Acta Entomology and Zoology

E-ISSN: 2708-0021
P-ISSN: 2708-0013
www.actajournal.com
AEZ 2024; 5(1): 118-123
Received: 08-01-2024
Accepted: 09-02-2024

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Mitochondrial DNA cytochrome oxidase 1 gene based genetic diversity analysis in ring-necked Korean Pheasant in Pakistan

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DOI: https://doi.org/10.33545/27080013.2024.v5.i1b.131

Abstract
The Ring-necked Pheasant (*Phasianus colchicus*) is a large, chicken-like bird native to North America and Asia, recognizable by its long, pointed tail, and small head with long neck. An important game bird in North America, it consumes a variety of foods including seeds, grains, grasses, leaves, roots, fruits, nuts, and insects. Recognizing the importance of the Ring-necked Pheasant, a genetic diversity analysis was conducted in Pakistan using mitochondrial DNA cytochrome oxidase 1 (COI) technique. Thirty different samples were collected, and after DNA extraction, quantification, primers optimization, and PCR, the sequences were aligned with reported sequences on NCBI. A phylogenetic tree was constructed using MEGA6 software, and the DNA barcode for the species was identified. The study provided insights into the genetic structure and phylogeny of the species. These unique COI sequences offer a reliable method for the quick identification of Ring-necked Pheasant, aiding in the prevention of illegal poaching and supporting conservation management efforts.

Keywords: Ring-necked Pheasant, COI, conservation, MEGA 6

Introduction
Pheasants are birds of the order Galliformes and the family Phasianidae. This family incorporates 177 types of Pheasants, quail and partridges from the old world (Ali *et al*., 2020) [1]. Most of Pheasants are woodland species and 23 species are considered all inclusive undermined (Anderson and Holmes, 2022) [2]. The Genus Phasianus incorporates two species, the regular bird *Phasianus colchicus* and the green fowl *Phasianus versicolor*. It is local to Asia and has been broadly presented somewhere else as a game fledgling. In parts of its range, specifically in places where none of its family members happen, for example, in Europe, where it is naturalized and it is essentially known as the bird. Ring-necked fowl is the name utilized for the species in general in North America and furthermore the aggregate name for various subspecies and their intergrades that have white neck rings (Ksepka *et al*., 2023) [10].

In the ever-advancing field of biotechnology, the most comprehensive genetic relationships can be discerned by examining the nucleotide sequence of the DNA chain, especially through mitochondrial DNA (mtDNA) nucleotide sequence polymorphisms, which provide high-resolution evolutionary data due to their rapid evolution, with the mitochondrial 16S rRNA and cytochrome C oxidase I (COI) genes being particularly well-conserved and informative markers, thus proving invaluable in delineating both intraspecific and interspecific relationships among various taxa, including marine mollusks and invertebrates at different taxonomic levels (Bucklin *et al*., 2021) [3].

DNA barcoding, utilizing the 5' end DNA sequences of the COI gene from the mitochondrial genome, is a valuable method for identifying and locating species, especially for parasites and multicellular organisms, due to its maternally inherited non-recombining nature, making it ideal for studying evolutionary relationships (Nehal *et al*., 2021) [13]. While COI barcoding is effective for animal species, plant species identification poses more challenges, as shown by comparing mitochondrial genes and homologous genes from various plants, fungi, and multicellular organisms, suggesting the mitochondrial genetic code for arginine (CGG) in animals and fungi corresponds to tryptophan in plants (Yang *et al*., 2020) [20].

In this study the genetic diversity of Korean ring-necked pheasants in Pakistan and their genetic relationship with individuals from other countries can be assessed by examining the Cytochrome Oxidase I gene.

Materials and Methods

Blood collection and Ethical clearance

The study was carried out at the Virtual University of Pakistan's 1-Davis Road, Lahore campus, in the Animal Genomics Laboratory of the Department of Molecular Biology. Ring-necked Pheasant blood samples were taken from the local fauna in Fazilpur, Rajanpur. 30 male and female Ring-Necked Korean Pheasants in Pakistan were venipunctured to obtain 10 mL blood samples, which were then promptly placed into tubes or vacuum-sealed containers containing ethylenediaminetetraacetic acid (EDTA), (Sambrook and Russell, 2006) [16]. To guarantee that the blood and EDTA were mixed and prevent clotting, the tubes were shook. The blood samples were moved to a lab, stored at -20°C until needed again, and put in an ice-chilled container. Without causing any stress or injury to the animals, sample collection was carried out in accordance with normal methods (Kumar *et al*., 2023) [11]. Every laboratory experiment was carried out at Pakistan's Virtual University. Prior to the trial, the Virtual University of Pakistan's Institutional Animal Care and Use Committee (IACUC) received ethical clearance.

