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Study of Cytochrome B Gene Variations in Avian Fauna of Sargodha

Ayesha Sajjad[1], Anisa Iftikhar[2], Shahid Hassan[3].Faiza Zubair[3], Shameen Shahid[3], Iqra Asif[3,4]

[1]Department of Zoology, The University of Lahore, Sargodha Campus,
[2]Department of Biology, Clarkson University, Potsdam, USA
[3]Department of Zoology, University of Sargodha, Sargodha Pakistan
[4]Department of Biological Sciences, Superior University Lahore, Pakistan

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Abstract

Pakistan is home to approximately 765 bird species, but molecular-level studies for species identification remain scarce. The Sargodha region, with its agricultural land, canal systems, and hilly terrain, provides ideal habitats for various bird species. A total of 34 species belonging to 29 different families have been morphologically identified in this area. However, morphological methods alone are often insufficient for accurate species identification, which is crucial for conservation, taxonomy, and ecological research. This study aimed to identify avian species using molecular tools, specifically the cytochrome b gene, which is widely used for phylogenetic and evolutionary studies due to its conserved nature within species and variation among species. Tissue samples from eight bird species (totaling 24 samples) were collected from various locations in Sargodha through local hunters. DNA was extracted using the Russell and Sambrook method, and cytochrome b was amplified using PCR. The PCR products were sequenced at Macrogen Laboratory. Sequence analysis was performed using BioEdit, and phylogenetic relationships were assessed using MEGA6, employing methods such as Maximum Likelihood (ML), Maximum Parsimony (MP), Neighbor Joining (NJ), Minimum Evolution (ME), and UPGMA. All tree-building methods consistently revealed the phylogenetic relationships between and within species.The results confirmed significant **genetic diversity** among the bird species of Sargodha. Homogeneity and heterogeneity across sequences were also identified and recorded in tabular format. The study demonstrated that **cytochrome b** is a reliable genetic marker for species differentiation and phylogenetic analysis. Additionally, the organic DNA extraction method proved to be efficient and economical. Overall, this research highlights the effectiveness of molecular tools in avian identification and underlines the rich avian biodiversity present in the Sargodha region, emphasizing the importance of integrating genetic methods into regional biodiversity assessments.

Keyword: Phylogenetic Analysis, Avian Fauna, Mitochondrial DNA, Cytochrome B Gene, PCR, Phylogenetic Tree, Pakistan

Introduction

Pakistan holds a significant geographic and ecological position, home to unique fauna like the endemic Indus dolphin and serving as a key stop on the fourth major bird migration route (Kazmi & Jan, 1997). With favorable climatic conditions, it supports over 765 bird species across 40 orders, including resident and migratory birds (Grimmett, 1998). Notable birds include the national bird (Chukar pheasant), Shaheen falcon, Asiatic peafowl, and rose-ringed parakeet (Bilal et al., 2025). Sargodha, an agriculturally rich region, hosts diverse bird life. However, urbanization and deforestation threaten this biodiversity. A study identified 34 species from 29 families, mostly Passeriformes, such as crows, sparrows, mynas, kites, and wagtails (Ashraf et al., 2018). Alexandrine parakeet, listed as near-threatened by IUCN, is also found here. Previous research focused on morphology; molecular identification was lacking. Species identification is key to biodiversity research. Traditional taxonomy relied on morphology, but molecular techniques are now vital,

especially in forensics (Ardura et al., 2011). DNA sequencing has advanced avian phylogeny studies, addressing relationships among higher bird orders and post-Cretaceous evolution (Jarvis et al., 2014). Birds evolved over 150 million years, with major shifts following the Cretaceous mass extinction (Butler et al., 2015). Fossils from China and elsewhere revolutionized understanding of avian evolution (Xu et al., 1999; Brusatte et al., 2015). Modern birds fall into two clades: Palaeognathae (flightless birds) and Neognathae (true flight birds, including ducks, parrots, and falcons) (Li et al., 2015). Phylogenetic data is essential to study speciation, migration, and ecology accurately; its absence may lead to misinterpretation (Duncan et al., 2007). Mitochondrial DNA (mtDNA) is widely used in genetic studies due to its small size (14.3–19.5 kb), lack of introns, and absence of recombination. It is maternally inherited, haploid, and mutates 5–10 times faster than nuclear DNA, making it useful for molecular identification (Wilson et al., 1985). Its high mutation rate, lack of repair mechanisms, and maternal inheritance make mtDNA effective for ancestry mapping, phylogenetics, and disease markers like cancer (Kujoth et al., 2017; West & Shadel, 2017). The mtDNA genome contains 37 genes: 13 for proteins (e.g., COI, ND1, Cyt b), 22 for tRNA, and 2 for rRNA. While gene arrangement is conserved across vertebrates, some variations exist (Shadel & Clayton, 1997). The control region (CR) regulates replication and transcription and includes three domains with differing evolutionary rates. Domains I and II evolve rapidly and are useful for intraspecific studies, while the utility of slower-evolving domains in phylogenetics remains debated (Lee et al., 1995; Saccone et al., 1991). Mitochondrial genomes of various eukaryotes (e.g., human, mouse, cow) help understand genome size, structure, divergence, and diversity. Gene order in mtDNA is generally conserved within phyla but varies between them (Wolstenholme et al., 1987). In birds, mtDNA aids in reconstructing evolutionary history and identifying genetic risks, low diversity, and dispersal patterns (Britten et al., 1986; Lyu et al., 2018). For phylogenetic studies, genes shared by all species with suitable evolutionary rates are selected (Miyata et al., 1980). Cytochrome b and ND2 are preferred for resolving relationships at species and genus levels (Edward et al., 1998). Control region domains vary in rate, but genes like cyt b—one-third of whose sites evolve rapidly—are reliable for phylogenetic inference (Sullivan et al., 1999). Cytochrome b, part of mitochondrial complex III, plays a key role in the electron transport chain. It contains 380 amino acids and is located in the conserved mtDNA region (Morais, 1990; Hatefi, 1985). Its full sequence has been characterized only in chicken among birds (Desjardins & Morais, 1990). The aims and objectives are: to check cytochrome b gene variation in birds of Sargodha, Primer optimization for cytchrome b region, to assess cytochrome b region as a marker for specie identification, to find out the avian fauna of Sargodha region and to build a phylogenetic relation between them.

