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Embarking on a Regal Voyage through the Avian Fauna of Sargodha District via DNA Barcoding

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Abstract

Pakistan contains about 792 bird species, while the Sargodha region contains about 34 bird species but no detailed DNA barcoding analysis of various bird species in Sargodha has been undertaken so far. The study was planned to generate the barcode data of Birds. Mitochondrial DNA is the latest method for studying the genetic diversity of birds. Complete genomic DNA extraction was done by using Organic DNA extraction method and Gel Electrophoresis is used for qualitative analysis. By using PCR purified mtDNA was amplified and for further processing amplified copies of DNA were obtained. Before to the process of PCR primers were designed. Multiple regions of amplified DNA were obtained and sent to Alpha Genomics, Rawalpindi for sequencing. Sequencing outcomes were analyzed to determine the genetic diversity of Avian Fauna inhibiting the Sargodha District. The construction of trees by using MEGA X explained all specific interactions between the bird's species under research and the related birds. The heterogeneity and homogeneity between bird species are elobrated in the form of tables that have been drawn along with tables of amino acids to identify potential similarities between avian fauna at different molecular levels of gene products. It is concluded thatMitrochondrial COI is a significant marker for identification of species and the study of molecular phylogenetics in birds. This analysis, however also states the need for expanded taxon sampling and for more genes to be analyzed in order to solve taxonic and phylogenetic problems concerning

birds. Furthermore, DNA Barcoding leads a great deal to the survival of endangered species and to the monetisation of their illicit trade.

Keyword: DNA Barcoding, Avian fauna, COI, PCR, Pakistan

INTRODUCTION

DNA barcoding is an advance, accurate and fastest method for the identification of many species. A standard portion of gene is used as a specie tag in DNA barcoding. As the taxonomist classify different organisms into groups and sub-groups, so DNA barcoding is very helpful tool for the taxonimist for naming many unknown specimens to the known species (J Tizard *et al*., 2019). Besides this DNA barcoding has played an important role in preservation of the natural resources, protecting those species which are threatened and endangered, agricultural pest which are very harmfull for the crops are also controlled by the DNA barcoding, identifing disease vectors, also moniter the quality of water. An important appilication of DNA barcoding is authentication of health products and also identify the medical products.DNA barcoding is very advantageous for checking meat adultration (A.C Dimitriou *et al*., 2017). Also a reliable tool for the authentication of commercial sea products (Nicole S *et al*., 2012).

Taxonomists use mitochondrial DNA with short genetic markers in an organism to determine that it belongs to a certain genus using DNA barcodes. By concentrating and analyzing a standardized genome in a short section of DNA, DNA barcodes ensure rapid and authentic species identifications (F. Pasha *et al*., 2020). Hebert *et al*., (2003) first revealed with evidence that DNA barcodes are used to differentiate species from certain phyla in the animal kingdom by analyzing short sequences of DNA (i.e. the subunit Cytochrome c oxidase I or COI) in closely related species.

Today, a well-established technique for identifying animal species, i.e. DNA barcode using cytochrome c oxidase I subunit of the mitochondrial gene (COI), is used, especially in mammals, birds, fish, and amphibians (S. Trivedi *et al*., 2020). The mitochondrial genome contains a 648 base pair fragment of the 5 'end of cytochrome c oxidase (COI) subunit I, which is an advanced method for organism identification and classification.

Instead of morphological research, taxonomic resolution by DNA barcoding is much easier and a partial solution to the decrease of conventional taxonomic awareness. DNA barcoding is also very useful for species delimitation, defining species borders as well as a very useful method for identifying new species.It can be very authentic and reliable tool as it has ability

to confirm the food and also has an important role for the protection of the biodiversity based on genetic resources and their wild relatives (Bondoc *et al*., 2013).It has been proved through various studies that COI sequence differentiation is very less within the species (generally less then 1-2%), but this differentiation is much more percent in closely related species in animals so it is a very advantageous role in species identification (A.Naseem *et al*., 2020).DNA barcoding also plays an significant role in the survival of endangered species.

Over the last century, the detection of species using DNA barcoding, a molecular technique, has become a rapidly evolving field.Through contrast to an open access website, DNA barcoding depends on a standardized area of the mitochondrial gene being translated, sequenced and analyzed.By using molecular taxonomy to classify animals, the core purpose of DNA barcoding is to establish a biological barcode and also to form a reference library for the identification of particular species (Kerr *et al*., 2007). Since ecosystems are increasingly declining, the biological consequences of the global warming make it necessary to classify animals for the survival of biodiversity. Millions of flora and fauna is living on the earth, out of which we are familiar with less then 2 million species.Due to different causes, the loss of animals and plants grows day by day, meaning thousands of them are killed each year, but most of them are not noticed. This habitat degradation and endangerment has led to an enhanced species recognition programe. An innovative and modern ecological approach, i.e. For the recognition of organisms and ecological analysis, DNA barcoding is used (Ball and J.R. deWaaed *et al*., 2003). DNA barcoding a rapid and accurate species identification tool has revolutionized the world (Jhon Waugh *et al*., 2007). The study was planned to generate the barcode data of Birds. Mitochondrial DNA is the latest method for studying the genetic diversity of birds.

MATERIALS AND METHODS

Sample Collection

Tissue samples of various birds were collected from locations within the District of Sargodha, including Bhalwal, Silanwali, Khushab, and others. A variety of bird species were chosen for the experiment. Tissue samples were taken from them. The samples were held at -20°C in the refrigerator. Freezing is the usual procedure for storing DNA samples, which can be stored at (-20°C) to avoid decay, although it is preferable to store at (-80°C) for longer periods of time or for archive samples.

Table 1: Collected Sample Data

Sr no.	Species Name	Sample ID	Sample type	Accession no.	source	Conservation status	Coordinates
1.	Bank myna (*Acridotheres ginginianus)*	BM1 BM2 BM3	Tissue	EU525242.1	Sillanwali	Least concern	31.848903, 72.651007
2.	Common Myna (Sturnus tristis)	CM1 CM2 CM3	Tissue	JF498821.1	Sillanwali	Least concern	31.808220, 72.616914
3.	Muscovy Duck (Cairina Moschata)	MD1 MD2 MD3	Tissue	FU755254.1	Bhalwal	Least concern	31.983960, 72.319848
4.	Hoopoe (Upupa epopos)	CH1 CH2 CH3	Tissue	MK060147.1	Sahiwal	Least Concern	31.983960, 72.319848
5.	Rock Pigeon (Columbia livia)	RC1 RC2 RC3	Tisssu	KF926376.1	Bhalwal	Least concern	31.983960, 72.319848
6.	Black drongo (*Dicrurus macrocercus)*	BD1 BD2 BD3	Tissue	KJ442635.1	Bhalwal	Least concern	31.983960, 72.319848
7.	Tricolored munia (Lonchura malacca)	TM1 TM2 TM3	Tissue	KC439323.1	Sargodha	Least concern	31.983960, 72.319848

8.	Scaly breasted munia (Lonchura punctulata)	SM1 SM2 SM3	Tissue	JF498872.1	Sargodha	Least concern	31.983960, 72.319848

Preservation

Freezing is the best method of preservation. Samples were preserved at -20 ℃ in refrigrator to prevent from degradation.