DNA Extraction

This procedure was used to extract DNA from the blood samples (Sambrook and Russell, 2006) [16]. A 200 µL sample of Ring-necked Pheasant blood was collected in an Eppendorf tube. It was mixed with 1000 µL of lysis buffer. It briefly experienced vortexing. For ten minutes, it was centrifuged at 10,000 rpm. After verifying the production of pellets, the supernatant was disposed of. After adding 1000 µL of lysis buffer, the centrifuge was run three times for ten minutes at 10,000 rpm. Twenty µl proteinase K, 80 µL 10% SDS, and 250 µL buffer A1 were added. To allow the protein to degrade, it was incubated at 58 °C for the entire night. The following day, each sample received 300 µL of PCI. It briefly experienced vortexing. For fifteen minutes, it was centrifuged at 13,000 rpm. There were three layers created. With caution, the top aqueous layer was moved into a different Eppendorf tube. A pipette was used to carefully combine 600 µL of isopropanol. For fifteen minutes, it was centrifuged at 13,000 rpm. The topmost layer was thrown away. A 1000 µL ethanol was added. For ten minutes, it was centrifuged at 13,000 rpm. The pellet was dried after the top layer was thrown away. After adding 150 µL of injection water, the pellet was dissolved in it. In order to be used later, DNA was kept at -20 °C. Inorganic method (Sambrook and Russell, 2006) was used for genomic DNA extraction. The final concentration of DNA was brought to 50 ng/uL and stored at -80 °C before further use.

Mitochondrial Genome analysis

A portion of the mitochondrial gene CO1 was amplified using a set of forward primers, MtC-CF2 (5'-GCAGAGTTTGAAGCTGCT 3') and MtCCR2 (5'-AGCTGACGTGAAGTAAGC-3'), in order to amplify the entire mitochondrial COI gene. A 25 µL reaction mixture was utilised for the PCR, which included 1 µL of template (each sample's genomic DNA was used as a template), 1 µL of each primer (10 pmol/µl), 12.5 µL of 2× Taq PCR MasterMix, and 9.5 µL of ddH_2O. In order to evaluate potential contamination, negative controls were consistently added to PCR experiments.

Polymerase Chain Reaction

A method for amplifying a target DNA segment, polymerase chain reaction (PCR) is also used in scientific research to detect viruses and bacteria. The touchdown PCR technique was employed in this study to amplify COI genes. The temperature is lowered by the given amount throughout each cycle in the touchdown method. Initial denaturation at 95 °C for 4 min was followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and final extension at 72 °C for 45 s after 10 min of final extension at 72 °C in the PCR process for COI. For PCR, 25 µL reaction mixtures with 1 unit Taq DNA polymerase and 50ng DNA as template were utilised. PCR product was purified by ethanol precipitation and sequenced using an automated 300 DNA sequencer ABI PRISM® 3130Xl Genetic Analyzer (Applied Biosystem Inc, Foster City, CA).

Gel Electrophoresis, DNA Sequencing and Analysis

On a 1.2% Agarose gel, the COI gene PCR product was run for 45 minutes at 120 volts. The sample is placed onto the gel with a 5:3 composition, meaning that 3 µL of bromophenol dye and 5 µL of DNA are combined. The COI's product size is 1035 bp. For this, a Thermo Scientific 1Kb ladder is utilised. The gel is filled with 3 µL of the ladder. The gel doc system was used to view the results. After being processed with 80% ethanol, PCR results were forwarded to be sequenced. From the Lab Genetix G-3 AL-Hafeez Business Centre, located at 89, Block B 3 Gulberg III, Lahore, Pakistan, 19 PCR products of COI were sequenced. Each sample's 35 µL PCR result was combined with 100 µL of COI-F primer sent for sequencing. The alignment editor for biological sequences, Bioedit 7.2.5, was used to analyse the Sanger sequencing products. A variety of bioinformatics tools, including MEGA X software, will be employed for phylogenetic analysis (Hipni *et al*., 2021) [9]. Using the programme MEGA X, the resulting sequences were examined for nucleotide analysis (Hipni *et al*., 2021) [9]. The evolutionary tree of COI was constructed using these alignments.