Material and Method

Sample Collection

Samples were collected from different areas of Bhalwal, Sahiwal, Kotmomin and Silanwali, Tehsils of Sargodha District. Samples were collected by contacting local hunters of these areas. 14 meat samples of different birds were kept in labelled plastic bags or zipper bags and taken to lab.

Preservation

After sample collection, the very first and most important step of research is to preserve the samples for future usage. The most simple and standard method of sample preservation is Freezing method. In

freezing method, sample is stored at -20ºC in refrigerator to prevent meat degradation. Samples, in lab, were freeze at -20ºC in lab refrigerator.

Table 1.: Collection of samples from different locations of Sargodha District.

Sr no	Specie name	Sample ID	No. of Samples	Sample type	Area	Conservation status	Coordinates
1	Bank Myna *(Acridotheres ginginianus)*	BM 1 BM 2 BM3	3	Tissue	Silanwali	Least concern	31º49′30N 72º32′20E
2	Common myna (*Acridotheres tristis)*	CM 1 CM 2 CM 3	3	Tissue	Silanwali	Least concern	31º49′30N 72º32′20E
3	Muscovy Duck (*Cairinamoschat)*	MD 1 MD 2 MD 3	3	Tissue	Bhalwal	Least concern	32º15′56N 72º53′58E
4	Common hoopoe (*Upupa epops)*	CH 1 CH 2 CH 3	3	Tissue	Sahiwal	Least concern	31° 58' 23"N 72° 19' 32"E
5	Rock pigeon *(Columba Livia)*	RP 1 RP 2 RP 3	3	Tissue	Bhalwal	Least concern	32º15′56N 72º53′58E
6	Black drongo *(Dicruarus macrocerus)*	BD 1 BD 2 BD 3	3	Tissue	Bhalwal	Least concern	32º15′56N 72º53′58E
7	Munia (*Lonchura Malacca)*	MU 1 MU 2 MU 3	3	Tissue	Sargodha	Least concern	32° 4' 56.8776" N 72° 40' 8.8608" E.
8	Scally breasted myna (*Lonchura punctulata)*	SBM 1 SBM 2 SBM 3	3	Tissue	Kot momin	Least concern	32º11′18N 73º01′43E

DNA Extraction Method

Phenol chloroform (Organic) method was used for DNA extraction. DNA extraction include following steps:

The DNA extraction was carried out using the phenol-chloroform (organic) method. Initially, 20g of each tissue sample was crushed using liquid nitrogen and transferred into labeled Eppendorf tubes. Each tube received 750 µl of lysis buffer (0.32mM Sucrose, 10mM Tris pH 7.5, 5mM $MgCl_2$, 1% Triton), followed by centrifugation at 13,000 rpm for 1 minute. An additional 500 µl of the same lysis buffer was added and centrifuged again. To ensure proper tissue digestion, 500 µl of another lysis solution (10mM Tris, 400mM NaCl, 2mM EDTA) was added and the samples were incubated at 60°C for 30 minutes. Next, 15 µl of Proteinase K and 20% SDS were added to each tube, and samples were incubated overnight at 56°C for complete digestion, resulting in lysate formation. For purification, 500 µl of phenol:chloroform:isoamyl alcohol (PCI) solution was added to each tube, mixed well, and centrifuged at 13,000 rpm for 10 minutes. The upper aqueous layer containing the DNA was carefully transferred to fresh tubes. In the precipitation step, 500 µl of chloroform:isoamyl alcohol (24:1) was added and centrifuged again, followed by transfer of the supernatant to new tubes. To precipitate DNA, 55 µl of sodium acetate and 500 µl of ice-cold isopropanol were added, and samples were incubated at -20°C for 45 minutes. After discarding the supernatant, the DNA pellets were washed with 500 µl of 70% ethanol and centrifuged at 7,500 rpm for 5 minutes. Ethanol was discarded, and the pellets were air dried. Finally, DNA pellets were eluted in TE buffer (Tris-EDTA) and stored at 4°C for further use. Clear solution was formed after heating. 7 µl Ethidium Bromide was added in gel solution.

Gell Electrophorasis

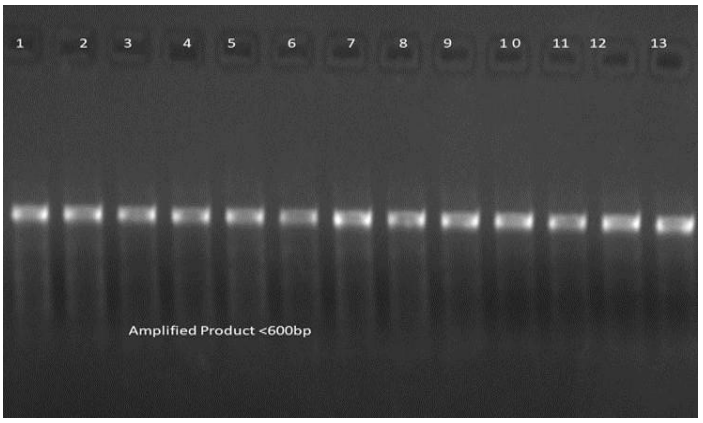
To separate DNA molecules based on size, charge, and density, agarose gel electrophoresis was used. The gel was prepared by dissolving 1 gram of agarose in 100 ml of 1X TAE buffer (Tris-Acetic acid-EDTA) and heating until a clear solution formed. Once cooled slightly, 7 µl of Ethidium Bromide was

added to stain the DNA. The gel solution was poured into a casting tray with combs inserted. After the gel solidified, it was transferred to an electrophoresis tank filled with 1X TAE buffer, and the combs were carefully removed to create wells. For loading, 2 µl of extracted DNA was mixed with 2 µl of 6X bromophenol blue loading dye and loaded into the wells. The gel was run at 110 volts and 500 mA for 35 minutes. DNA bands were visualized using a UV Trans-Illuminator with a Bio Doc Analyzer, and the results were compared with a 1KB DNA ladder. For further analysis, amplified products were also run on a 2% agarose gel and visualized under UV light.

