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Research Article

Diversity and molecular identification of bird species from South Punjab, Pakistan

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Abstract

The present one-year study was conducted in selected sites in South Punjab, Pakistan. A total of 2634 bird specimens were recorded belonging to 33 species 12 orders and 23 families. The avifauna recorded was a combination of bird species found in both aquatic and terrestrial habitats. Passeriformes was the most dominant order followed by Columbiformes and Falconiformes. Shannon-Weiner diversity index was calculated as 3.261, Simpson's diversity index was 0.9562 and Evenness was 0.8149 showing homogenized species distribution. DNA of a few species was successfully extracted from whole blood by phenol-chloroform method and quantification was done on Thermo Scientific NanoDrop 2000. DNA was amplified using a COI primer set and after trimming ambiguous bases, the obtained COI fragment was 670 bp. The obtained DNA sequences were submitted to NCBI Genbank and accession numbers were obtained. The neighbor-joining tree was constructed based on p-distance using MEGA 10. The overall, genetic divergence was 0.154±0.009. It can be concluded that compiling a public library of DNA barcodes associated with identified specimens could contribute to a new token for species identification. The present study confirmed the efficacy of COI barcodes in the identification of avifauna from the study area. High bootstrap values in the N-J tree indicate that most of the South Punjab bird species seem to have a related genetic structure. It is recommended that further comprehensive studies on genetic diversity should be conducted across more diverse geographical locations in Pakistan. This would help identify cryptic species and provide a clearer understanding of the phylogenetic relationships among avian species in the region.

Keywords: COI, 16S rRNA, Cytb, Cryptic species, phylogenetic lineage

Introduction

The estimated diversity of planet Earth ranges from 5 to 100 million species while 1.5 million species have not been categorized yet. Hence, many of the species are still unexplored while the authentication of the known species is questioned. The dilemma of identification among cryptic taxa is globally accepted and advanced molecular techniques assist in resolving the issue by comparing amino acid and nucleotide sequences of species to determine genetic divergence and their phylogenetic lineage. Long-term persistence of the species is directly linked with genetic diversity as diversity implies adaptability towards changing environments (Slate & Pemberton, 2002; Mattila & Seeley, 2007).

A crucial component of sustainable agro-ecosystems is biodiversity. Numerous ecological services, such as biological control, which form the basis of bird biodiversity programs, are provided by diverse avian groups. A novel approach for tracking and assisting in the prediction of how management will affect the functioning of different bird communities is the genetic study of a bird's diet (Schmidt et al., 2021). The avian diversity of any ecosystem is the indicator of the health of that ecosystem (Czech *et al.,* 2000) and bird species in Pakistan are as diverse as any other country having similar geographical and climatic conditions (Roberts, 1991). Birds from European and Asian countries migrate annually towards the wetlands of Pakistan to avoid extreme weather conditions. However, their populations in the country are declining at an unprecedented rate due to many anthropogenic activities viz. introduction of non-native species, urbanization, overexploitation, and habitat destruction (Ali et al., 2015). Birds are considered bio-indicators of environmental changes and are used as models to understand the effects of urbanization. They also rapidly respond to landscape transformation, configuration, and ecosystem functioning. Hence, are used to monitor long-term land use changes, environmental disturbances, and urbanization (Turner, 2003). Habitat degradation and improper land use are considered important factors for the loss of biodiversity (Flynn et al., 2009). The increase in human population puts a lot of pressure on natural resources while poverty also fastens exploitation of biodiversity at faster rates in Pakistan (Baig et al., 2009).

Pakistan is home to a pantropical diversity of environments, which attracts avian species to exploit their resources. Pakistan has about 650 bird species, and they are found to be unprecedented in

three zoogeographical zones (Oriental, Palearctic, and Ethiopian areas). For the designation of biodiversity hotspots, species abundance, habitat suitability, and endemism play the main role (Myers et al., 2000; Prendergast et al., 1993). The main purpose of conservation is to identify threats to the species and to eliminate them (Caughley, 1994) however it depends upon precise species identification (Mace, 2004). In a population, the genetic diversity of individuals influences the range of evolutionary and ecological aspects. Previous studies indicated that an individual's fitness is linked with genetic diversity (Hughes et al., 2008), permitting a species to maintain and adapt in alternating environments (Lenoormand, 2002; Garant et al., 2007). Within populations, genetic diversity may have environmental significances and the variations in genetic diversity can be derived by ecological factors (Vellend & Geber, 2005).