Multiple Sequence Analysis

The Pakistani Ring-Necked Korean Pheasant's recently discovered full mitochondrial COI sequences have been uploaded to GenBank. Multiple sequence alignment (MLSA) is a technique used in research to identify closely related genes or proteins by revealing similar patterns across functionally or structurally related genes as well as the evolutionary links between genes. For additional study, the various alignments were stored in FASTA format. After being aligned with the downloaded FASTA files of other Ovis Aries sequences on MEGA X, the sequences of the Ring-Necked Korean Pheasant in Pakistan exhibited the highest similarity ratio with our query sequences from Gen-Bank. When a biological polymer sequence (DNA, protein) is uploaded to a sequence database, its accession number serves as a unique identification which was used in this analysis.

Phylogenetic analysis

The Ring-Necked Korean Pheasant in Pakistan's numerous COI sequence alignments were used to create phylogenetic

trees using MEGA X software. Bootstrap techniques were employed to generate a phylogenetic tree using the Maximum Likelihood (ML) statistical method. In addition, the Jukes-Cantor model was employed in this phylogenetic study. By modifying the 500 replicate values, the bootstrap test method was applied. The branch-es length indicates the genetic distance. The amount of genetic variation or change is shown by the scale 0.02 (2%) at the base of the phylogenetic tree. Tree topology is strongly supported when the Bootstrap value is greater than 0.6.

Results

Phylogenetic analysis

Phylogenetic tree constructed with 1000 bootstrap repetitions using NJ models (Figures 1 and 2). As an out group, ring-necked pheasant species were obtained from GenBank (NCBI). Pheasants were grouped into two major groupings (clades) on the tree, with bootstrap values ranging from 54 to 100%. Group I included five species with bootstrap values ranging from 54 to 99%: red junglefowl, chukar, francholin, pheasant, and monal partridge. With bootstrap 88-100%, two Anthracoceros members *A. Malayanus* and *A. Albirostris*-formed a distinct Group II. The genetic distance of 0.059 (5.90%) was found to support the two groupings. It is evident that the pheasant in Indonesia and Pakistan are distinct species.