Gel electrophoresis analysis:

Amplified product was run on 2 % agarose gel and visualized under UV.

Fig 1.Visualizing DNA after Gel of 13 Samples



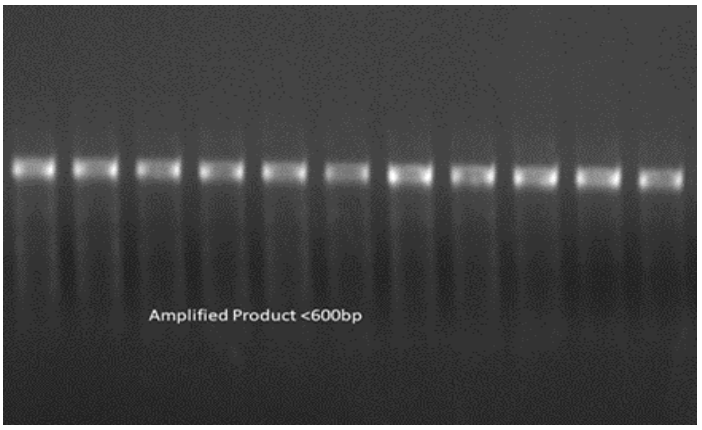


Fig:2 Visualizing DNA after Gel of 11 samples

Prime Selection

Table 2: Primer Sequences

F1484B	**5´-ATCCAACATCTCAGCATGATGAAA-3´**
R1485B	**5´- TCAGTTTTTGGTTTACAAGAC-3´**

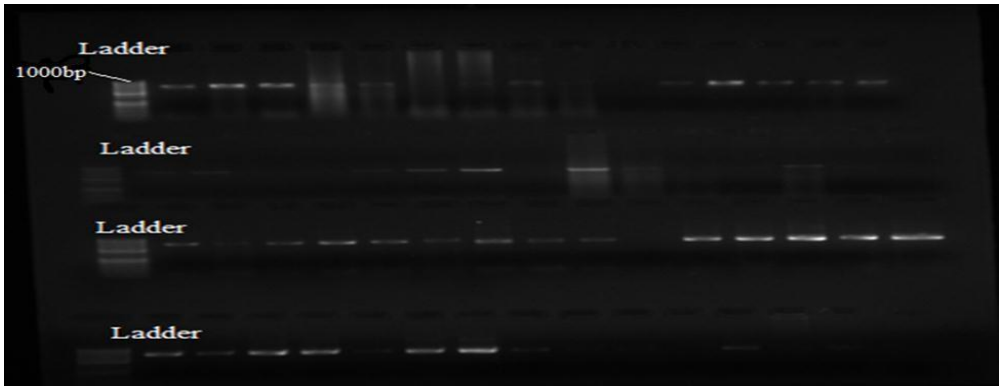
PCR Polymerase chain reaction

PCR is a technique used to amplify specific DNA sequences. VFID and VRID primers were used to target the tissue samples. DNA was denatured at 94°C to form single-stranded templates, primers annealed to these templates, and DNA polymerase extended the strands, producing new double-stranded DNA. This cycle was repeated to obtain the desired number of copies. The PCR master mix contained DNA template, dNTPs, forward and reverse primers, $MgCl_2$, Taq DNA polymerase, PCR buffer, and deionized water, with reactions performed using a Galaxy XP Thermal Cycler. Optimized PCR conditions included an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), and extension (72°C, 1 min), with a final extension at 72°C for 10 minutes and hold at 4°C.

Analysis of PCR product

Amplified PCR products were analyzed using 1.5% agarose gel electrophoresis. The gel was prepared in 0.5X TBE buffer with ethidium bromide, poured into a casting tray, and allowed to solidify. PCR products were mixed with bromophenol blue loading dye and loaded into the wells alongside a DNA ladder. The gel was visualized under a gel documentation system, and the results were photographed for analysis.

Fig3. Amplified PCR



Gene Sequencing

The PCR products were sent to Macrogen lab, Korea for sequencing where DNA synthesis and DNA gene typing facilities are provided. Sequencing of various species was obtained from BLAST tool.

Results

DNA Extraction and Quantification

The genomic DNA was extracted from 8 different species of birds of total 24 samples as each specie hold 2 of its organisms, visualized and confirmed on 1% agarose gel. A 20mg of meat was used from each sample for genomic DNA extraction. Total genomic DNA was extracted from meat sample was optimized with careful adjustments in optimization of Protinase K concentration, phenol-chloroform method. The results of genomic DNA were visualized by agarose gel documentation system and they were also recorded.