DNA Extraction Method

Phenol chloroform (Organic) method was used for DNA extraction.

Lysis

200mg of heart tissues were picked and homogenized by using steel homogenizers in 500 µl of lysis buffer and incubated for 20-30 minutes at room temperature. Samples were mixed gently to ensure the proper homogenization. Samples were centrifuged for 3 minutes at 13000 rpm for phase separation. The supernatant was discarded while the pellet containing DNA was further processed, multiple washings with lysis solution were done to prevent contamination. Pellet was again treated with 400 µl lysis solution, 13 µl of 20% SDS (Sodium Dodecyl sulphate) and 25 µl proteinase K.

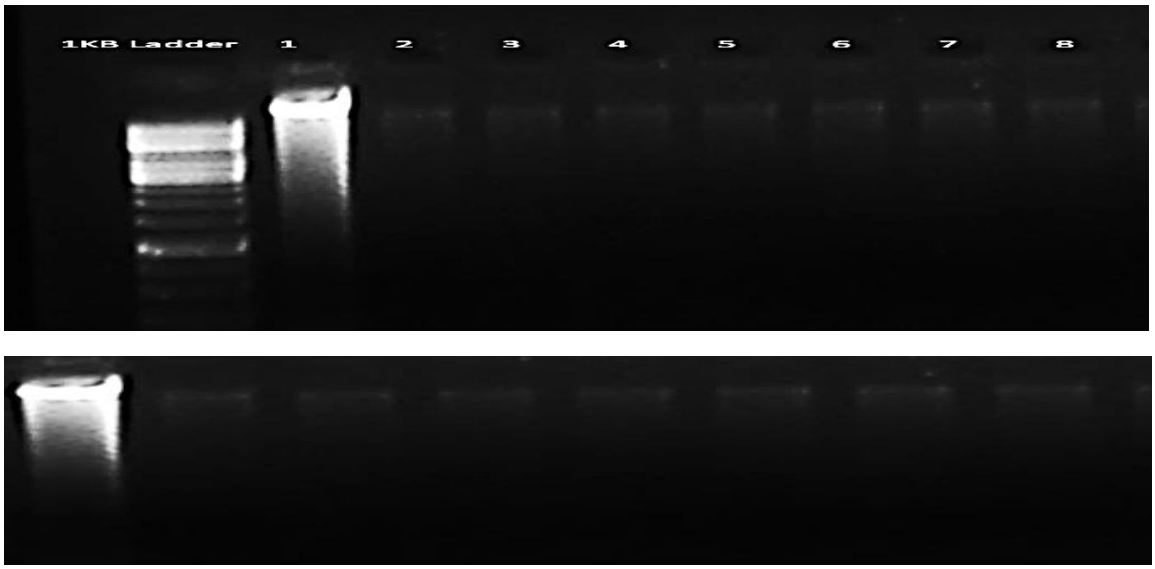
Figure 1: Addition of lysis solution in eppendrop

Then eppendrops were incubated at 56 ℃ for whole night in water bath (Digital Constant Water Bath, Jiangsu Zhenghi Instruments Co. Ltd. China).

Figure 2: Water bath

Agarose Gel Electrophoresis

Gel electrophoresis was performed using 1% agarose gel and the composition included 1 gram of agarose which was dissolved in 100 ml of 1X TAE buffer (Tris Acetic acid EDTA). Clear solution was formed after heating. 7 µl Ethidium Bromide was added in gel solution. Gel was poured into the gel casting tray with inserting combs. After solidification, gel caster was transferred to gel tank filled with 1X TAE buffer and combs were removed carefully. 2 µl of extracted DNA was mixed with 2 µl of 6X bromophenol blue dye (loading dye) and it was loaded in wells. The gel was run under specific parameters which included 500 mA of current with 110 volts for 35 minutes. Gel was visualized under UV Trans-Illuminator bio Doc Analyzer. Following gel picture is showing representative DNA bands with comparison to 1KB Ladder:



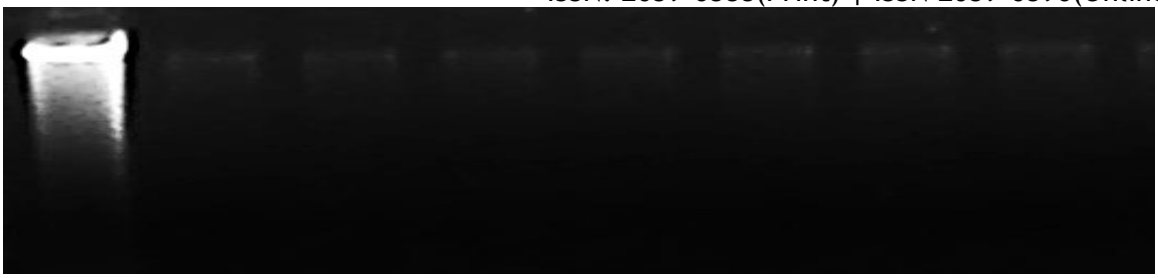


Figure 3: Gel electrophoresis image

1KB ladder was loaded in first well with DNA samples in next wells. DNA is highly intact and of more than 20kb size.

Polymerase Chain Reaction (PCR)

PCR is a molecular biology technique used to amplify a single copy or a specific sequence of DNA. COX1 primers were used to amplify the DNA samples.

The experiment utilized the following chemicals at specified concentrations: template DNA, forward & reverse primers (Macrogen Company), Taq polymerase enzyme, PCR buffer, MgCl2, dNTPs, and PCR water.

Table 2: PCR Master Mix

PCR Reagents	Stock Conc.	Working Conc.	Vol/Rec	Vol. x (12)
DNA template	-	-	4 µL	
Pf	10 µM	0.2 µM	0.4 µL	4.8µL
Pr	10 µM	0.2 µM	0.4 µL	4.8µL
DNTPs	10 mM	0.2 Mm	0.4 µL	4.8µL
Buffer	10X	1X	2 µL	24µL
$MgCl_2$	25 mM	2.5 Mm	2 µL	24µL
taq Polymerase	5U/ µL	1.5 U	0.3 µL	3.6µL
PCR H_2O			10.5 µL	126µL
Final Volume			20 µL	

"n" would be any number for which you are making master mix.