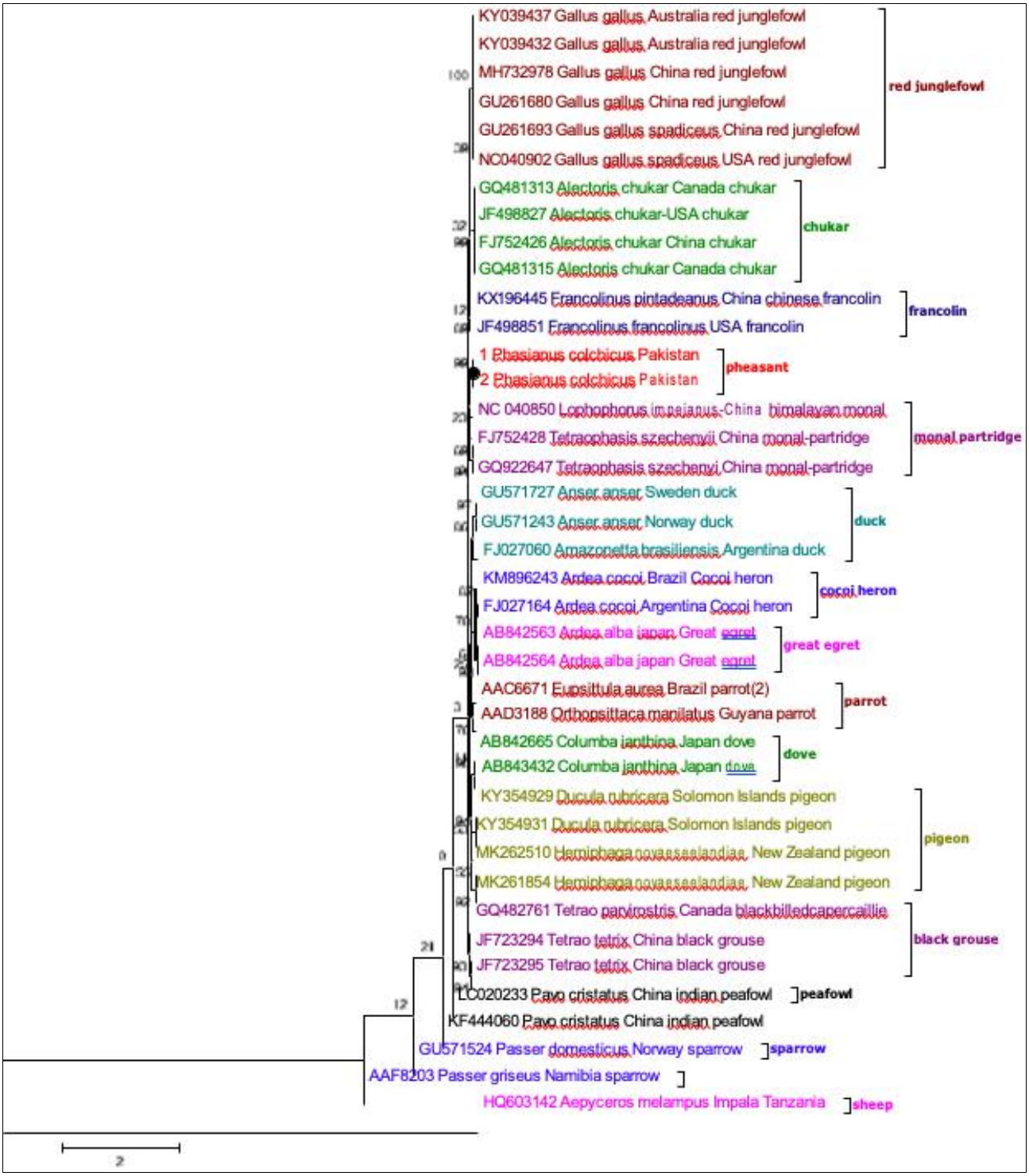

Fig 1: Neighbour-Joining (NJ) phylogenetic tree of Pheasant with other species based on partial sequence of COI gene.

Fig 2: Neighbor-joining (NJ) phylogenetic tree of Pheasant based on partial sequence of COI gene.

Genetic Distance: The quantity of base replacements per site from averaging over all succession matches between bunches are appeared. Standard mistake estimate (s) are appeared over the askew. Investigations were directed utilizing the Clustal W program (Hipni *et al*., 2021) [9] (Table 1). The investigation included 38 nucleotide groupings. All positions containing holes and missing information were disposed of. There were a sum of 670 situations in the last dataset. Developmental investigations were directed in MEGAX (Hipni *et al*., 2021) [9] (Table 2).

Table 1: Estimates of evolutionary divergence over sequence pairs between groups

Gp_1		0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.1	0.1	0.1	0.1
Gp_2	0.0		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gp_3	0.0	0.0		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gp_4	0.0	0.0	0.1		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gp_5	0.0	0.0	0.0	0.0		0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gp_6	0.0	0.0	0.0	0.1	0.0		0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Gp_7	0.0	0.0	0.0	0.1	0.0	0.0		0.0	0.0	0.0	0.0	0.0	0.1	0.1
Gp_8	0.0	0.0	0.0	0.1	0.0	0.0	0.0		0.0	0.0	0.0	0.0	0.1	0.1
Gp_9	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0		0.1	0.0	0.0	0.1	0.1
Gp_10	0.0	0.1	0.0	0.1	0.1	0.1	0.0	0.0	0.0		0.1	0.0	0.1	0.1
Gp_11	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0		0.0	0.1	0.1
Gp_12	0.0	0.1	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0		0.1	0.1
Gp_13	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.0	0.1		0.0
Gp_14	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1	0.1	0.1	0.1	0.1	0.0	

Table 2: Estimates of evolutionary divergence over sequence pairs of pheasant

Gp_1		0.016	0.005	0.008	0.014	0.016	0.015
Gp_2	0.104		0.015	0.015	0.016	0.012	0.016
Gp_3	0.016	0.096		0.007	0.012	0.014	0.013
Gp_4	0.052	0.087	0.043		0.012	0.014	0.013
Gp_5	0.089	0.093	0.077	0.074		0.016	0.006
Gp_6	0.115	0.080	0.102	0.100	0.104		0.016
Gp_7	0.092	0.093	0.082	0.081	0.023	0.106	

Discussion

Little nucleotide variation was discovered at the species level, but a substantial amount of variation was discovered at the genus and family levels. According to de Melo *et al*. (2020) [5], the length of the COI gene utilised for the barcode is approximately half of the overall length found at the beginning of the COI gene in all animal species. However, very short barcode sequences (109-208 bp) are equally useful for identifying the species (Macher *et al*., 2021) [12]. In our study, the COI gene was amplified to a remarkable length of 746 base pairs. It is longer than the one that Salonna *et al*. (2021) [15] recommended for DNA barcode purposes. However, it is shorter than the COI gene of cockatoos (Psittaciformes) (807 bp) (Salonna *et al*., 2021) [15]. *Penelopides panini, Aceros waldeni, A. coronatus, Rhabdotorrhinus* (*Aceros) corrugatus*, and R. leucocephalus are the five species of hornbills that were found within their ranges. These species are members of the Bucerotidae family, specifically the Bucerotiformes order (Poonswad *et al*. 2013) [14].