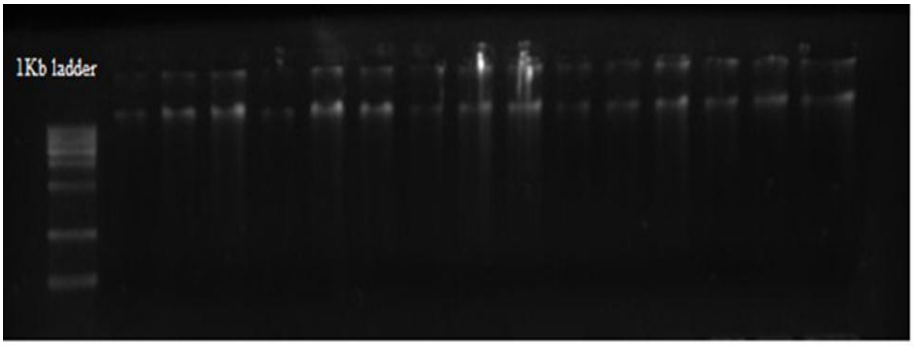


Fig 4. Genomic DNA

Polymerase Chain Reaction (PCR) Results

The Polymerase Chain Reaction (PCR) of the extracted genomic DNA of birds was carried in order to amplify the desired region of Cytochrome b gene. For the analysis of samples, the products were obtained and base pairs of Cytochrome b were amplified by primers and the results are as below:

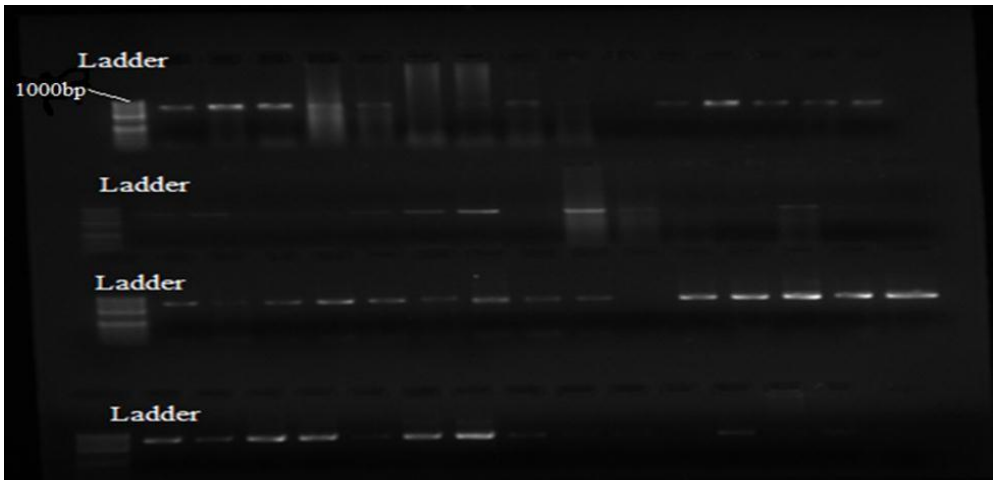


Fig 5 PCR gel results of extracted DNA samples

The genomic data of other birds of different genus was retrieved from GenBank to find out evolutionary analysis. Accession number and source of different species of birds are as follow:

Table 2. Species names, accession numbers and data source of different birds acquired from GenBank.

Common Name	Scientific Name	Accession Number	Source
Little egret	Egretta garzetta	KJ190950	GenBank
House sparrow	***Passer domesticus***	NC_025611	GenBank
Red-vented bulbul	***Pycnonotus cafer***	MG762208	GenBank
House crow	***Corvus splendens***	NC_024607	GenBank
Cattle egret	***Bubulcus ibis***	KJ722534	GenBank
Purple sunbird	***Nectarinia asiatica***	MT683497	GenBank
Black-winged stilt	***Himantopus himantopus***	LC541436	GenBank
Eurasian collared dove	***Streptopelia Decaocto***	NC_037513	GenBank
White wagtail	***Motacilla alba***	NC_029229	GenBank
Temminck's stint	***Calidris temminckii***	JN601815	GenBank
Indian roller	***Coracias benghalensis***	KT240053	GenBank
Common moorhen	***Gallinula chloropus***	HQ896036	GenBank
Greater coucal	***Centropus sinensis***	KT947122	GenBank
Black Kite	***Milvus migrans***	NC_038195	GenBank
Spotted owlet	***Athene brama***	KR779894	GenBank

Table 3-A: Sampling details and comparison of morphological vs molecular identification

Morphology	**Taxonomy**				**Mitochondrial Cyt-b gene**			
					BLAST			
Common Name	**Order**	**Family**	**Genus**	**Species**	**Similarity**	**Identification with identity %**	**Difference**	**Accession no**
Bank Myna	Passeriformes	Sturnidae	Acridotheres	ginginianus	100%	A. ginginianus (100%)	0%	KJ456174.1
Common Myna	Passeriformes	Sturnidae	Acridotheres	Tristis	100%	A. tristis 1143/1143 (100%)	0%	KJ456175.1
Muscovy Duck	Anseriformes	Anatidae	Cairina	Moschata	99%	C. moschata 1141/1143 99%	1%	L08385.1
Common Hoopoe	Bucerotiformes	Upupidae	Upupa	Epops	99%	U. epops 1142/1143 (99%)	1%	KY689872.1
Rock pigeon	Columbiformes	Columbidae	Columba	Livia	99%	C. livia 1141/1143 (99%)	1%	KP319029.1
Black drongo	Passeriformes	Dicruridae	Dicruarus	macrocerus	100%	D. macrocerus 1026/1026 (100%)	0%	JQ864501.1
Munia	Passeriformes	Estrididae	Lonchura	Malacca	99%	L.. malacca 1129/1143 (99%)	1%	MN991592.1
Scally breasted myna	Passeriformes	Estrididae	Lonchura	punctulata	99%	L. punctulata 1141/1143 (99%)	1%	KJ456325.1

Table 3-B: Sampling details and comparison of morphological vs molecular identification

Taxonomy					
Phylum	**Class**	**Identification method**	**Identifier name**	**Taxonomic status**	**Identifier email**
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com
Chordata	Aves	Morphological	Asif Naseem	Least concern	asifnaseem@live.com