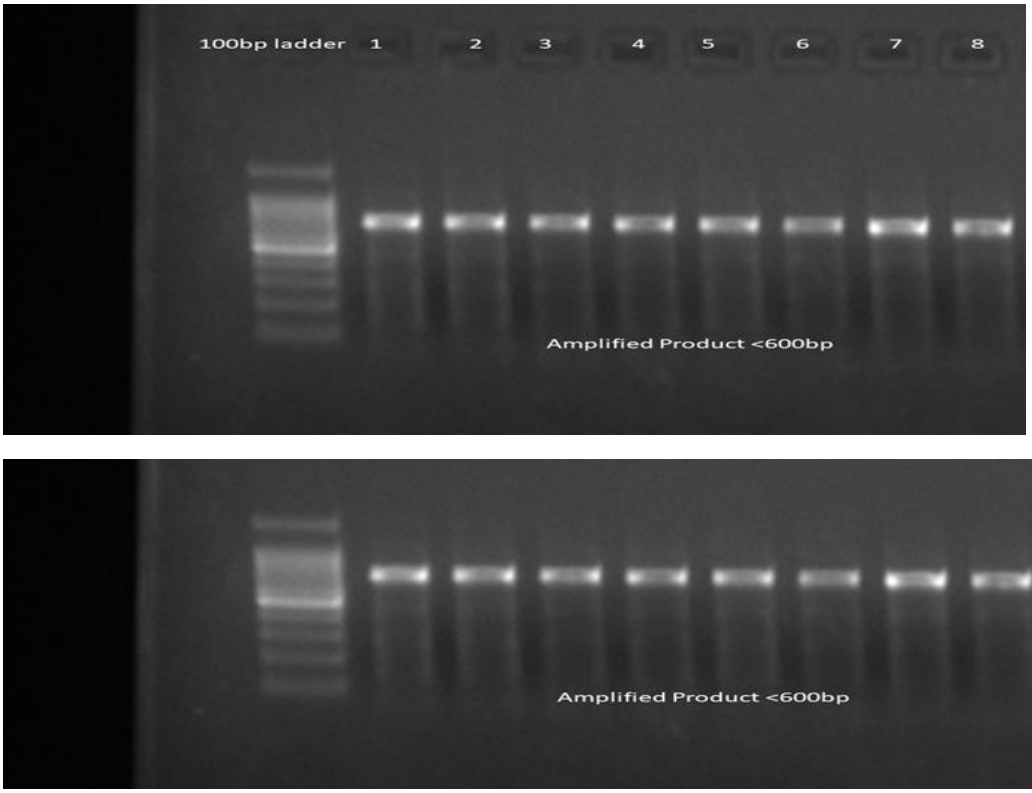
Polymerase chain reactions were performed on a Galaxy XP Thermal Cycler (BIOER, PRC). Optimized PCR conditions were shown in table.

Table 3: Optimized PCR conditions

Steps	Sub-cycles	Conditions	PCR cycles
Initial Denaturation		95 °C, 10 min	1
PCR Cycles	Denaturation	95 °C, 1 min	40
	Primer annealing	60 °C, 1 min	
	Primer extension	72 °C, 1 min	
Final extension		72 °C, 10 min	1
Hold		04 °C, ∞	1

Gel Electrophoresis Analysis

Amplified product was run on 2 % agarose gel and visualized under UV. Following is the representative picture of PCR, 50 bp ladder (Thermo scientific) was run in first lane against amplified samples.



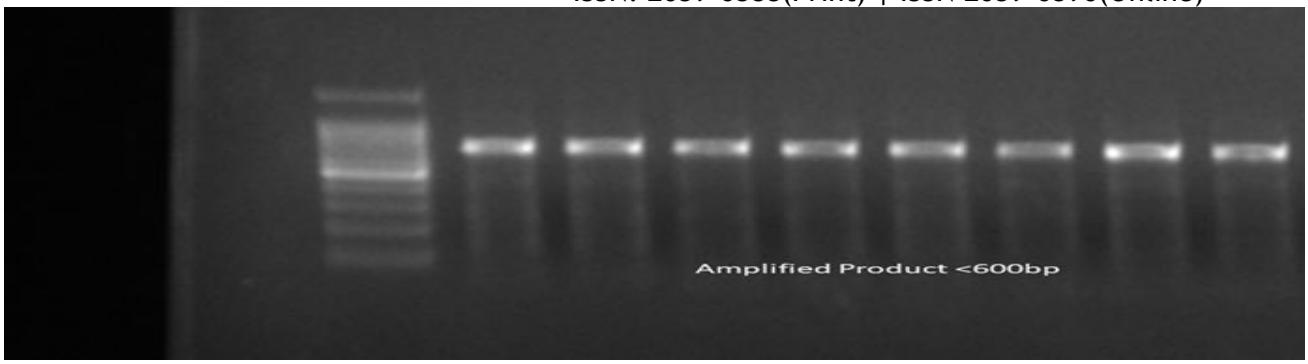


Figure 4: Gel Electrophoresis Analysis

Primer Selection

Primer No.	Primer Name	Primer Sequence	Length	Temperature
1.	VF1D	TTCTCAACCAACCACAARGA[Y]AT[Y]GG	26	57.6 ℃
2.	VR1D	TAGACTTCTGGGTGGCCRAARAA[Y]CA	26	59.2℃

Table 4: A list of Primers

RESULTS

DNA Extraction and Quantification

The genomic DNA was extracted from 8 tissue samples of different bird species. Visulized and confirmed on *1% Agarose Gel.* 200mg of tissue used Russell and Sambrook method. The protocol was optimised by carefull adjustment in optimisation of *Protease K* concentration, *Standerd phenol chloroformextraction technique.* The results of genomic DNA were visualized by gel documantation system and recorded. The result of DNA extraction and Gel electrophoresis are given bellow:

