, most of which hybridize regularly.

Further, Priscila *et al*. (2015) analysed *CO1* gene of parrot samples, described that illegal trading can be investigated with help of DNA Barcoding. This DNA barcode data of Pakistani species will contribute in the International DNA barcode data bank BOLD (Barcode of Life Databank). Moreover, the present study reporting DNA barcode of Pakistani bird species using 650 bp *CO1* genetic marker provides a molecular reference that is of high value in biodiversity conservation. It also act as a powerful tool to improve the identity and control of avian products including their trade (Awan *et al*., 2013). This study devised new orientations in taxonomic classification of bird species of Pakistan. Our results accurately identify different Pakistani bird species by making use of DNA barcoding.

CONCLUSION

In conclusion, the nucleotide sequence of partial (650bp) segment of cytochrome oxidase I (*COI*) gene successfully discriminated the 20 avian species depicting effectiveness of COI barcoding as a powerful tool for phylogenetic inference of the avian fauna from Pakistan. This DNA barcoding will lead to develop a DNA Data Bank which would help scientists to study genetic and phenotypic evaluation of Pakistani avian fauna, species identification, biodiversity of the region, taxonomic classification. This will also provide solutions to investigate forensic cases of

illegal wildlife trade hunting and poaching.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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