Table 4: Codon usage on the basis of cytochrome b gene

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU
UUU(F)	4	0.33	UCU(S)	1.2	0.4	UAU(Y)	1.9	0.33	UGU(C)	0.6	0.34
UUC(F)	20.2	1.67	UCC(S)	6.8	2.26	UAC(Y)	9.5	1.67	UGC(C)	3	1.66
UUA(L)	3.8	0.45	UCA(S)	8.5	2.84	UAA(*)	0.3	0.12	UGA(*)	8.4	2.88
UUG(L)	0.3	0.03	UCG(S)	0.4	0.13	UAG(*)	0	0	UGG(W)	0.6	1
CUU(L)		0.43	CCU(P)	2.1	0.44	CAU(H)	1.9	0.39	CGU(R)	0.6	0.57
CUC(L)		1.49	CCC(P)	7.3	1.56	CAC(H)	8	1.61	CGC(R)	1.5	1.44
CUA(L)		3.26	CCA(P)	8.7	1.86	CAA(Q)	5.5	1.76	CGA(R)	4.1	3.85
CUG(L)	3.1	0.35	CCG(P)	0.7	0.15	CAG(Q)	0.7	0.24	CGG(R)	0.2	0.14
AUU(I)	5.3	0.54	ACU(T)	2.9	0.52	AAU(N)	2.1	0.28	AGU(S)	0.1	0.03
AUC(I)	19.1	1.91	ACC(T)	10.1	1.78	AAC(N)	12.8	1.72	AGC(S)	1	0.34
AUA(I)	5.5	0.55	ACA(T)	9.2	1.62	AAA(K)	6.1	1.83	AGA(R)	0	0
AUG(M)	1	1	ACG(T)	0.4	0.08	AAG(K)	0.6	0.17	AGG(R)	0	0
GUU(V)	1.4	0.36	GCU(A)	3.3	0.58	GAU(D)	0.7	0.23	GGU(G)	1.5	0.28
GUC(V)	5.5	1.4	GCC(A)	12	2.14	GAC(D)	5.1	1.77	GGC(G)	7.3	1.38
GUA(V)	8.2	2.1	GCA(A)	6.9	1.23	GAA(E)	4.8	1.74	GGA(G)	10.3	1.94
GUG(V)	0.5	0.14	GCG(A)	0.3	0.05	GAG(E)	0.7	0.26	GGG(G)	2.1	0.4

Average codons = 305
All the frequencies are average over all taxa
Relative synonymous codon usage is given in parentheses following the codon frequencies

Table 5: Test of the Homogeneity of Substitution Patterns between Sequences

U. epops																									
P. barbatus	1.00																								
N. jugularis	0.07	0.01																							
M. migrans	1.00	0.27	1.00																						
L. rohita	0.19	0.27	0.05	0.14																					
G. chloropus galeata	1.00	0.26	1.00	0.22	0.04																				
Egretta eulophotes	0.00	0.00	1.00	0.25	0.01	1.00																			
D. aeneus	0.03	0.02	0.00	0.13	0.21	0.25	0.00																		
C. canorus	1.00	0.30	0.00	0.33	1.00	0.36	0.00	0.37																	
C. albicolis	1.00	0.27	0.01	0.06	0.36	1.00	0.00	0.37	1.00																
C. livia	0.09	0.03	1.00	1.00	0.07	1.00	0.18	0.00	0.00	0.04															
C. alpina	1.00	1.00	0.02	0.05	0.19	1.00	0.00	0.03	1.00	1.00	0.02														
A. ginginianus	1.00	0.12	0.29	0.27	0.00	0.19	0.06	0.02	0.04	0.11	1.00	0.06													
A, ginginianus	0.12	0.08	0.07	0.02	0.14	0.20	0.01	0.04	0.36	0.04	0.00	0.03	0.00												
A. ginginianus	0.16	0.08	0.13	0.01	0.15	0.21	0.01	0.02	0.28	0.05	0.01	0.03	0.00	1.00											
L. punctulata	1.00	0.14	1.00	1.00	0.02	1.00	0.08	0.02	1.00	0.04	0.12	0.19	0.26	1.00	1.00										
L. malacca sinensis	0.06	0.01	1.00	1.00	0.13	1.00	0.16	0.01	0.01	0.01	0.30	0.02	1.00	0.16	0.24	1.00									
D. macrocercus	0.12	0.32	0.00	0.13	0.29	0.37	0.00	1.00	1.00	1.00	0.00	0.27	0.04	0.21	0.17	0.05	0.02								
C. livia	0.13	0.02	1.00	1.00	0.06	1.00	0.19	0.00	0.00	0.05	1.00	0.01	1.00	0.00	0.01	0.30	0.30	0.00							
U. epops	1.00	1.00	0.11	1.00	0.21	1.00	0.00	0.03	1.00	1.00	0.08	1.00	1.00	0.14	0.18	1.00	0.05	0.11	0.15						
C. moschata	0.00	0.01	0.04	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.01	0.00					
A. tristis	1.00	0.38	1.00	0.36	0.07	1.00	0.03	0.00	0.08	0.06	0.12	0.13	0.22	0.37	1.00	1.00	1.00	0.01	0.22	1.00	0.0				
L. punctulata	1.00	0.16	1.00	1.00	0.01	1.00	0.15	0.03	1.00	0.03	0.19	0.17	0.29	1.00	1.00	1.00	1.00	0.04	0.26	1.00	0.0	1.00			
L. malacca sinensis	0.05	0.01	1.00	1.00	0.16	1.00	0.21	0.01	0.01	0.00	1.00	0.01	1.00	0.08	0.11	1.00	1.00	0.01	1.00	0.04	0.0	0.30	1.00		
D. macrocercus	0.12	0.34	0.00	0.14	0.29	1.00	0.00	1.00	1.00	1.00	0.00	0.30	0.04	0.24	0.14	0.03	0.00	1.00	0.00	0.13	0.0	0.02	0.03	0.01	