Despite knowing importance of biodiversity, human pressure on biodiversity is increasing and maintenance of genetic variability in declining populations is the major concern of the conservation biologists. Biological diversity is studied at three levels i.e. genetics, ecosystems and species in a given geographical region. Genetic diversity is measured as intraspecific genetic variation and provides valuable data regarding historical pattern of a population fragmentation, population subdivision, evolutionary background of a population and gene flow (Bates et al., 2003). By eating arthropod pests, birds can serve as particularly significant providers of ecosystem services to farmers. However, certain bird species can occasionally behave pestily by eating crops. Additionally, changes in the make up of the bird community can affect the balance of ecosystem benefits and negative effects provided by birds, which is influenced by on-farm management practises (such as crop diversification and vegetative structure) and the complexity of the surrounding environment (Garcia et al., 2023). Furthermore, rate of mutation and number of individuals that can contribute to the next generation are helpful in determining levels of genetic diversity (Bazin et al., 2006). However, problems in collection of genetic data and expensive techniques make it difficult to measure genetic diversity in animal phyla while conservation demands measuring species on genetic basis (Moritz & Faith, 1998).Each method's precision can differ between animal species and can overlook some groups, leading to sampling bias (Prendergast & Hogendoorn, 2021). Conservation genetics emphasizes assessment of the populations, species and sub-species on molecular basis for proper management and conservation of declining taxa. In this context, development of permanent genetic resources like autosomal microsatellites is of importance. For conservation genetics, microsatellites, commonly named as

simple sequence repeats (SSRs) are considered ideal markers (Sunnucks, 2000; Selkoe & Toonen, 2006).

Native species are considered major genetic resources for maintenance of genetic variations introducing valuable characteristics with changing environmental factors; hence the fitness of the individuals is affected by loss of genetic variability (Fulton & Delany, 2003). 668 from Pakistan and 2700 are reported from asia. Natural vegetation has been changed to agricultural fields over the last 10,000 years, and generalist species have adapted to new environments while habitat-specific species have wiped out (Di Giulio et al., 2009).

Morphological identification of species requires confirmation through molecular analysis as many resembling taxa have been identified as separate species through molecular analysis. Hence, DNA barcoding or DNA taxonomy has gained attention of the biologists to exactly identify a species (Vences et al., 2005). Identification of species is essential for successful ecological protection and management, but it can be difficult to achieve for physically cryptic species. Rapid advancements in molecular techniques have provided tools for determining similarity between apparently similar species and determining morphological traits that can be used to distinguish them (Herbert et al., 2004).

Human population is increasing at an unprecedented rate, crossed 7 billion people and supposed to cross 9 billion by 2050. This population increase is blamed to lead many species to extinction while others are struggling hard for their survival. However, the loss of genetic variability also negatively affects species' adaptabilities towards changing climates. There is dire need to conserve avifauna present in the country. The present research was therefore planned to understand the relationship between genetic diversity and fitness in selected avian species.

Material and Methods

Study area

This one year study was carried out in selected districts of South Punjab, Pakistan. Southern Punjab is administratively divided into Multan, Vehari, Khanewal, Lodhran, Bahawalpur, Bahawalnagar, Rahim Yar Khan, Dera Ghazi Khan, Layyah and Rajanpur. The details of sub-sampling sites, habitat types, GPS coordinates and elevation is given in Table 1. The study area face extremely hot climate in summer (March-August) to severe cold in winter (December to January). Annual precipitation is around 500 mm on average.

Habitat types

The habitat types included urban habitat (UH): consisted of houses, roads and have almost no plantation, cropland habitat (CLH) consisted of agriculture land and plantations, Desert/forest land habitat (DFH) consisted of sand and as well as forest plantations. Prominent vegetation in study area includes gum arabic acacia (*Acacia senegal*) and euphorbia (*Aleurites moluccana*), khajri tree (*Prosopis cineraria*), athel (*Tamarixaphylla*), jand(*Prosopis cineraria*), kans grass (*Saccharumspontaneurn*), goose grass (*Eleusinecompressa*), shisham (*Dalbergiasissoo*), Indian plum (*Zizyphusmauritiana*) and Kikar or thorn-tree (*Acacia nilotica*).

Sample collection

Each captured bird species was identified following (Grimmett et al., 2008). Non-invasive methods were used to extract total genomic DNA from feathers and blood samples. Blood samples were collected in EDTA tube from each captured species and then released into their natural habitat after sampling. Blood and feathers samples were brought to the Postgraduate Lab, Department of Wildlife and Ecology, University of Veterinary and Animal Sciences Ravi Campus for molecular characterization.

Table 1. Details of study area, sub-sampling sites, habitat type and GPS coordinates.

Sampling stations	Habitat Types	Location	Elevation (ft)
Multan			
Chakbhedda	Forest/desert	N 30º10.297,EO71º30.443	365
Hassan Wali	Crop land	N 30º.07.792.¨E071.27.594	367
Jinnah Park	Urban area	N 30º 10.297.E071°30,451	423
Bahawalpur			
Cholistan 69F	Desert area	N29º.39.890,E072º 28.85	456
Head Islam	Urban area	N29º196.,E072,32.942	466
Bahawalpur Zoo	Urban area	29o23'44 N, 71o41'01 E	388
Bahawalnagar			
TilokaLona	Crop land	N29º 56”392.E072º 53.538	533
Latif Abad	Desert area	N29º24”.497,E072º.50.556	510
BWN Zoo	Urban area	N30º.00.192,E072º,16.352	535
Rahim Yar Khan			
Chak 46/P	Desert area	N28º,26.275,EO70º31.197	245
City	Urban area	N28º.25,175,EO70.26,020	260
Allaabad	Crop land	N28º,55,125.EO70º52,240	272
Dera Ghazi Khan			
GOVT College	Urban area	N30º.193,EO70,38.206	6447
ChakPaigha	Crop land	N29º,59.994”E070º38,988	6423
Rajanpur			
Noushera East	Cropland area	N29º,12.946”E070,31.034	4822
MouzaSaidpur	Desert area	N29º,12,699,”E070,33,2865	4856
Fazalpur	Urban area	N29º,17.490,”EO70,27.034	4834

DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted by phenol chloroform method. Purity of DNA was checked through agarose gel electrophoresis and quantified using Thermo Scientific NanoDrop (Ali et al.,2017). DNA was amplified using primers set mentioned in (Table 2). PCR amplification was done following Ali et al. (2020). PCR products were checked on 1% agarose gel. Purification of PCR products was done using the GeneJET PCR purification kit and each DNA sample was Sanger sequenced from Macrogen Korea.