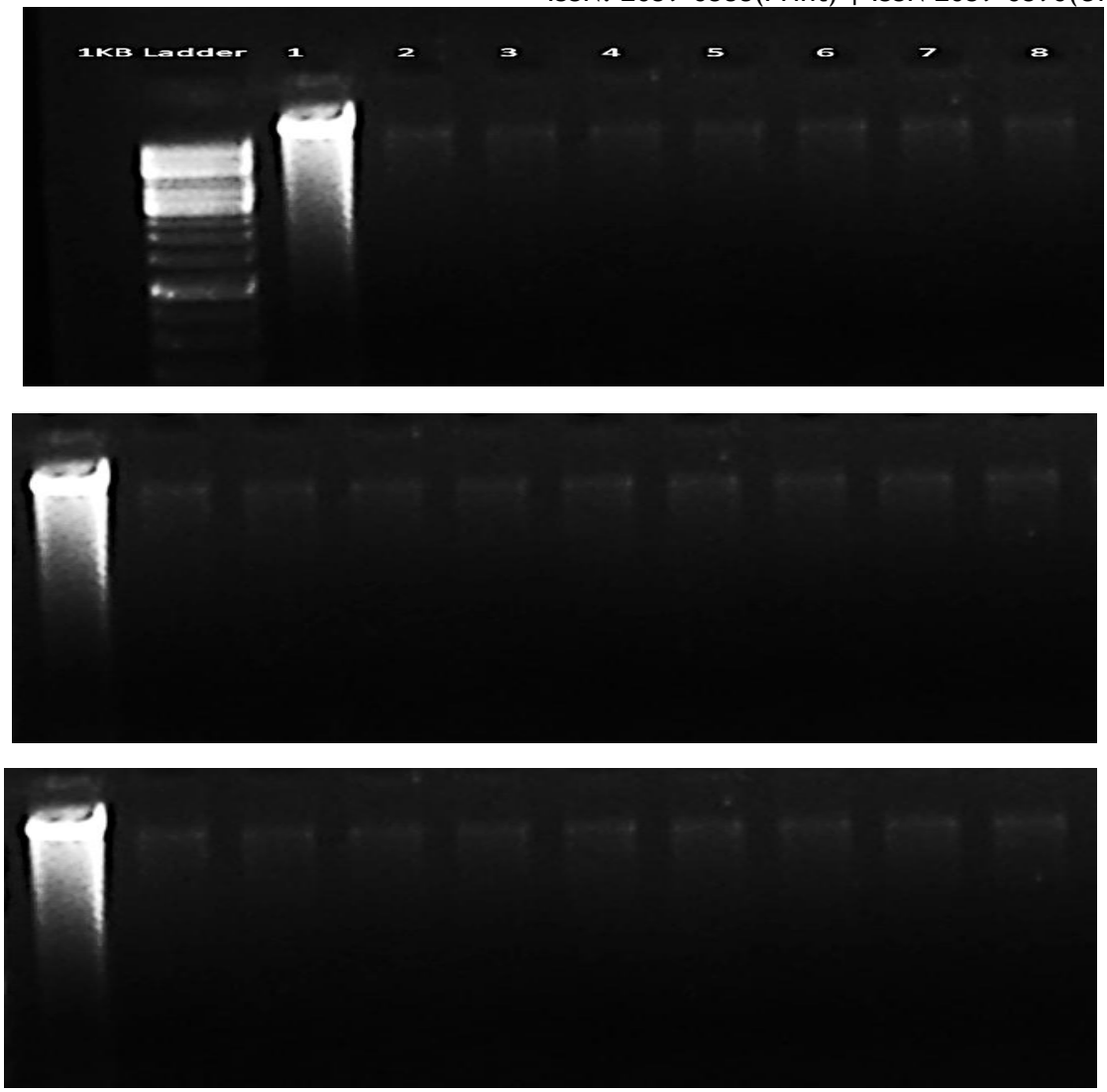
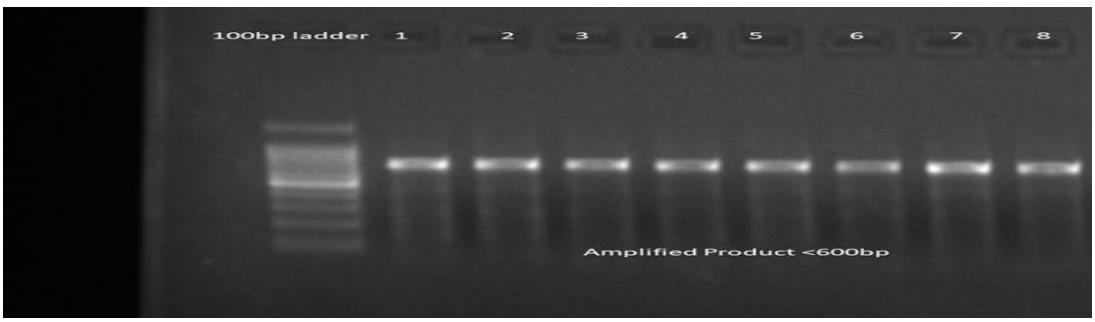


Figure 5: gel electrophoresis image

PCR results

The PCR of extracted genomic DNA of different birds was carried out in order to amplifiy COI region.



