Molecular Identification and Phylogenetic relationship of Corvus splendens (Common House Crow) by Cytochrome c oxidase subunit I gene

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Research Article

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Abstract

The traditional taxonomic approach for identification of species and their phylogenetic relationship is handicapped due to its limitations. The present study is carried out to evaluate the genetic identification and phylogenetic status of Corvus splendens (Common house crow) using mitochondrial cytochrome c oxidase subunit 1 (COX1). We amplified 690bp region of COX I from crow specimens collected from seven cities of Punjab. The PCR product was got sequenced and submitted to NCBI. Multiple sequence alignment was done among 10 Pakistani specimens and 18 species of common crow belonging to different families obtained from GenBank database using Gyps indicus as an outgroup. Phylogenetic tree was constructed among various Crow species by Neighbor Joining method (NJ) in MEGA X. It shows that KAS2 & NAR2 were very similar and shared evolutionary relationship with Common house crow from India. While KAS3 and LHR5 showed very close resemblance and shared common ancestry with the NAR3. The relative time tree shows the SKT2 evolved earlier than BHR1, PKT2, BHR2 & GUJ3 while the later were evolved at the same time. Further, the entire crow species irrespective of regions evolved almost at the same time with very little time difference. The genetic distance among the Common house crow from different localities showed very low degree of sequence divergence despite long physical distances among them. Overall, our analysed Corvus splendens were closely related to each other and evolved at same time. Moreover, these crows were closely related to other crow species dispersed all over the world.

Introduction

The Common House Crow *(Corvus Spenders)* is the inborn of the subcontinent. It has shown swift extension of the habitat ranging all over the Europe, Africa, East Asia, Australia, America and most of the Arabian countries[1]. There are four subspecies namely *Corvus splendens splendens, Corvus splendens insolens, Corvus splendens protegatus, Corvus splendens Zugmayeri* found in native range. An average sized crow has 40 cm length and 245-371 g weight with long bill and legs[2]. Besides its origin in Indian subcontinent, it shows great ecological plasticity by its tolerance of wide range of temperature ranges from -8°C to 50°C[3].

The modern-day world has enormous biodiversity and its classification is very challenging task. Scientists have explored biological details of only 5 % of the species[4,5]. Some species are morphologically alike but genetically different and in some cases they are morphologically transformed at different stages of their life[6]. The modern world biodiversity together with traditional approaches call for the scientists to find new strategies and methods of identification of species.

Species identification and characterization is the basic part of exploring biodiversity. Previously, the taxonomists generally considered the morphological characters to delineate the animals and plants [7]. But the phenotypic flexibility and character inconsistency (used for species identification) can lead to the improper species recognition. This approach overlooks the morphological cryptic taxa which are common in most of the animal and plant groups [8]. Therefore, new techniques of species identification and

phylogenetic relationship are required to describe the biodiversity[9]. One of the powerful molecular tools is to use DNA barcoding for identifying species and studying biodiversity. This is very rapid and cost-effective method to accelerate the discovery and identification of new species[10]. This technique generally scans mitochondrial DNA genes like cytochrome-C or cytochrome oxidase subunit I (COX1) to identify the species and evolutionary relationship[11-12]. Cytochrome-C and COX1 are protein coding genes present in all types of eukaryotes. Only the mitochondrial genes are preferred to use as a marker in the animal DNA barcoding because they are highly conserved and free of the recombination and lacks the introns [6, 9]. The mitochondrial DNA has been widely used in phylogenetic studies because it evolves much faster than the nuclear DNA, resulting in the collection of the differences between the closely related species [13]. Cytochrome-c oxidase subunit1 (COX1) gene ensure the fast and reliable identification of the wide range of biological samples. This gene spans a region of 648 nucleotides and enjoys special status due to its particular location. Further, a tree diagram (derived from particular sequences of mitochondrial markers) shows the temporal patterning of deviations among or within groups [14].

In present study, we amplified and sequenced 690-bp region of mitochondrial COX I gene from *Corvus splendens* of various cities, like Narowal, Lahore, Gujranwala, Pakpattan, Kasure, Bhawalpur of the Punjab (Pakistan) to evaluate sequence alignment in comparison with published COX I sequence for *corvus splendens* of other regions of the world. As no substantial information on evolutionary relationship and divergence of crow is available, this study may contribute to clarifying the evolutionary status and divergence of *corvus splendens* at regional and local scale.