Data analysis

The obtained DNA sequences were analyzed and edited in Bioedit software 7.0. Each DNA sequence was subjected to BLAST analysis to download the closely matched sequences from NCBI genbank. The closely related sequences were incorporated in the neighbor-joining (NJ) tree analyses using100 bootstrap replicates in MEGA 10. Genetic diversity was calculated by Mega 10 based on p-distance (Ali et al., 2020). Sequence Identity matrix was plotted using Bioedit software 7.0.

Table 2. List of primers and annealing temperature of primers for PCR amplification used during present study.

Marker	**Primer Pair**	**Sequence (5′–3′)**	**Annealing condition**
COI	LCO1490	GGTCAACAAATCATAAAGATATTGG	50°C for 40 seconds
	HCO2198	TAAACTTCAGGGTGACCAAAAAATCA	
	BirdF1	TTCTCCAACCACAAAGACATTGGCAC	51°C for 40 seconds
	BirdR1	ACGTGGGAGATAATTCCAAATCCTG	
	CO1F	TTCTCGAACCAGAAAGACATTGGCAC	48°C for 45 seconds
	CO1R	ACTTCTGGGTGGCCAAAGAATCAGAA	
Cytb	L14841	AAAAAGCTTCCATCCAACATCTCAGCATGATCAAA	55°C for 1 min
	H15149	AAACTGCAGCCCCTCAGAATGATATTTGTCCTCA	
	L14816	CCATCCAACATCTCAGCATGATGAAA	50°C for 45 seconds
	H15173	CCCCTCAGA ATGATATTT GTC CTCA	

Results

Diversity and relative abundance

Present one year study was conducted at selected districts of South Punjab, Pakistan namely Multan, Vehari, Khanewal, Lodhran, Bahawalpur, Bahawalnagar, Rahim Yar Khan, Dera Ghazi Khan, Layyah and Rajanpur.Map of the study area was created by using ArcGIS software at GIS and Remote Sensing Lab of the Department of Wildlife and Ecology, University of Veterinary and Animal Sciences Ravi Campus (Figure 1). During present study a total of 2634 birds specimens were recorded belonging to 33 species 12 order and 23 families. The avifauna recorded was a combination of bird species found in both aquatic and terrestrial habitats. The detail of recorded birds species and relative abundance is mentioned in Table 3.