Note: The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Disparity Index test). A Monte Carlo test (1000 replicates) was used to estimate the P-values, which are shown above the diagonal. P-values smaller than 0.05 are considered significant (marked with yellow highlights) the estimates of the disparity index per site are shown for each sequence pair below the diagonal. This analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd. There were a total of 1143 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

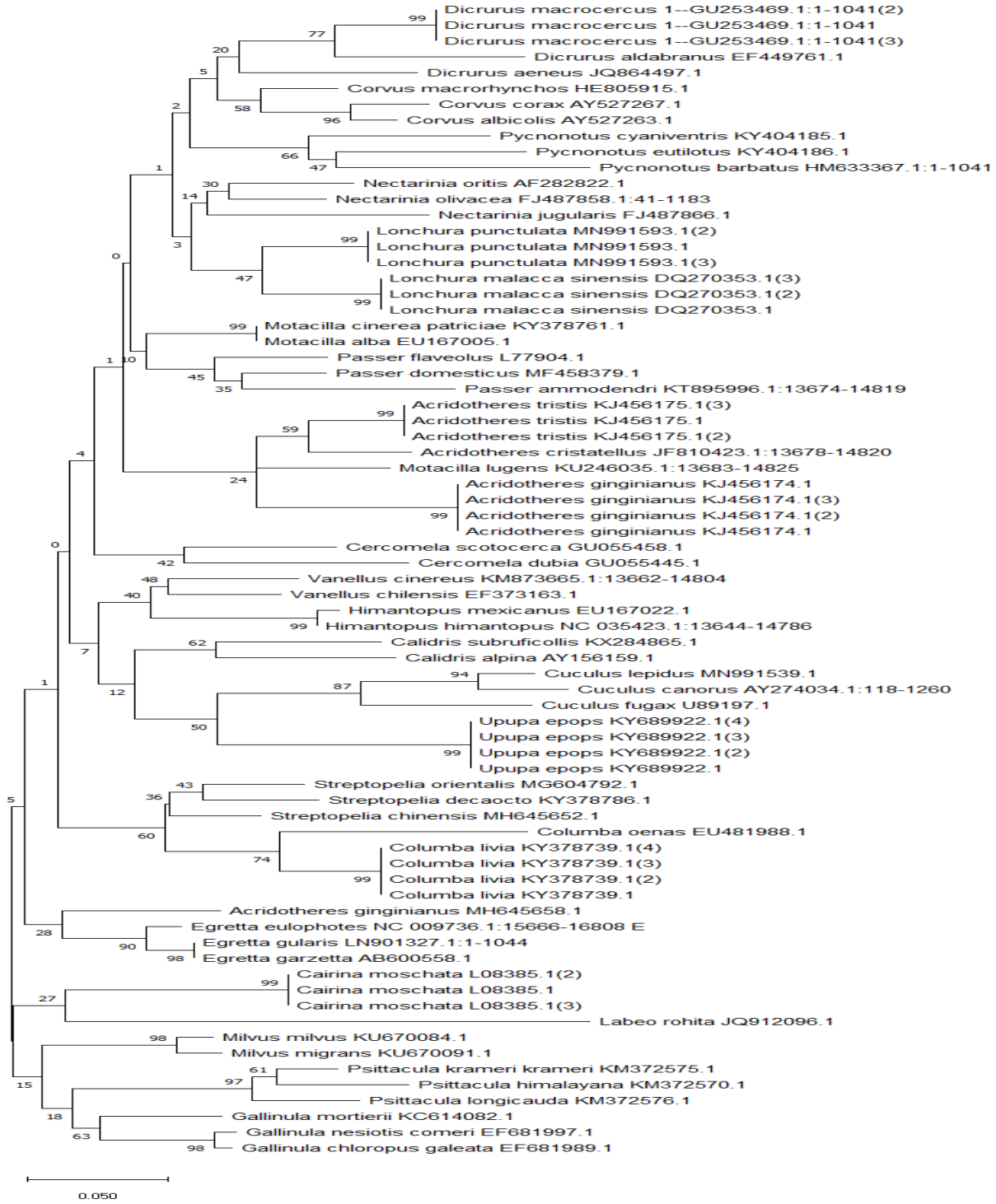


Fig 6. Minimum Evolution analysis of taxa

Note: The evolutionary history was inferred using the Minimum Evolution method. The optimal tree with the sum of branch length = 3.65800118 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite

Likelihood method and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The ME tree was searched using the Close- Neighbor-Interchange (CNI) algorithm at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree. This analysis involved 73 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 1146 positions in the final dataset. Evolutionary analyses were conducted in MEGA X.

Table 6.: Nucleotide Composition Based on Cytochrome B Gene All frequencies are given in percent