Materials And Methods

Blood Collection

Corvus splendens (Common house crow) were captured by using special net trap from seven cities (Lahore, Kasur, Sialkot, Narowal, Pakpattan, Gujranwala, Bahawalpur) of Punjab (Pakistan). Whole blood samples (5 ml) were collected aseptically in sterilized vacutainer tubes containing EDTA as anticoagulant, stored at -20°C until DNA extraction.

DNA extraction

Genomic DNA from crow blood was extracted using Gene JET Whole Blood Genomic DNA purification Mini Kit (K0781) (Thermo Fisher Scientific, USA) following the manufacturer's protocol. The quality and quantity of DNA was assessed using 1% agarose gel electrophoresis. The intact DNA was selected for further analysis.

PCR amplification and gel electrophoresis

Cytochrome c oxidase subunit-I *(COX1)* gene was amplified from genomic DNA by using pair of customized primers: Bird F1 (5'-TCTCAACCAACCACAARGAYATYGG-3') Bird R1 (5'-TAGACTTCTGGGT-GGCCRAARAAYCA-3'). PCR amplification reactions were performed in a total volume of 25 µl. Each

reaction mixture contained 3µl template DNA, 3µl MgCl, 2µl PCR Buffer, 2.5µl DNTPs, 0.5µl Taq pol., 0.5µl of each Primer (1 µl), 13µl distilled water free from DNAse and RNAse. PCR was carried out in a thermal cycler. The cycling conditions included a single initial denaturation at 95°C for 5 min. followed by 14 cycles of 95°C for 30s, annealing temperature of 60°C for 45s, extension at 72°C for 45s, with the touch down of – 0.3°C, followed by the 22 cycles at 95°C for 30s, 56°C for 40s, 72°C for 45s and a final extension step at 72°C for 10 min. PCR Products (~700 bp) were fractionated by 1.2% agarose gel electrophoresis. The resulting DNA fragments were visualized by UV trans illumination and analyzed using Gel Documentation System (Bio Doc Analyse, Biometra, Germany). The PCR product (15 µl) of 10 samples was sent to the Beijing Genomic Institute (BGI) for sequencing.

Sequence alignment and phylogenetic analysis

The phylogenetic analyses and multiple alignment was conducted using MEGA X Neighbour-Joining method[15]. The reliability of nodal support was assessed through bootstrap method with 1000 bootstrap replications [16]. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The bootstrap values are shown at each node and the values above the 60% well support the nodes. The evolutionary distances were computed using the p-distance method [17] and was in the units of the number of base differences per site. Our query sequences (Cox I) were edited manually and then imported into nucleotide BLAST N (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to retrieve and align closely similar sequences from NCBI GenBank. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 618 positions in the final dataset.

Results

The study was conducted in accordance with rules and regulations of Ethical Committee of University of Education, Lahore (Pakistan). The PCR amplification of COX1 gene using customized primer yielded a single amplification product, when fractionated on a 1.2% agarose gel. The size of the product is approximately 700 bp. The PCR products of 10 Common house crow from various cities of Punjab were got sequenced. The query sequences were aligned and compared against the published sequence of *Corvus splendens* via BLAST. The query Cox I sequences from *corvus splendens* were subsequently submitted to NCBI GenBank. Some query sequences on 5́ & 3́ ends and the gaps therein were trimmed. The sequence length, accession no. and other details of query sequences submitted to NCBI are given in Table 1.

Phylogenetic relationship and sequence divergence of common house crow

Phylogenetic tree was constructed among 10 Common house crow specimens (from Punjab) & 18 taxa of house crow from other countries and a species of vulture (Gyps indicus) having accession no KX012839 as an out group.

Figure 1 shows that there were 4 clades where bootstrap values were below 60 i.e. 39, 47, 49, & 52 which implied that these clades were not closely related with the other clades investigated. In the first clade

from the top, two query sequences named, KAS2 & NAR2 shared the evolutionary history with the Common house crow taken from India. But our all other specimens (eight) evolved distantly from these two taxa.