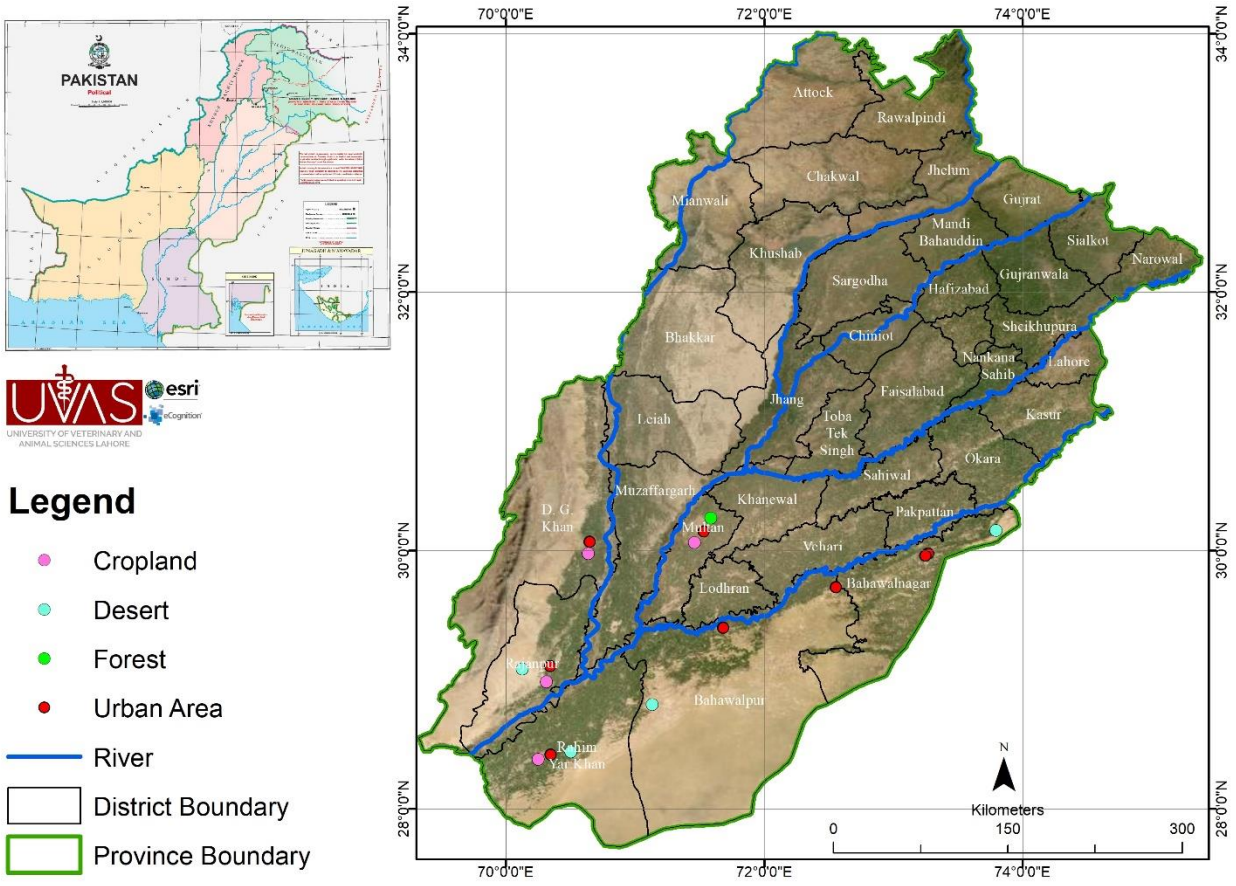

Figure 1. GIS based map of the study area showing different habitat types.

Diversity index

Passeriformes was the most dominant order represented by 16 species belonging to 11 families, followed by Columbiformes and Falconiformes, which were represented by 3 species belonging

to 1 family each. Order Coraciiformes was represented by 2 species with 2 families and Ciconiformes was represented by 2 species belonging to 1 family. Order Galliformes, Anseriformes, Piciformes, Cuculiformes, Gruiformes, Psittaciformes and Charadiiformes were represented by single species each belonging to single family. Different studies was conducted in Pakistan on different species of avifauna and the number of studies are increasing by each passing year (Fig. 2).

Table 3. Details of recorded birds species from study area and their relative abundance.

Order	**Family**	**Common Names**	**ScientificName**	**Number of specimens recorded (n)**	**Relative abundance (Pi)**
Galliformes	Phasianidae	Grey Francolin	*Francolinus pondicerianus*	50	0.018983
Anseriformes	Anatidae	Mallard	*Anas platyrhynchos*	118	0.044799
Piciformes	Picidae	Black-rumped flame back	*Dinopium benghalense*	60	0.022779
Psittaciformes	Psittacidae	Rose-ringed parakeet	*Psittacula krameri*	17	0.006454
Coraciiformes	Upupidae	Eurasian hoopoe	*Upupa epops*	69	0.026196
	Ancidinidae	Brown-breasted kingfisher	*Halcyon gularis*	111	0.042141
Cuculiformes	Cuculidae	Crow peasant	*Centropus sinensis*	121	0.045938
Gruiformes	Rallidae	White-breasted water hen	*Amaurornis phoenicurus*	34	0.012908
Columbiformes	Columbidae	Common pigeon	*Columba livia*	153	0.058087
		Eurasian collared dove	*Streptopelia decaocto*	98	0.037206
		Laughing dove	*Streptopelia senegalensis*	81	0.030752
Charadiiformes	Charadriidae	Red-wattled lapwing	*Vanellus indicus*	191	0.072513
Ciconiformes	Ardeidae	Indian pond heron	*Ardeola grayii*	132	0.050114
		Cattle egret	*Bubulcus ibis*	99	0.037585

Falconiformes	Accipitridae	Black kite	*Milvus migrans*	37	0.014047
		Shikra	*Accipiter badius*	31	0.011769
		Long-legged buzzard	*Buteo rufinus*	10	0.003797
Passeriformes	Corvidae	House crow	*Corvus splendens*	161	0.061124
		Rufous treepie	*Dendrocitta vagabunda*	67	0.025437
	Dicruridae	Black drongo	*Dicrurus macrocercus*	60	0.022779
		Black redstart	*Phonicurus ochruros*	15	0.005695
	Muscicapidae	Brown rock chat	*Cercomela fusca*	45	0.017084
	Sturnidae	Bank myna	*Acridotheres ginginianus*	188	0.071374
		Common myna	*Acridotheres tristis*	165	0.062642
	Hirunidinidae	Barn swallow	*Hirundo rustica*	89	0.033789
		Wire-tailed swallow	*Hirundo smithii*	13	0.004935
	Pycnonotidae	Red vented bulbul	*Pycnonotus cafer*	46	0.017464
	Cisticolidae	Rufous-fronted prinia	*Prinia buchanani*	25	0.009491
	Tmaliidae	Jungle babbler	*Turdoides striatus*	22	0.008352
	Nectariniidae	Purple sunbird	*Nectarinia asiatica*	61	0.023159
	Passeridae	Eurasian tree sparrow	*Passer montanus*	78	0.029613
		House sparrow	*Passer domesticus*	136	0.051632
	Motacillidae	White wagtail	*Motacilla alba*	51	0.019362

The order of abundance was Red-wattled lapwing (*Vanellus indicus*), Bank myna (*Acridotheres ginginianus*), Common myna (*Acridotheres tristis*), House crow (*Corvus splendens*), Common pigeon (*Columba livia*) and Mallard (*Anas platyrhynchos*). The diversity indices of avifauna calculated from study area is mentioned in Table 4. Evenness of bird species was 0.8149 showing homogenized species distribution. Shannon-Weiner diversity index value was estimated to be 3.261. Simpson's diversity index value was estimated to be 0.9562.