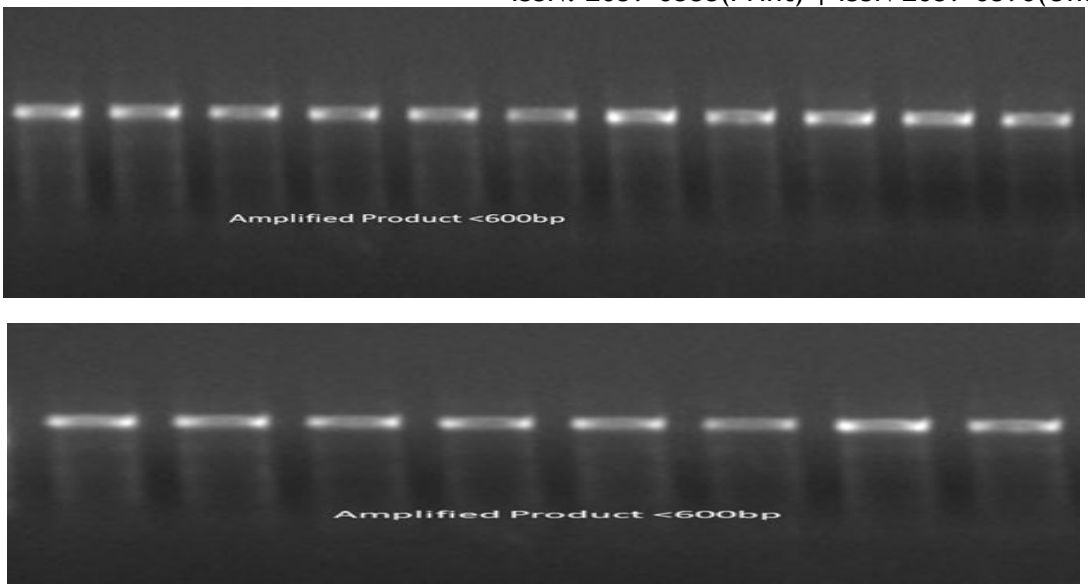


Figure 6: Gel electrophoresis analysis

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Meta data table

Common Name	Sample ID	BLAST Result	Phylum	Class	Order	Family	Genus	Species	Identification method	Taxonomic status
Bank Myna	BM 1 BM 2 BM 3	720/720 (100%)	Chordata	Aves	Passeriformes	Sturnidae	Acridotheres	ginginianus	Morphological	Least concern
Common Myna	CM 1 CM 2 CM 3	1143/1143 (100%)	Chordata	Aves	Passeriformes	Sturnidae	Acridotheres	tristis	Morphological	Least concern
Muscovy Duck	MD 1 MD 2 MD 3	1141/1143 (99%)	Chordata	Aves	Anseriformes	Anatidae	Cairina	moschata	Morphological	Least concern
Common Hoopoe	CH 1 CH 2 CH 3	1142/1143 (99%)	Chordata	Aves	Bucerotiformes	Upupidae	Upupa	epops	Morphological	Least concern
Rock pigeon	RP 1 RP 2 RP 3	1141/1143 (99%)	Chordata	Aves	Columbiformes	Columbidae	Columba	livia	Morphological	Least concern
Black drongo	BD 1 BD 2 BD 3	1026/1026 (100%)	Chordata	Aves	Passeriformes	Dicruridae	Dicruarus	macrocerus	Morphological	Least concern
Munia	MU 1 MU 2 MU 3	1141/1143 (99%)	Chordata	Aves	Passeriformes	Estrididae	Lonchura	malacca	Morphological	Least concern
Scally breasted myna	SBM 1 SBM 2 SBM 3	1129/1143 (99%)	Chordata	Aves	Passeriformes	Estrididae	Lonchura	punctulata	Morphological	Least concern

Conserved sites	985/1577
Variable sites	575/1577
Parsim value	337/1577
Singleton sites	235/1577
0-fold degenerate sites	964/1577
2-fold degenerate sites	116/1577
4-fold degenerate sites	141/1577

Table 4: Sequence analysis of Avian Fauna byusing MEGA X

Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU	Codon	Count	RSCU

Table 5: Codon usage on the bases of Cytochrome Oxidase I gene

UUU(F)	2.5	0.53	UCU(S)	4.6	1.15	UAU(Y)	3.5	0.9	UGU(C)	1.2	0.66
UUC(F)	7.1	1.47	UCC(S)	4.2	1.05	UAC(Y)	4.2	1.1	UGC(C)	2.5	1.34
UUA(L)	1.8	0.42	UCA(S)	3.8	0.94	UAA(*)	2.3	0.93	UGA(*)	3.2	1.28
UUG(L)	0.9	0.2	UCG(S)	1.3	0.33	UAG(*)	2	0.8	UGG(W)	2.7	1
CUU(L)	6.4	1.48	CCU(P)	9.2	1.49	CAU(H)	5.2	1.02	CGU(R)	2.3	0.84
CUC(L)	5.6	1.29	CCC(P)	7.3	1.17	CAC(H)	5	0.98	CGC(R)	2.8	1.01
CUA(L)	8.2	1.88	CCA(P)	6.1	0.98	CAA(Q)	4.6	1.31	CGA(R)	2	0.71
CUG(L)	3.2	0.73	CCG(P)	2.2	0.36	CAG(Q)	2.4	0.69	CGG(R)	2.7	0.98
AUU(I)	4.5	0.84	ACU(T)	6.3	1.42	AAU(N)	5.8	1.01	AGU(S)	3.6	0.92
AUC(I)	6.4	1.19	ACC(T)	5.6	1.27	AAC(N)	5.7	0.99	AGC(S)	6.4	1.61
AUA(I)	5.1	0.97	ACA(T)	5	1.14	AAA(K)	2.9	1.46	AGA(R)	2.6	0.93
AUG(M)	2.2	1	ACG(T)	0.8	0.17	AAG(K)	1.1	0.54	AGG(R)	4.2	1.53
GUU(V)	1.8	0.79	GCU(A)	2.9	0.93	GAU(D)	1.5	0.65	GGU(G)	1.8	0.63
GUC(V)	2.3	1.03	GCC(A)	5.2	1.67	GAC(D)	3.1	1.35	GGC(G)	2.9	1.04
GUA(V)	4	1.84	GCA(A)	4	1.29	GAA(E)	2	1.22	GGA(G)	5	1.78
GUG(V)	0.8	0.34	GCG(A)	0.3	0.11	GAG(E)	1.3	0.78	GGG(G)	1.6	0.56

MAXIMUM LIKELYHOOD ESTIMATE OF TRANSITION/TRANSVERSION (ML)

The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model [1]. The tree with the highest log likelihood (-6761.69) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 1.5106)). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 52.81% sites). This analysis involved 37 nucleotide sequences. Codon positions included were 1st+2nd+3rd. There were a total of 1559 positions in the final dataset. Evolutionary analyses were conducted in MEGA X