Every tested participant had a similarity score below 97.0%. This fact was due to the BOLD System database not having the nucleotide sequences for the COI genes of these seven pheasant species. The BOLD System's pheasant database differed from the hornbill database by 7.11%. This difference is significantly more than the animal species interspecific threshold (> 3.0%) (Van der Hoek *et al*., 2013) [18]. As a result, the COI gene nucleotide sequences that were examined provide new information and may be consulted for identifying Indonesian hornbills.

Variations in nucleotides within genus and family were found in this study. The COI gene sequence alignment results from 31 hornbill samples showed 45 single nucleotide polymorphism (SNP) sites within the genus family species, which ranged in number from 84 to 737 sites. Three pheasants (*Anthracoceros malayanus, A. albirostris, and Aceros cassidix*) were shown to have the largest variation at the species level, whilst the remaining species lacked variation. At the genus level, Buceros had the lowest COI gene variation and Anthracoceros had the largest variance (10 sites). The diversity of mtDNA COI gene sequences in 648 bp regions may possibly function as animal species, as variations in COI gene sequences can be used to differentiate closely related species in all animal groups barcodes (Hebert *et al*. 2004) [8] and each species has a specific nucleotide sequence in the COI gene. Modern molecular technology allows for the identification of genetic diversity at the DNA level. K2P techniques were used to calculate the genetic distance within the Pheasant species, genus, and family. The average genetic distance fell between 0.002 (0.2%) and 0.008 (0.8%) within species, 0.045 (4.5%) within genus, and 0.046 (4.6%) within family. The genetic distance used to discriminate between species was less than that found in earlier research (Wang *et al*., 2017) [19]; nonetheless, it is still the same at the genus and family levels, particularly in comparison to what Hebert *et al*. (2004) [8] found. Genetic distance within species is typically less than 1% and rarely exceeds 2% (Waugh 2007) [22]. Examples of this include average genetic distances of up to 0.3% within species of Korean birds and Phasianidae (Yoo *et al*. 2006) [21], between 0 and 13.7% in Neotropical birds (Tavares *et al*. 2011) [17] and between 0.1 and 0.7% in parrots (Gonçalves *et al*. 2021) [7].

There seems to be variation in the mean genetic distance within a genus. The interspecific genetic difference between the two genera (Buceros and Rhyticeros) was less than the 3.0% threshold that separates species. The COI gene variation amongst species within the Laridae (Sternini) family varied from 0.25 to 10.51%, according to Efe *et al*. (2009) [6]. *Rhyticeros undulatus* and *R. plicatus* also share certain morphological characteristics. The colour of their neck, head, and neck pouch vary from person to person. The two rhinoceroses, *Buceros rhinoceros* and *B. bicornis*, are similar in that they have the same caque shape and similar neck and wing colours (Poonswad *et al*. 2013) [14]. In comparison, the genetic distance between *Anthracoceros malayanus* and *A. albirostris* was 3.2%, or 0.032, since the Anthracoceros genus has interspecific COI sequence differences that are higher than the threshold. In order to correctly identify a species, the genetic distance needs to be greater than 5% (Waugh 2007) [22]. The average divergence of the COI gene within the genus of Ardeidae was reported by Waugh to be 13.08%; it was 8.2% within the genera of Korean birds (Yoo *et al*. 2006) [21]; it was 4.8-15.6% within the Thamnophilidae (Passeriformes); it was 5.35% within the Phasianidae (Cai *et al*. 2010) [4]; it was 7.95% within the Scandinavian birds and 9.52% within the Green Bee-eater (Merops orientalis). The genetic distance within the Bucerotidae family is 0.046 (4.6%), which is less than what has been discovered in other research. According to Yoo *et al*. (2006) [21], the mean genetic distance on Phutananidae was 15.63%, but it was 13.8% within Korean bird groups (Cai, *et al*. 2010) [4].

Conclusion

This study elucidates the genetic diversity of Korean ring-necked pheasants in Pakistan and their genetic relationships with individuals from other countries through analysis of the Cytochrome Oxidase I gene. By employing advanced biotechnological techniques such as DNA barcoding and mitochondrial genome analysis, insights into the evolutionary relationships within the Phasianidae family were gained. The phylogenetic analysis revealed distinct groupings among pheasant species, underscoring the significance of genetic variation at both species and genus levels. Notably, the COI gene's substantial length

amplification in this study contributes to expanding the understanding of genetic markers in avian species identification. These findings underscore the importance of genetic studies in wildlife conservation and taxonomy.

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