The Simpson diversity index (SDI) is an important measure of diversity that provides ecologists with a powerful tool for understanding and managing ecosystems. The Simpson index considers both the number of species and their relative abundance in a group. Because it takes into account more factors than just the number of species, it is a more accurate indicator of diversity than simple species richness. The measured SDI for this study was noted as 0.0303 (Table 5).

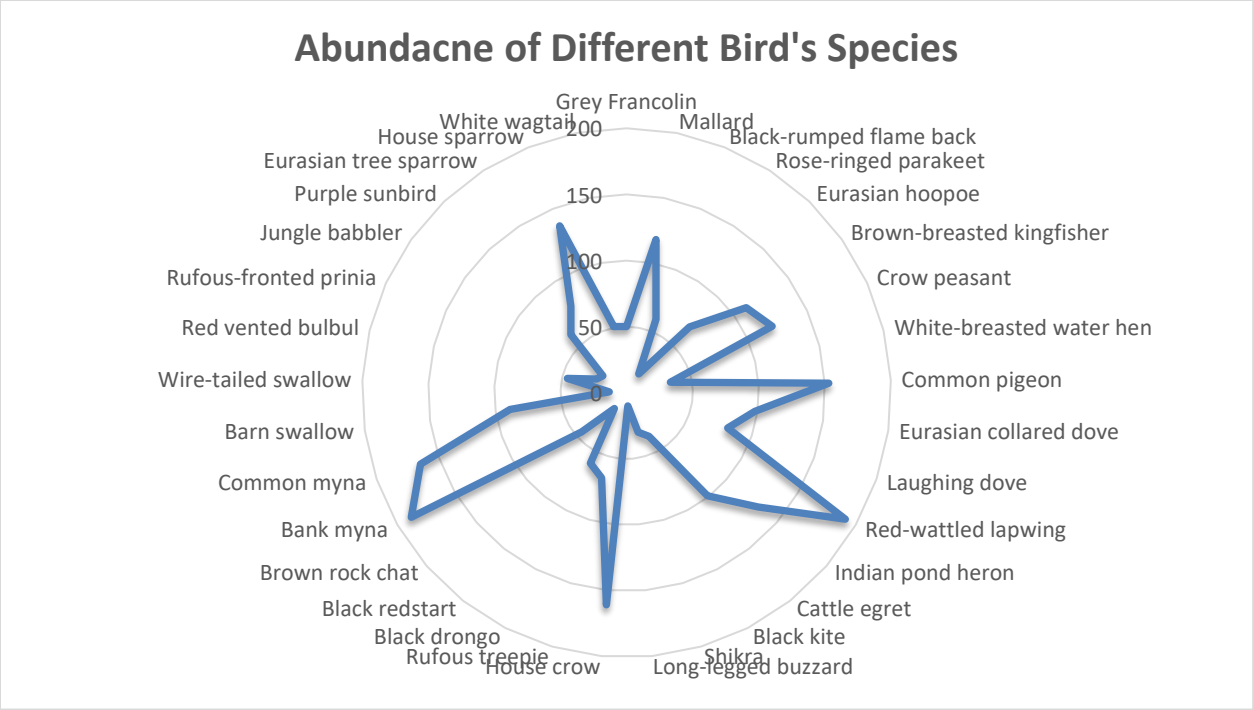

Figure 2. Abundance of Different Bird's Species from the studied area

DNA Extraction

During present study, DNA of common myna (*Acridotheres tristis*), common pigeon (*Columba livia*), house crow (*Corvus splendens*), black rumped flame back (*Dinopium benghalense*), house sparrow (*Passer domesticus*), rose-ringed parakeet (*Psittacula krameri),* red vented bulbul (*Pycnonotus cafer),* laughing dove (*Streptopelia Senegalensis*), eurasian hoopoe (*Upupa Epops*), and grey francolin (*Francolinus pondicerianus)*successfully extracted from whole blood by phenol chloroform method. Purity of DNA was checked through 1% agrose gel (Figure 3). DNA quantification was done on Thermo Scientific NanoDrop 2000 (Table 6).

Table 4. Diversity indices of avifauna from the study area.

Diversity Indices	**Recorded Diversity Indices**	**Lower**	**Upper**
Taxa	32	-	-

Individuals	2634	2634	2634
Dominance D	0.04375	0.04262	0.04567
Simpson 1-D	0.9562	0.9543	0.9573
Shannon H	3.261	3.232	3.277
Evenness e^H/S	0.8149	0.7917	0.8282
Margalef	3.939	3.939	3.939

Table 5. Simpson's Diversity index of avifauna from the study area.