The second clade of this phylogenetic tree shows four species *Lanius tigrinus*, *Corvus ruficollis*, *Struthidea cinerea* and *Monarcha melanopsis* which belong to different families but share common ancestry lineage with the first clade. The Common house crow did not fall in this clade and these species were quite divergent within themselves. The third clade includes 10 taxa belonging to 5 different families. The specimens KAS3 and LHR5 show very close resemblance and share common ancestor with the NAR3. The later (NAR3) evolved first than KAS3 and LHR5 as shown. The fourth clade shows that the two taxa such as *Lanus minor* and *Manucodia ater* overlaps with our query house crow and share common origin. These birds existed between SKT2 and BHR2. Both these species evolved earlier than our query house crow (Fig. 1).

Fig 2 shows the relative time tree to indicate the divergence time among Common house crow from Pakistan and other representative taxa of the world. The time tree shows that *Coloeus monedula* evolved earlier than all other taxa analysed and *Corvus splendens* evolved later when compared with outgroup (*Gyps indicus*). Our entire group of house crow from different localities showed no divergence time difference with one and another which means that they evolved at the same time. Moreover, there was little time difference between divergence of common crow species belonging to Pakistan and India. The relative time tree also shows that the SKT2 evolved earlier than BHR1, PKT2, BHR2 & GUJ3 while the latter were evolved at the same time.

To evaluate the genetic divergence among the Common house crow and the other published species, genetic distances were computed and assessed. The genetic distance (p distance) among the Common house crow from different localities of Punjab (Pakistan) ranges from 0.0000-0.0049 showing very low degree of sequence divergence despite long physical distances among them. The p distance differences among the Pakistani crow specimens and the other published specimens from various parts of the world, based on COXI gene sequences, are quite short (0.0016-0049) showing close relationship among the species despite immense geographical distances (Fig. 3).

Discussion

Genetic identification and phylogenetic status of *Corvus splendens* is an obscured issue as very few publications are available. The traditional taxonomic approach is handicapped due to its limitations to fully elaborate the issue. In this context genome exploration through mitochondrial COX1 gene (DNA Barcoding) provides molecular diversity and phylogenetic analysis of various animals worldwide. We undertook present study to elaborate the evolutionary relationship among various representatives of common house crow from Punjab (Pakistan) and the other regions of the world using mitochondrial marker (COXI). The use of COX I gene for identification and phylogeny is based on the findings of the Herbert and his colleagues (2003)[6]. He reported that utilization of the DNA barcoding being sub-atomic

instrument help in the segregation of species based on COX1 gene. Our approach to understand the molecular phylogeny was supported by the findings of Herbert, where COX1 gene was used to discriminate various species. DNA barcoding is a very successful tool for identification of species and exploration of biodiversity. But, DNA barcoding cannot solve all issues as raised by some scientist[18] and other earlier critics[19].

This study compares the COX1 sequences of the Corvus splendens from 9 different destinations of the world (Pakistan, India, Djibouti, Australia, Kenya, Bangladesh, Malaysia, South Africa, and One from NCBI GenBank) to assess the sequence divergence and evolutionary relationship. By analysing the available data, it may be inferred that *Corvus splendens* is a native of the Indian Subcontinent; from where it is spread to other continents either by man or some other agency. Ryall (1995) [20] has reported the distribution of Common house crow in various regions of the world such as Europe, Holland, Africa, America, Dubai and Middle East. The spread of *corvus splendens* to various continents is well supported by the phylogenetic tree with node offshoots having bootstrap values more than 60%.

Our phylogenetic tree and alignment results indicate that all the analysed specimens of *Corvus splendens* from Pakistan were closely related to other representative of common house crow found in various parts of the world despite long physical distances/ geographical barriers among them. Our two specimens, KAS2 & NAR2 were very similar and phylogenetically, evolved from Indian Common house crow. Both these cities of Pakistan are very close to India and probably these two taxa or their parents came from India having common ancestor. The bootstrap value (100%) validates the phylogenetic relationship and evolutionary lineage among these three taxa. Moreover, no major genetic divergence occurred despite the presence of geographical distances to gene flow among various populations of Common house crow of the continents. Similarly, the other specimens from various areas of Pakistan, such as KAS3, NAR3, LHR5, BHR1, PKT2, SKT2, showed no genetic divergence as they evolved almost at the same time. Moreover, NAR3 and SKT2 appeared as ancestors to these crows. Similar studies were carried out in Netherlands where mean intraspecific divergence found in the birds (0.29%) was congruent with that of Argentina (0.24%), North America (0.23%) and the Holarctic (0.24%) despite long geographical distances[21-22].