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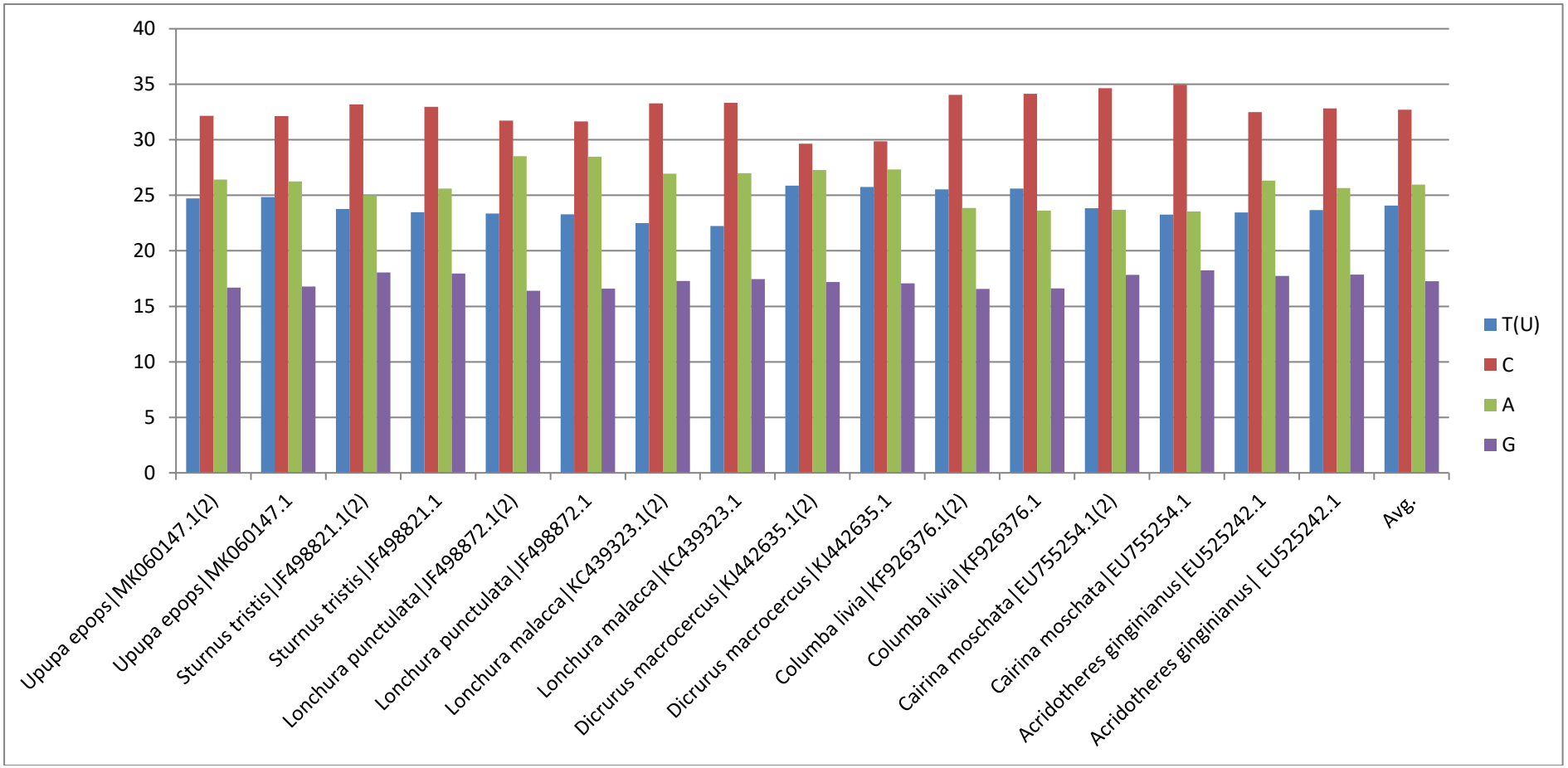
Figure 6: neucleotide composition based on COI gene



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Maximum Likelihood Estimate of Substitution Matrix

	A	T/U	C	G
A	-	*4.06*	*5.98*	**6.33**
T/U	*4.24*	-	**34.74**	*0.75*
C	*4.89*	**27.16**	-	*0.34*
G	**9.77**	*1.11*	*0.64*	-

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 General Time Reversible model (+G+I) (Nei and Kumar, 2000). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories, [+*G*], parameter = 0.9513). The rate variation model allowed for some sites to be evolutionarily invariable ([+*I*], 50% sites). 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 = 26.13%, T/U = 24.98%, C = 31.95%, and G = 16.94%. For estimating ML values, a tree topology was automatically computed. The maximum Log likelihood for this computation was -10446.245. This analysis involved 61 nucleotide sequences. Codon positions included were 1st+2nd+3rd. There were a total of 1561 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

Table 6: Comparison similarity of avian fauna on BOLD and BLAST

Sr#	Common name	Scientific name	Similarty On BLAST	Similarity on BOLD
1	Bank myna	*Acridotheres ginginianus*	99%	99.85%
2	Common Myna	*Sturnus tristis*	99%	100%
3	Muscovy Duck	*Cairina Moschata*	99%	100%
4	Hoopoe	*Upupa epopos*	99%	99.68%
5	Rock Pigeon	*Columbia livia*	98%	99.36%
6	Black drongo	*Dicrurus macrocercus*	99%	99.68%
7	Tricolored munia	*Lonchura malacca*	97%	96.79%
8	Scaly breasted munia	*Lonchura punctulata*	99%	99.83%

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Table 7: Comparison of BLAST results of avian fauna with other members of different genus (Source : GenBank) based on COI gene

Birds	Similarity index	Gaps	COI gene difference
Acridotheres ginginianus	649/655(99%)	3/655(0%)	1%
Sturnus tristis	644/645(99%)	0/645(0%)	1%
Cairina moschata	650/655(99%)	1/655(0%)	1%
Upupa epops	635/640(99%)	0/640(0%)	1%
Columba livia	642/653(98%)	2/653(0%)	2%
Dicrurus macrocercus	628/631(99%)	1/631(0%)	1%
Lonchura malacca	606/627(97%)	1/627(0%)	3%
Lonchura punctulata	653/658(99%)	1/658(0%)	1%

DISCUSSION

DNA barcoding is also very useful for species delimitation, defining species borders as well as a very useful method for identifying new species. It can be very authentic and reliable tool as it has ability to confirm the food and also has an important role for the protection of the biodiversity based on genetic resources and their wild relatives (Bondoc *et al*., 2013).It has been proved through various studies that COI sequence differentiation is very less within the species (generally less then 1-2%), but this differentiation is much more percent in closely related species in animals so it is a very advantageous role in species identification (A.Naseem *et al*., 2020). DNA barcoding also plays an significant role in the survival of endangered species.

The study was performed to identify the birds of Distruct Sargodha, by the use of DNA barcoding method. Samples were randomly collected from District Sargodha. This study includes 24 samples of different species. The DNA was extracted through total genomic DNA extraction method.The COI gene was amplified by using universal primers. Sequencing was done from MicroGen lab, USA. All the species were correctly identified. It is concluded that COI is significant marker for the identification of species. It is a useful tool for the prevention of illegal trade. So, DNA barcoding has played an important role for the survival of endangered species. So, it is a reliable marker at species level.

By using molecular taxonomy to classify animals, the core purpose of DNA barcoding is to establish a biological barcode and also to form a reference library for the identification of particular species (Kerr *et al*., 2007). Since ecosystems are increasingly declining, the biological consequences of the global warming make it necessary to classify animals for the survival of biodiversity. Millions of flora and fauna is living on the earth, out of which we are familiar with less then 2 million species.Due to different causes, the loss of animals and plants grows day by day, meaning thousands of them are killed each year, but most of them are not noticed. This habitat degradation and endangerment has led to an enhanced species recognition programe. An innovative and modern ecological approach, i.e. For the recognition of organisms and ecological analysis, DNA barcoding is used (Ball and J.R. deWaaed *et al*., 2003). DNA barcoding a rapid and accurate species identification tool has revolutionized the world(Jhon Waugh*et al*., 2007) .

Due to such a great variety, it is difficult to distinguish many species on the basis of their morphological characters but due to the advance technology and modern lab facilities we can easily identify the species accurately on the basis of their molecular approach. On the basis of the morphology the species could be wrongly distributed in different classes. To

avoid all these problems many scientist prefer the molecular approach based on their DNA. Mostly mitochondrial DNA is preferred for this study due to its unique characters (J. Tizard *et al*., 2019).