Diversity Indices	**No. of Individuals**	**(N – 1)**	**N(N – 1)**
Taxa	32	31	992
Individuals	2634	2633	6935322
Dominance D	0.04375	-0.95625	-0.04184
Simpson 1-D	0.9562	-0.0438	-0.04188
Shannon H	3.261	2.261	7.373121
Evenness e^H/S	0.8149	-0.1851	-0.15084
Margalef	3.939	2.939	11.57672
	ΣN= 2675.015	Σ = 2668.015	Σ = 6936333

*SDI – 0.0303

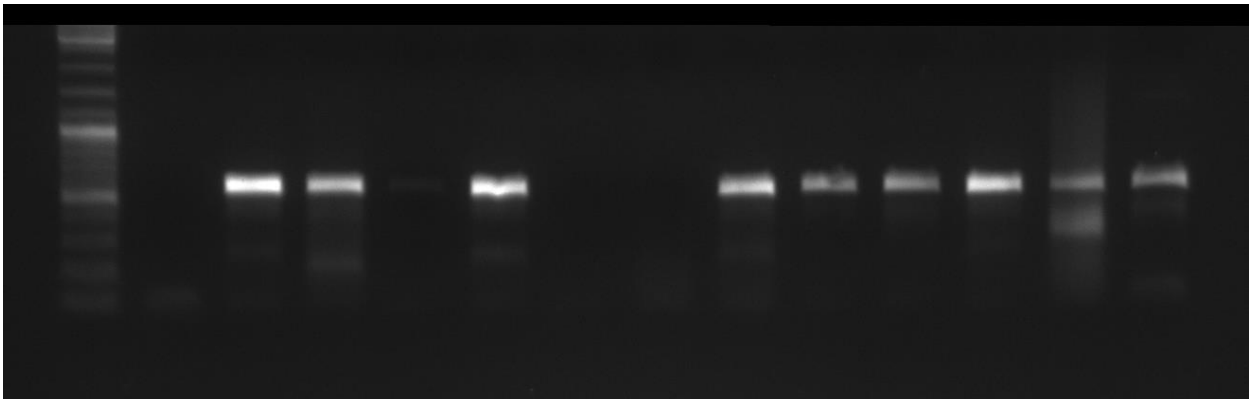

Figure 3. Successful amplified DNA samples.

Amplification and sequencing

DNA of common myna (*Acridotheres tristis*), common pigeon (*Columba livia*), house crow (*Corvus splendens*), black rumped flame back (*Dinopium benghalense*), house sparrow (*Passer domesticus*), rose-ringed parakeet (*Psittacula krameri),* red vented bulbul (*Pycnonotus cafer),*

laughing dove (*Streptopelia Senegalensis*), eurasian hoopoe (*Upupa Epops*) and grey francolin (*Francolinus pondicerianus)* was amplified using COI primer set mentioned in Table 2. The obtained DNA sequences checked and ambiguous bases were removed in MEGA 10. After trimming ambiguous bases, the obtained COI fragment was 670 bp.The obtained DNA sequences have shown reliable and clear species identification of all the recorded specimens. The DNA sequences were submitted to NCBI Genbank and Accession numbers were obtained. The detail of voucher numbers and NCBI accession numbers is given in Table 7.

Table 6. Quantification of DNA samples using NanoDrop.

Species	**Nucleic Acid (ng/uL)**	**A260/A280**	**A260/A230**	**A260**	**A280**	**Corrected (ng/uL)**
Acridotheres tristis	468.601	1.618	0.934	9.372	5.791	259.45
Columba livia	468.541	1.561	0.823	9.173	5.391	258.99
Corvus splendens	468.311	1.489	0.876	9.272	5.611	258.68
Dinopium benghalense	467.987	1.698	0.912	9.283	5.587	259.11
Passer domesticus	468.891	1.576	0.983	9.865	5.765	258.87
Psittacula krameri	324.5	1.618	0.934	9.372	5.791	259.45
Pycnonotus cafer	289.0	1.561	0.823	9.173	5.391	258.99
Streptopelia senegalensis	207.52	1.549	0.89	4.15	2.679	103
Upupa epops	207.11	1.489	0.87	4.11	2.589	102
Francolinus pondicerianus	210.13	1.549	0.89	4.15	2.679	104

Table 7. The detail of voucher specimens numbers and NCBI accession numbers of birds species.

Common Name	**Scientific Name**	**Voucher Number**	**Accession numbers**
Common myna	*Acridotheres tristis*	ZMUVAS100	OL468633.1
Common pigeon	*Columba livia*	ZMUVAS102	OL468685.1

House crow	*Corvus splendens*	ZMUVAS103	OL468735.1
Black-rumped flameback	*Dinopium benghalense*	ZMUVAS104	OL468738.1
House sparrow	*Passer domesticus*	ZMUVAS105	OL468742.1
Rose-ringed parakeet	*Psittacula krameri*	ZMUVAS106	OL468764.1
Red vented bulbul	*Pycnonotus cafer*	ZMUVAS107	OL468766.1
Laughing dove	*Streptopelia senegalensis*	ZMUVAS108	OL468798.1
Eurasian hoopoe	*Upupa epops*	ZMUVAS109	OL468799.1
Grey Francolin	*Francolinus pondicerianus*	ZMUVAS110	OL468807.1

Phylogenetic analysis

Each obtained DNA sequence was subject to BLAST analysis at NCBI to check the similar sequences. Closely matched sequences were downloaded and incorporated in Neighbor joining tree analysis. Neighbor joining tree was constructed based on p-distance using MEGA 10 (Figure 4). Next to the branches is the percentage of replicate trees in which the corresponding taxa assembled together in the bootstrap test (100 replicates).The evolutionary distances were obtained using the p-distance method and are represented in base differences per site.