In the phylogenetic tree, the taxa from Turkey (KX283124 *Lanius minor*) evolved earlier and appeared as an ancestor to our house crow from Gujranwala and Bahawalpur. Both destinations are thousands km away and this linkage is supported by the more than 70% of bootstrap value. While JN 801029 *Corvus frugilegus* from Iran evolved earlier than Indian and Pakistani representative specimens and emerged as ancestor to them. This lineage is also supported by the more than 70% of bootstrap value.

Taken together, the present study reports that the specimens of Common House Crow from various localities of Punjab (Pakistan) like Bahawalpur, Gujranwala, Lahore, Narowal, Kasur, Pakpatan and Sialkot, based on COX1 sequences, have close similarity with rest of specimens dispersed across various continents whether they were evolved at different time points. These findings further substantiate that the *Corvus splendens* is the native of the Indian subcontinent and from here they were spread in the world.

Moreover, additional investigation with other molecular markers is required to support the findings of present study.

Declarations

Author's contribution

Muhammad Mansha conceived the idea, analysed the results, wrote the manuscript, Muhammad Arbab Khan collected the specimens, performed the experimental work, Tanveer Hussain, supervised the research work, helped in analysis of literature review and the results.

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Conflict of interest

There is no conflict of interest among the authors.

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Tables

COX1 Sequences of *Corvus splendens* from various cities of Pakistan submitted to NCBI

Sequence ID	Sequence Length	Reading Frame (Gene)	Reading Frame (CDS)	Accession no
B1	642	+3	+1	MN138045
B2	633	+3	+1	MN138046
G3	633	+3	+1	MN138047
K2	648	+1	+1	MN138048
K3	642	+2	+1	MN138049
L5	642	+2	+1	MN138050
N2	642	+1	+1	MN138051
N3	642	+2	+1	MN138052
P2	648	+3	+1	MN138053
S2	642	+3	+1	MN138054

Table 1: Sequence ID, length, reading frames and Accession No. assigned by GenBank to our query sequences from *Corvus splendens* of Punjab (Pakistan).