	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
C. dubia	24.1	33.5	28.1	14.2	701	23.1	28.7	23.6	24.4	233	41.8	24.7	18.3	14.9	234	7.2	47	42.3	3.4	234
C. familiaris	25.5	33.7	27.1	13.6	983	22	30.5	22.9	24.4	327	42.3	25	19.8	12.8	328	12.1	45.7	38.4	3.6	328
C. scotocerca	23.2	35.8	26.9	13.9	898	23	29.6	22.3	25	300	39.4	27	20	13.3	299	7.3	50.8	38.4	3.3	299
C. livia	24.7	36	26.4	12.7	1066	22.2	30.4	25.3	21.9	355	40	27.3	19.4	13.2	355	12	50.2	34.5	3	356
C. albicolis	25.6	32.5	28.7	13	925	21.7	31.1	23.7	23.3	308	41.2	25.6	18.8	14.2	308	13.9	40.7	43.6	1.6	309
C. corax	25.4	32.4	29.1	12.9	925	21.7	30.8	23.7	23.7	308	40.9	25.9	18.8	14.2	308	13.5	40.4	44.9	0.9	309
D. aeneus	25.2	31.1	30.8	12.6	1143	20.4	31.2	26.7	21.5	381	40.9	25.1	20.7	13.1	381	14.4	37	45.1	3.4	381
C. macrorhynchos	24.9	32.9	29.1	12.9	1140	21.3	30.7	25.2	22.6	380	40.9	25.7	20.4	12.8	381	12.4	42.4	41.6	3.4	379
D. aldabranus	26.7	28.1	20	16	441	26.5	21.7	24.4	27.2	147	38	25.8	18.3	17.6	147	15.6	36.7	44.2	3.4	147
D. annectans	25.8	30.3	30.9	12.8	1070	20.7	29.9	26.6	22.6	357	41.4	24	21.2	13.1	357	15.4	37	44.9	2.5	356
E. eulophotes	22.6	37.2	27	13	1143	21.2	30.9	26.2	21.5	381	38.5	28	20.4	12.8	381	8.1	52.7	34.3	4.7	381
E. garzetta	24.4	34.5	25.4	15.6	307	25.4	25.4	24.5	24.5	102	36.2	25.4	20.5	17.6	102	11.6	52.4	31	4.8	103
L. punctulata	23	32.8	28.8	15.2	603	22.3	26.3	25.3	25.9	201	37.8	25.8	19.4	16.9	201	8.9	46.2	41.7	2.9	201
L. malacca sinensis	23.1	35.4	28.1	13.2	869	21.7	30.6	23.1	24.4	290	40.3	27.2	19.3	13.1	290	7.2	48.4	42.2	2	289
D. macrocercus	25.3	31.7	29.5	13.4	999	21.9	28.8	26.1	23.1	333	40.8	26.4	19.5	13.2	333	13.2	39.9	42.9	3.9	333
A. ginginianus	22.7	32.2	30.5	14.4	685	23.6	25.4	25.8	25	228	37.2	25	21.9	15.7	228	7.4	46.2	43.6	2.6	229
C. livia	25.1	35.8	26	12.9	1026	22.2	30.7	24.8	22.2	342	40.6	26.9	19	13.4	342	12.5	50	34.2	3.2	342
U. epops	25.5	33.5	27.8	13.1	1097	22.9	28.4	25.6	22.9	366	40.2	27.3	19.1	13.1	365	13.3	44.8	38.5	3.2	366
C. moschata	24.3	34.9	24.5	16.2	1080	23	28.8	23.3	24.7	360	40	27.2	19.7	13	360	10	48.6	30.5	10.8	360
A. tristis	23.9	34.6	27.4	13.9	1048	22.5	29.7	23.7	24	350	40.1	27.7	18.6	13.4	349	9.1	46.4	40.1	4.2	349
Avg.	24.6	33.7	27.9	13.5	911	22.2	29.5	24.7	23.4	303.4	40.2	26.3	19.7	13.7	303.4	11.5	45.3	39.5	3.6	303.5

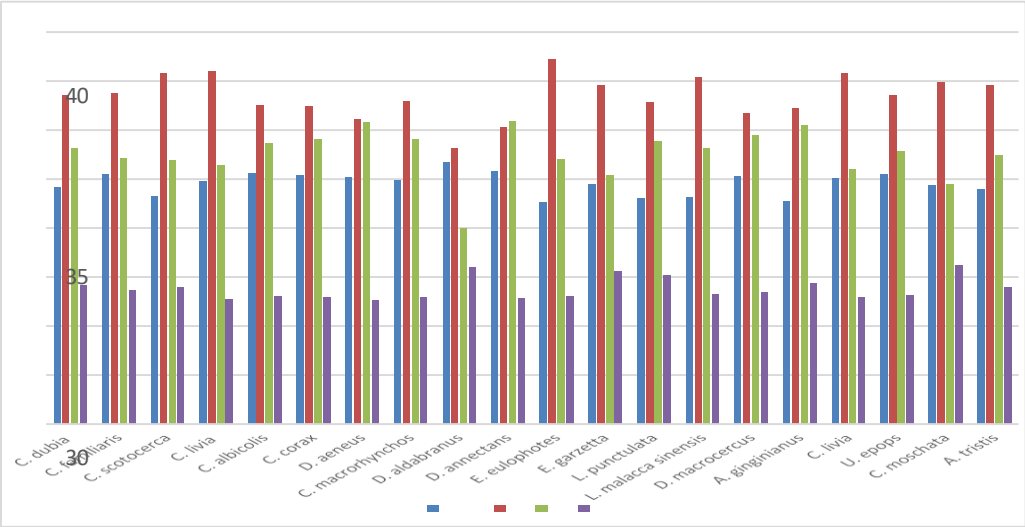


Fig 7: Nucleotide Composition based on Cytochrome b gene

Table 7: Maximum Likelihood Estimate of Substitution Matrix

	A	**T/U**	**C**	**G**
A	-	*3.33*	*4.58*	**9.72**
T/U	*3.79*	-	**24.84**	*1.80*
C	*3.79*	**18.02**	-	*1.80*
G	**20.41**	*3.33*	*4.58*	-

NOTE: Each entry is the probability of substitution (*r*) from one base (row) to another base (column). Substitution pattern and rates were estimated under the Tamura-Nei (1993) model (+G) [1]. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*], parameter = 0.3245). Rates of different transitional substitutions are shown in **bold** and those of transversionsal substitutions are shown in *italics*. Relative values of instantaneous *r* should be considered when evaluating them. For simplicity, sum of *r* values is made equal to 100, the nucleotide frequencies are A = 28.05%, T/U = 24.64%, C = 33.95%, and G = 13.36%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -23329.074. This analysis involved 120 nucleotide sequences. Codon positions included were 1st+2nd+3rd. There were a total of 1146 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Tamura *et al*., 2018).