It is concluded that mitrochondrial COI is an efficient marker for species identification as well as for study of phylogenetic relationship of birds. However, study also specifies the requirement of increased taxon sampling as well as studying more genes in order to resolve taxonic and phylogenetic issues that surround the birds. In addition, because DNA Barcoding contributes a lot in the conservation of endangered species and monetring their illegal trades.

The general consensus of mtDNA trees with species trees means that it should be used in reverse, i.e. to classify organisms by evaluating their DNA, instead of examining the DNA of morphologically classified organisms. Past uses for the detection of DNA-based species range from the reconstruction of trophic networks via the analysis of stomach fragments (Symondson *et al*., 2002) to the classification of items originating from endangered species (Palumbi and Cipriano *et al*., 1998) and the resolution of mosquito complexes transmitting malaria and dengue (Phuc *et al*., 2003).

CO1 is chosen primarily since it is easy to amplify as a high copy number per cell, it is also chosen because it is much quicker than the nuclear DNA because the reactive oxygen release during the oxidative phosphorylation damage of the mtDNA also lacks the mechanism of proof reading. The CO1 gene is located at 648 bp and is used as a barcode called "folmer region" or "tagging region". At the species level, it has a distinguishing ability, so CO1 is used as a barcode for the easy and accurate identification of the species level. Using the mitochondrial gene cytochrome c oxidase subunit I, barcoding is now a well-established technique for identifying animal species, especially in mammals, birds, fish, and amphibians (COI). COI sequence variability is lowest among species (usually less than 1-2%), but varies by several percent in closely related species, according to a significant number of studies.

Innovations of molecular methods provide significant ways to study evolution and genetic links in diverse populations. The DNA barcode as a genetic tool explores the notion of organisms rather than their relationship (Hajibabaei *et al*., 2007; Bilal[a,b,c] *et al*., 2024). Using a quicker way to identify species is critical for assessing the ecology of these areas and protecting rare, endemic, and endangered species. However, it should be noted that the DNA barcode is not a substitute for taxonomy, but it is a useful method for producing data on unidentified taxa. Ebach and Holrege (Ebach and Holrege, 2005). In the context of

domestication and phylogeography, mitochondrial DNA (mtDNA) has become one of the most common markers because it has certain advantages over maternal genomic DNA, such as the ability to monitor animal distributions and a faster rate of evolution.(Wu *et al*., 2009).

Other regions of the gene loci may serve as a base for species identification, according to researchers. CO1 has been established as a barcode region in animal species. Mitochondrial markers, with the exception of CO1, have been used to classify samples at the species level. Short sequences of the mtDNA cytochrome b gene (CYTB) containing (150 bp) were used to detect canned sardine animals (Jerome *et al*., 2003; Bilal, 2021; Shah *et al*., 2022). COI, on the other hand, has the benefit of having a vast variety of primers available to a diverse range of taxa, and genetic variations grow more slowly than in Cyt b (Folmer *et al*., 1994; Lynch and Jarrell *et al*., 1993).

From the last century, the recognition of organisms by DNA barcoding, which is a molecular tool, has become a very developing field. Through contrast to an open access website, DNA barcoding depends on a standardized area of the mitochondrial gene being translated, sequenced and analyzed. By using molecular taxonomy to classify animals, the core purpose of DNA barcoding is to establish a biological barcode and also to form a reference library for the identification of particular species (Kerr *et al*., 2007). Since ecosystems are increasingly declining, the biological consequences of the global warming make it necessary to classify animals for the survival of biodiversity. Millions of flora and fauna is living on the earth, out of which we are familiar with less then 2 million species. Due to different causes, the loss of animals and plants grows day by day, meaning thousands of them are killed each year, but most of them are not noticed. This habitat degradation and endangerment has led to an enhanced species recognition programe. An innovative and modern ecological approach, i.e. For the recognition of organisms and ecological analysis, DNA barcoding is used (Ball and J.R. (deWaaed *et al*., 2003). DNA barcoding a rapid and accurate species identification tool has revolutionized the world (Jhon Waugh *et al*., 2007).

Conclusions

It is concluded that mitrochondrial COI is an efficient marker for species identification as well as for study of phylogenetic relationship of birds. However, My study also specifies the requirement of increased taxon sampling as well as studying more genes in order to resolve taxonic and phylogenetic issues that surround the birds. In addition, because DNA

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REFERENCES

Dimitriou, A. C., Forcina, G., Papazoglou, C., Panayides, P., Guerrini, M., Crabtree, A., ...& Sfenthourakis, S. (2017). DNA barcoding of bird species in Cyprus: a tool for conservation purposes. *Bird Conservation International*, *27*(4), 483.

Nei M. and Kumar S. (2000). *Molecular Evolution and Phylogenetics*. Oxford University Press, New York.

Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. *Molecular Biology and Evolution* ***35***:1547-1549.

Field, D. J., Hanson, M., Burnham, D., Wilson, L. E., Super, K., Ehret, D., ...& Bhullar, B. A. S. (2018). Complete Ichthyornis skull illuminates mosaic assembly of the avian head. *Nature*, *557*(7703), 96-100.

Bilal, A., Iftikhar, A., Ali, U., Naveed, N., Anjum, M. I., Fatima, U., ... & Sajjad, M. K. (2021). Comparison of different Covid-19 vaccines globally: *An overview. J Gynecol Women's Health*, 21, 556071.

Bilal, A., Tanvir, F., Ahmad, S., Kanwal, N., Zulfiqar, H., & Ishaq, R. (2024). Pharmacokinetic Properties of Bioactive Compounds of Aloe vera against Pregnancy-Associated Plasma Protein A (PAPP-A) inducing Triple-Negative Breast Cancer. *Kurdish Studies*, *12*(5), 157-168.

Folmer, R. H. A., Nilges, M., Folkers, P. J. M., Konings, R. N. H., & Hilbers, C. W. (1994). A model of the complex between single-stranded DNA and the single-stranded DNA binding protein encoded by gene V of filamentous bacteriophage M13. *Journal of molecular biology*, *240*(4), 341-357.

Folmer, R. H. A., Nilges, M., Folkers, P. J. M., Konings, R. N. H., & Hilbers, C. W. (1994). A model of the complex between single-stranded DNA and the single-stranded DNA binding protein encoded by gene V of filamentous bacteriophage M13. *Journal of molecular biology*, *240*(4), 341-357.

Bilal, A., Tanvir, F., Ahmad, S., Azam, A. R., Qasim, M., & Zafar, H. (2024). THERAPEUTICAL EVALUATION OF BIOACTIVE COMPOUNDS OF NIGELLA SATIVA FOR HER2-POSITIVE BREAST CANCER TREATMENT. Journal of Population Therapeutics & Clinical Pharmacology. 31(9), 3149-3164

Hajibabaei, M., dewaard, J. R., Ivanova, N. V., Ratnasingham, S., Dooh, R. T., Kirk, S. L., ...& Hebert, P. D. (2005). Critical factors for assembling a high volume of DNA barcodes. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*(1462), 1959-1967.