Figures

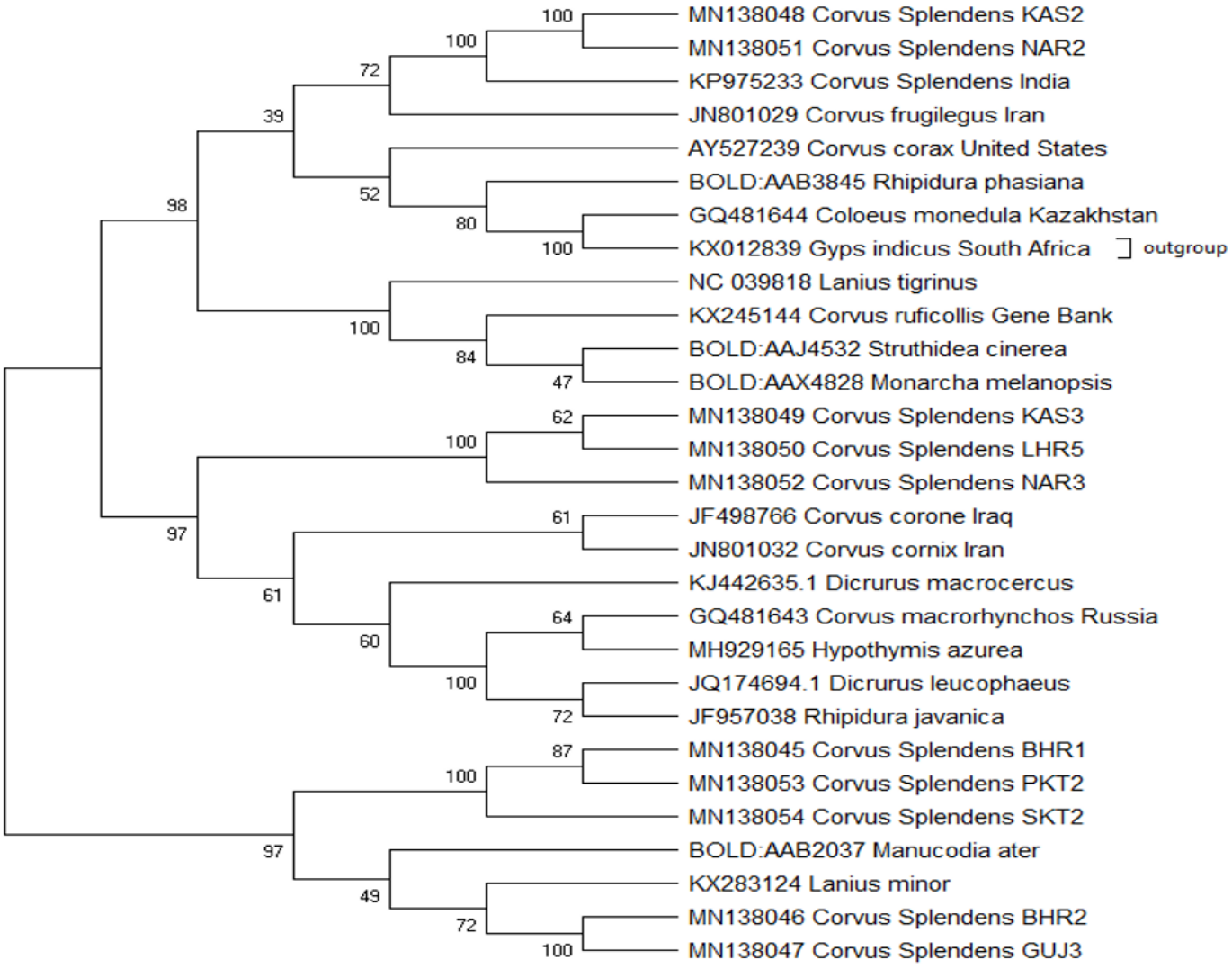


Figure 1

Phylogenetic tree based on COX1Sequences of Corvus splendens collected from Pakistani localities and compared with other representative taxa of the world. Bootstrap values are shown on each node. The specimen, accession number and the regions are shown along each leaf.