Table 8.Comparison of BLAST Results of all the species based on Cytochrome b gene

Sample ID	**Common Name**	**Species**	**Accession no.**	**Similarity Index**	**Gaps**	**Cytochrome B Gene Difference**
BM 1 BM 2 BM 3	Bank Myna	***Acridotheres ginginianus***	KJ456174.1	720/720 (100%)	0/720 (0%)	0 %
CM 1 CM 2	Common Myna	***Acridotheres tristis***	KJ456175.1	1143/1143 (100%)	0/1143 (0%)	0%

CM 3						
MD 1 MD 2 MD 3	Muscovy Duck	***Cairina moschata***	L08385.1	1141/1143 (99%)	0/1143 (0%)	1%
CH 1 CH 2 CH 3	Common Hoopoe	***Upupa epops***	KY689872.1	1142/1143 (99%)	0/1143 (0%)	1%
RP 1 RP 2 RP 3	Rock Pigeon	***Columba livia***	KP319029.1	1141/1143 (99%)	0/1143 (0%)	1%
BD 1 BD 2 BD 3	Black Drongo	***Dicrurus macrocercus***	JQ864501.1	1026/1026 (100%)	0/1026 (0%)	0%
MU 1 MU 2 MU 3	Munia	***Lonchura Malacca***	MN991592.1	1129/1143 (99%)	0/1143 (0%)	1%
SBM 1 SBM 2 SBM 3	Scally Breasted Munia	***Lonchura punctulata***	KJ456325.1	1141/1143 (99%)	0/1143 (0%)	1%

Discussion

Accurate species identification plays a crucial role in forensic science, conservation biology, and biodiversity monitoring. This study focused on the molecular identification of bird species in the Sargodha region using the mitochondrial cytochrome b gene, a well-established genetic marker for distinguishing closely related species. Traditional morphological identification can often be misleading, especially in cases where visual differences are minimal—as seen with the common myna (*Acridotheres tristis*) and bank myna (*A. ginginianus*), which are often considered the same by non-experts due to subtle differences in beak color and eye patches. However, molecular analysis revealed a 93% similarity between these two species, confirming that they are genetically distinct members of the genus *Acridotheres*. The cytochrome b gene, known for its reliability in species-level differentiation and phylogenetic analysis, was successfully amplified and sequenced in 24 samples representing eight bird species (Shahin et al., 2024). The resulting sequences were compared with existing data in the NCBI database, verifying that most of the species are already genetically documented. Despite this, the study adds to the growing body of genetic data and confirms cytochrome b as a powerful tool for resolving taxonomic ambiguities and revealing genetic relationships. A comprehensive phylogenetic tree was constructed using the Neighbor-Joining method in MEGA X software, which provided evolutionary insights into the relationships among the studied species (Bilal et al., 2025). Besides species identification, the study also addressed the broader issue of declining avian biodiversity in the Sargodha region. Although the area hosts a rich diversity of bird species, rapid urbanization, deforestation, pollution, and pesticide usage are threatening their natural habitats. Increasing human population pressure has led to habitat fragmentation, tree cutting, and environmental degradation, significantly reducing bird populations year after year. Vehicles and industrial pollution further contribute to habitat loss by increasing smog and air pollutants. This highlights the urgent need for conservation efforts based on scientific evidence, including molecular data (Basharat et al., 2024). The use of mitochondrial DNA, especially the control region (CR), has proven instrumental in

studying genetic variability and evolutionary patterns. The control region consists of three domains that evolve at different rates, with Domains I and II showing higher mutation rates and thus being more suitable for population-level studies. Although earlier studies speculated that slower-evolving domains were better for higher-level phylogenetics, this study and others have shown that rapidly evolving domains can also provide valuable insights. Mitochondrial studies have become increasingly popular for understanding avian evolution, especially given the slow molecular evolution rates in birds (Akbar et al., 2025). In a broader evolutionary context, the study aligns with previous research showing that bird evolution spans over 150 million years, with roots in the Mesozoic and Cretaceous periods. Molecular data have helped resolve key questions about the origins of major bird orders and their divergence before and after the Cretaceous extinction. Studies by Jarvis et al. (2014) and others have shown the power of genetic data in building the avian tree of life. Relationships between aquatic and terrestrial birds, such as flamingos, grebes, pigeons, and sandgrouse, reflect convergent adaptations rather than shared ancestry. This study used *Labeo rohita* as an out-group to root the phylogenetic tree and validate evolutionary relationships. Evolutionary analyses were based on disparity index tests and nucleotide substitution models using MEGA X. Base composition and substitution bias were statistically analyzed, revealing significant variation in sequences. The final dataset included 1143–1146 base pairs across multiple codon positions, providing a robust platform for evolutionary inference (Bilal et al., 2024).

Conclusion

24 samples of 8 different birds were collected from different Tehsils of Sargodha and their mitochondrial genes were used for construction of phylogenetic trees among different members of birds of Sargodha and the genus of Pakistan bird fauna. Cytochrome b gene is most similar in similar birds and remain conserved within specie and differ among different species. The study showed that cytochrome b gene form reliable and accurate genetic tree. The tree showed 616/1143 conserved site and 527/1143 variable sites. Results showed that Cytochrome b gene is also good tool for specie identification. Cytochrome b gene is useful for phylogenetic analysis. In conclusion, the cytochrome b gene is a highly effective molecular marker for identifying bird species, resolving taxonomic uncertainties, and analyzing genetic variation. It not only supports phylogenetic classification but also aids in understanding evolutionary history and biodiversity conservation. Given the environmental challenges and biodiversity loss in the Sargodha region, molecular tools such as DNA barcoding can play a pivotal role in developing targeted conservation strategies. This study reaffirms that mitochondrial markers, particularly cytochrome b, are essential for accurate species identification, evolutionary research, and biodiversity preservation in both local and global contexts.

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