Genetic diversity and variation

The overall, genetic divergence was 0.154±0.009. Simpson's diversity is also measured for the said data to know about the accurate biodiversity of the species. The significant values in range of $\Sigma N = 2675.015$ and $\Sigma = 6936333$ were noted for this study (Table 5). The amino acid sequence was maintained that was used for primer formation and the molecular identification of studied species of birds (Table 8).

Validation of Sequence Similarity is determined by using the blast results, the validation of the sequence similarity with known sequences was measured in public databases. This demonstrates the credibility of this research and supports the current work. The evolutionary relationships, functional annotations, and structural motifs of this study was demonstrated for the deeper study.

Table 8. The used amino acid sequence for the molecular identification

Marker	Primer Pair	Sequence (5′–3′)
COI	LCO1490	Leu-Thr-Gln-Gly-Asp-Pro-Lys-Lys-Ile

	HCO2198	Stop Thr Ser Gly Stop Pro Lys Lys Ser
	BirdF1	Lys Arg LeuValPhenLeuStop ProCys
	BirdR1	Thr Trp Glu Lle Iie Pro Asn
	CO1F	Lys Ser Leu Val Leu Ser Val Thr Val
	CO1R	Thr-Ser-Gly-Trp-Pro-Lys-Asn-Gln-Stop
Cytb	L14841	Phe-Phe-Glu-Gly-Ser-Val-Glu-Ser-Tyr.
	H15149	Lys-Leu-Gln-Pro-Leu-Arg-Met-Ile-Phe-Val-Pro-Ser
	L14816	Gly-Arg-Leu-Met-Ser-Val-Leu-Phe.
	H15173	Pro-Leu-Arg-Met-Ile-Phe-Val-Leu-Stop