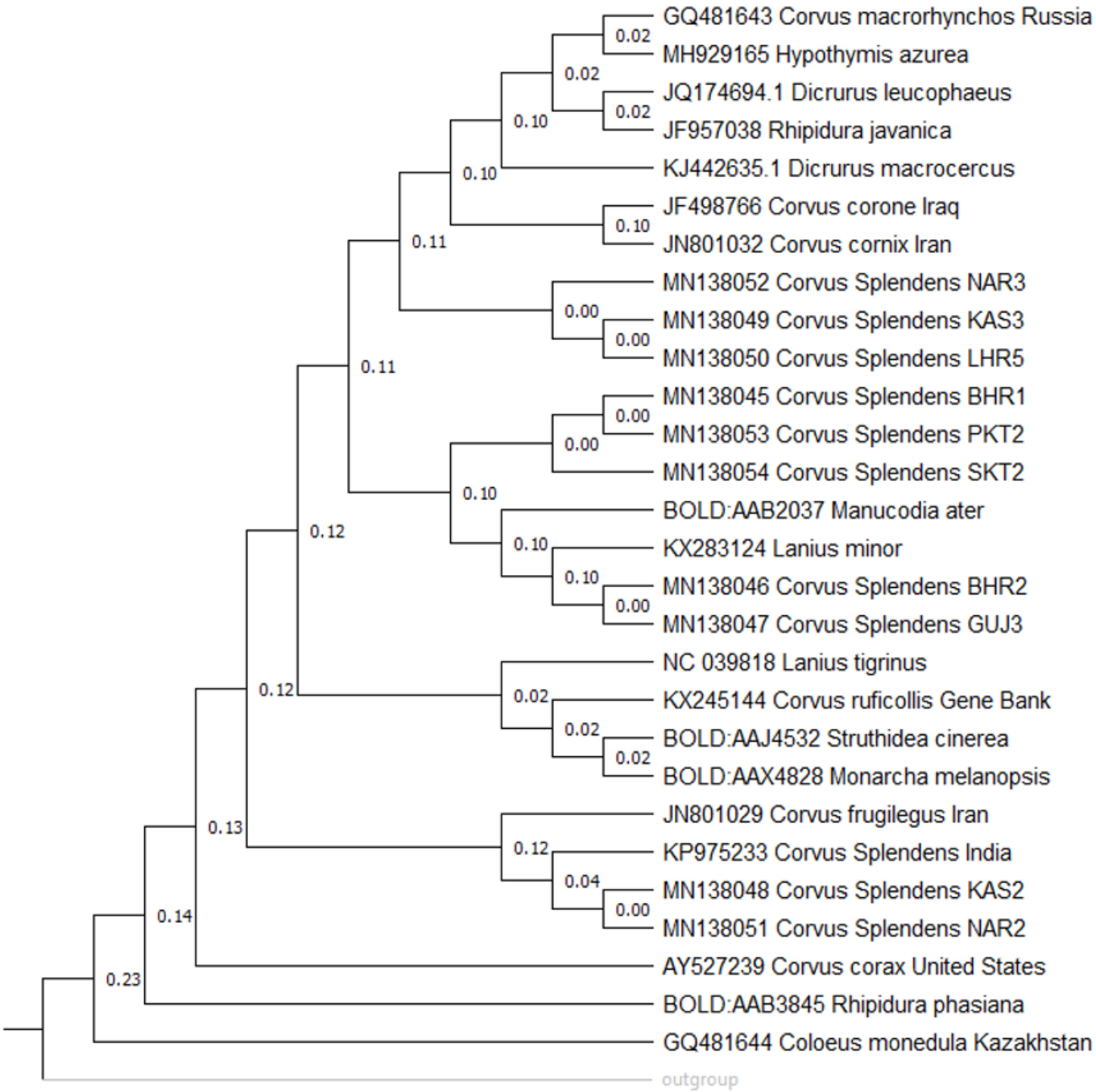
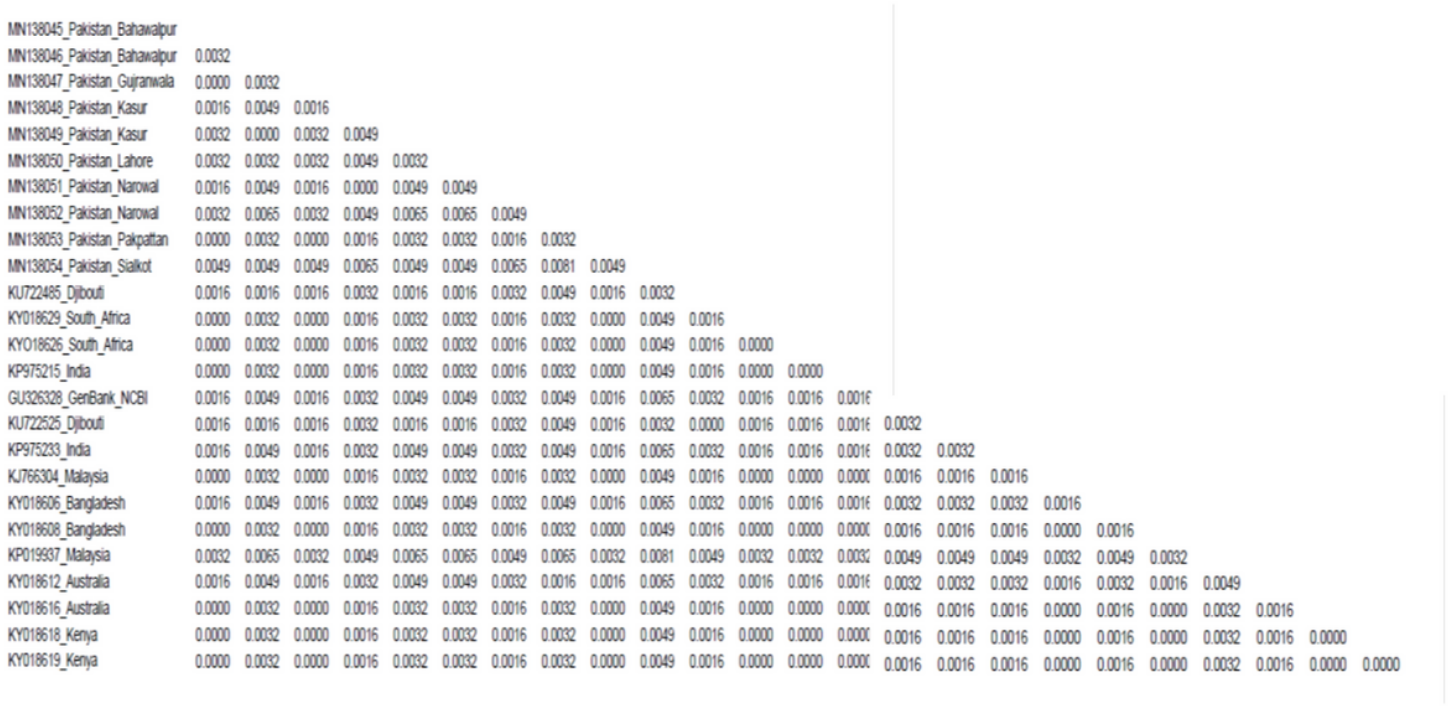