Hebert, P. D., & Gregory, T. R. (2005).The promise of DNA barcoding for taxonomy. *Systematic biology*, *54*(5), 852-859.

Hebert, P. D., Cywinska, A., Ball, S. L., & Dewaard, J. R. (2003).Biological identifications through DNA barcodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *270*(1512), 313-321.

Hebert, P. D., Stoeckle, M. Y., Zemlak, T. S., & Francis, C. M. (2004).Identification of birds through DNA barcodes. *Plos biol*, *2*(10), e312.

Bilal, A., Tanvir, F., Ahmad, S., Shah, S. H. A., Ahmad, H. A., & Kanwal, N. (2024). Pre-clinical study of the bioactive compound Asiaticoside against the proteins inducing human mammary carcinoma using molecular docking and ADME analysis. Remittances Review, 9(2), 3543-3576.

Kerr, K. C., Birks, S. M., Kalyakin, M. V., Red'kin, Y. A., Koblik, E. A., & Hebert, P. D. (2009). Filling the gap-COI barcode resolution in eastern Palearctic birds. *Frontiers in Zoology*, *6*(1), 29.

Kerr, K. C., Lijtmaer, D. A., Barreira, A. S., Hebert, P. D., & Tubaro, P. L. (2009). Probing evolutionary patterns in Neotropical birds through DNA barcodes. *PLoS One*, *4*(2), e4379.

Kerr, K. C., Stoeckle, M. Y., Dove, C. J., Weigt, L. A., Francis, C. M., & Hebert, P. D. (2007).

Comprehensive DNA barcode coverage of North American birds. *Molecular ecology notes*, *7*(4), 535-543.

Kerr, K. C., Stoeckle, M. Y., Dove, C. J., Weigt, L. A., Francis, C. M., & Hebert, P. D. (2007). Comprehensive DNA barcode coverage of North American birds. *Molecular ecology notes*, *7*(4), 535-543.

Kinebuchi, T., Idota, N., Tsuboi, H., Takaso, M., Bando, R., & Ikegaya, H. (2019). The genome profiling method can be applied for species identification of biological materials collected at crime scenes. *BMC genetics*, *20*(1), 1-7.

Li, J., Cui, Y., Jiang, J., Yu, J., Niu, L., Deng, J., ...& Yue, B. (2017). Applying DNA barcoding to conservation practice: a case study of endangered birds and large mammals in China. *Biodiversity and Conservation*, *26*(3), 653-668.

Lynch, M., & Jarrell, P. E. (1993). A method for calibrating molecular clocks and its application to animal mitochondrial DNA. *Genetics*, *135*(4), 1197-1208.

Naseem, A., Batool, S., & Abbas, F. I. (2020).Utility of mitochondrial CO I gene for identification of wild ungulate species of conservational importance from Pakistan. *Mitochondrial DNA Part B*, *5*(2), 1924-1928.

Palumbi, A. R., & Cipriano, F. (1998). Species identification using genetic tools: the value of nuclear and mitochondrial gene sequences in whale conservation. *Journal of Heredity*, *89*(5), 459-464.

Bilal, A. (2021). Rabies is a zoonotic disease: a literature review. Occup. Med. Health Aff, 9(2).

Shah, S. A. H., Bilal, A., Ahmad, M. M., & Bukhari, S. S. (2022). Deforestation is causing a great loss in avian diversity in Pakistan. American Journal of Zoology, 5(3), 24-29.

Pasha, F. (2020).DNA Barcoding in Avian Species with Special Reference to Taxonomically Wide Biogeographic Studies. In *DNA Barcoding and Molecular Phylogeny* (pp. 191-201). Springer, Cham.

Ratnasingham, S., & Hebert, P. D. (2007). BOLD: The Barcode of Life Data System (http://www. barcodinglife. org). *Molecular ecology notes*, *7*(3), 355-364.

Round, P. D., Pierce, A. J., Saitoh, T., & Shigeta, Y. (2016).Addition of Kamchatka Leaf Warbler Phylloscopus examinandus and Sakhalin Leaf Warbler P. borealoides to Thailand's Avifauna. *Bulletin of the Japan Bird Banding Association*, *28*, 9-21.

Safety of meloxicam to critically endangered Gyps vultures and other scavenging birds in India. *Animal Conservation*, *10*(2), 192-198.

Sanger, F., & Coulson, A. (1996).A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. *Sel. Pap. Frederick Sanger Comment*, *94*, 382.

Satoh, M., & Kuroiwa, T. (1991).Organization of multiple nucleoids and DNA molecules in mitochondria of a human cell. *Experimental cell research*, *196*(1), 137-140.

Symondson, W. O. C. (2002). Molecular identification of prey in predator diets. *Molecular ecology*, *11*(4), 627-641.

Tizard J, Patel S, Waugh J, Tavares E, Bergmann T, Gill B, Norman J, Christidis L, Scofield P, Haddrath O, Baker A. DNA barcoding a unique avifauna: an important tool for evolution, systematics and conservation. BMC evolutionary biology. 2019 Dec 1;19(1):52.

Tizard, J., Patel, S., Waugh, J., Tavares, E., Bergmann, T., Gill, B., ...& Baker, A. (2019). DNA barcoding a unique avifauna: an important tool for evolution, systematics and

conservation. *BMC evolutionary biology*, *19*(1), 52.

Tizard, J., Patel, S., Waugh, J., Tavares, E., Bergmann, T., Gill, B., ...& Baker, A. (2019). DNA barcoding a unique avifauna: an important tool for evolution, systematics and conservation. *BMC evolutionary biology*, *19*(1), 52.

Trivedi, S., Rehman, H., Saggu, S., Panneerselvam, C., & Ghosh, S. K. (2020). Closing Shots: DNA Barcoding and Molecular Phylogeny. In *DNA Barcoding and Molecular Phylogeny* (pp. 265-267). Springer, Cham.

Waugh, J., Evans, M. W., Millar, C. D., & Lambert, D. M. (2011). Birdstrikes and barcoding: can DNA methods help make the airways safer?. *Molecular Ecology Resources*, *11*(1), 38-45.

Wu, Y., Li, H., Zou, F., & Hsu, Y. (2011). Lineages differentiation and population structure of the light-vented bulbul Pycnonotus sinensis in China based on mitochondrial DNA and microsatellite analysis. *Integrative zoology*, *6*(4), 387-398.