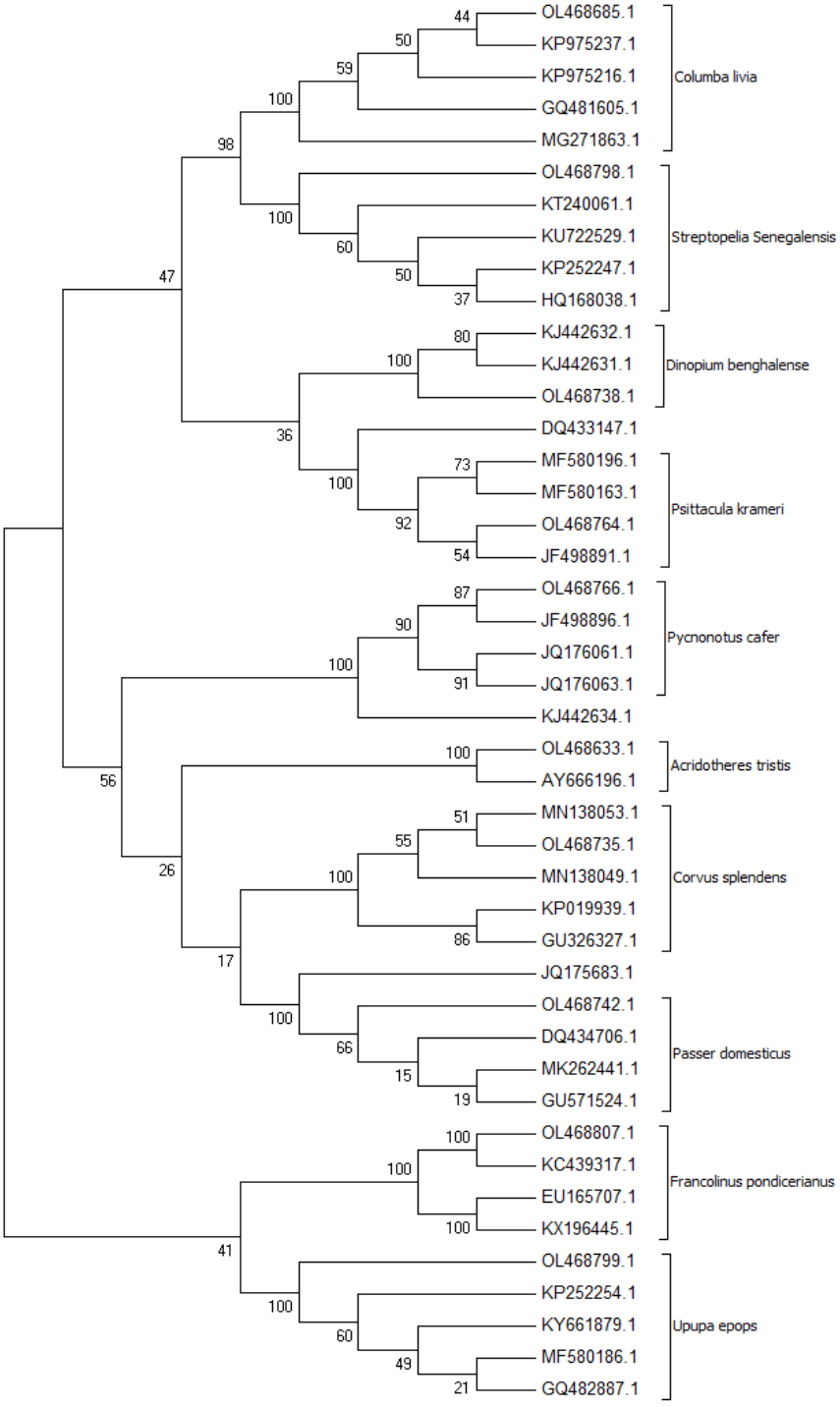

Figure 4. Neighbor-Joining tree of COI gene.

Discussion

This one year study was conducted in selected districts of South Punjab, Pakistan. The study area face extremely hot climate in summer (March-August) to severe cold in winter (December to January). Annual precipitation is around 500 mm on average. Food resources abundant in the research area, including small invertebrates, fruits, grains and human excrement which nourish most Passeriformes species such as the common myna, barn swallow, and house sparrow. During present research a total of 2634 birds specimens were recorded belonging to 33 species 12 order and 23 families. The avifauna recorded was a combination of bird species found in both aquatic and terrestrial habitats. Passeriformes was the most abundant order represented by 16 species belonging to 11 families, followed by Columbiformes and Falconiformes, which were represented by 3 species belonging to 1 family each. For example, common myna scavenges among grasses for insects, especially grasshoppers, which is why it is given the generic name Acridotheres, "grasshopper hunter."

The research study on biodiversity and molecular identification of bird species from South Punjab, Pakistan, contributes significantly to our understanding of the avian diversity in this region. By employing molecular techniques, the study enhances the accuracy of species identification, which is crucial for conservation planning and management. Similar to this study COI barcodes were created for 260 different bird species in North America and discovered that identifying species was often easy by using the molecular technique.

Prominent vegetation in study area was providing food as well as protection and also serves as a habitat for various species' nests including house sparrow, common myna and jungle Babbler. This strongly favors the Passeriformes order. Diverse plant species have a significant relationship with the provision of shelter, which includes nesting area, nourishment, and water, scorching, and brooding facilities (Mahboob et al., 2013).

The relative abundance of birds was also recorded, and a table was constructed that indicates the dominating bird species in the research area. The order of abundance was red-wattled lapwing (*Vanellus indicus*), bank myna (*Acridotheres ginginianus*), common myna (*Acridotheres tristis*), house crow (*Corvus splendens*), common pigeon (*Columba livia*) and mallard (*Anas platyrhynchos*).The relative abundance of birds may be correlated to the reproductive cycle, environmental factors, and diet supplementation (Bibi & Ali, 2013). Seasonal fluctuations in bird species abundance are caused by the varied seasonality of precipitation and annual fluctuation in the amount of food resources (Gaston et al., 2000). The composition of the flora that makes up a

large part of many bird species' habitats determines their distribution and abundance. A single bird species can arise, fluctuate in abundance, and disappear as flora varies along multiple biotic and abiotic gradients (Lee & Rotenberry, 2005).

Hunting and shooting of migratory birds reach at maximum as the arrivals and departure starts in the area ultimately effect migratory bird's population. The major threats, emerging in the recent years, are thought to account for the accelerating migratory bird's population decline. Some major threats include the habitat loss, illegal and excessive shooting, use of pesticide etc. Habitat loss can be attributed primarily to the ever increasing pressure on wetlands. Unregulated human activities such as farming, livestock grazing, and settlements have led both to the reduction in overall wetland size and the fragmentation of wetlands, resulting in less suitable habitat (Harris & Mirande, 2013).

Conclusion

The present study provides an in-depth analysis of the diversity and molecular identification of bird species from South Punjab, Pakistan. A total of 2634 bird specimens representing 33 species, 12 orders, and 23 families were recorded across various terrestrial and aquatic habitats. Passeriformes emerged as the most dominant order followed by Columbiformes and Falconiformes. Molecular analysis of selected species using COI primers yielded clear and reliable DNA barcodes for species identification. The obtained genetic sequences were submitted to NCBI GenBank, contributing to the creation of a reference library for future avian studies. The genetic divergence observed was 0.154±0.009, indicating that the bird species in South Punjab exhibit distinct genetic structures, with some species showing relatedness. High bootstrap values in the constructed neighbor-joining tree further support the validity of the molecular identification process, highlighting the potential of COI barcodes for accurate species recognition.

It is recommended that further comprehensive studies on the genetic diversity should be conducted across more diverse geographical locations in Pakistan. This would help identify cryptic species and provide a clearer understanding of the phylogenetic relationships among avian species in the region. This will help in establishing a DNA barcode database of avian species that could aid in the quick and accurate identification of birds.

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