Figure 2

Phylogenetic tree based on COX I sequences considering time divergence among specimens of Corvus splendens collected from Pakistani localities and other representative taxa of the world. The specimen, accession number and the regions are shown along each leaf. The time divergence among different sequence of corvus splendens is shown on each node.



MN138045_Pakistan_Bahawalpur																									
MN138046_Pakistan_Bahawalpur	0.0032																								
MN138047_Pakistan_Gujranwala	0.0000	0.0032																							
MN138048_Pakistan_Kasur	0.0016	0.0049	0.0016																						
MN138049_Pakistan_Kasur	0.0032	0.0000	0.0032	0.0049																					
MN138050_Pakistan_Lahore	0.0032	0.0032	0.0032	0.0049	0.0032																				
MN138051_Pakistan_Narowal	0.0016	0.0049	0.0016	0.0000	0.0049	0.0049																			
MN138052_Pakistan_Narowal	0.0032	0.0065	0.0032	0.0049	0.0065	0.0065	0.0049																		
MN138053_Pakistan_Pakpattan	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032																	
MN138054_Pakistan_Sialkot	0.0049	0.0049	0.0049	0.0065	0.0049	0.0049	0.0065	0.0081	0.0049																
KU722485_Djibouti	0.0016	0.0016	0.0016	0.0032	0.0016	0.0016	0.0032	0.0049	0.0016	0.0032															
KY018629_South_Africa	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016														
KY018626_South_Africa	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000													
KP975215_India	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000	0.0000												
GU326328_GenBank_NCBI	0.0016	0.0049	0.0016	0.0032	0.0049	0.0049	0.0032	0.0049	0.0016	0.0065	0.0032	0.0016	0.0016	0.001											
KU722525_Djibouti	0.0016	0.0016	0.0016	0.0032	0.0016	0.0016	0.0032	0.0049	0.0016	0.0032	0.0000	0.0016	0.0016	0.001	0.0032										
KP975233_India	0.0016	0.0049	0.0016	0.0032	0.0049	0.0049	0.0032	0.0049	0.0016	0.0065	0.0032	0.0016	0.0016	0.001	0.0032	0.0032									
KJ766304_Malaysia	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000	0.0000	0.000	0.0016	0.0016	0.0016								
KY018606_Bangladesh	0.0016	0.0049	0.0016	0.0032	0.0049	0.0049	0.0032	0.0049	0.0016	0.0065	0.0032	0.0016	0.0016	0.001	0.0032	0.0032	0.0032	0.0016							
KY018608_Bangladesh	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000	0.0000	0.000	0.0016	0.0016	0.0016	0.0000	0.0016						
KP019937_Malaysia	0.0032	0.0065	0.0032	0.0049	0.0065	0.0065	0.0049	0.0065	0.0032	0.0081	0.0049	0.0032	0.0032	0.003	0.0049	0.0049	0.0049	0.0032	0.0049	0.0032					
KY018612_Australia	0.0016	0.0049	0.0016	0.0032	0.0049	0.0049	0.0032	0.0016	0.0016	0.0065	0.0032	0.0016	0.0016	0.001	0.0032	0.0032	0.0032	0.0016	0.0032	0.0016	0.0049				
KY018616_Australia	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000	0.0000	0.000	0.0016	0.0016	0.0016	0.0000	0.0016	0.0000	0.0032	0.0016			
KY018618_Kenya	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000	0.0000	0.000	0.0016	0.0016	0.0016	0.0000	0.0016	0.0000	0.0032	0.0016	0.0000		
KY018619_Kenya	0.0000	0.0032	0.0000	0.0016	0.0032	0.0032	0.0016	0.0032	0.0000	0.0049	0.0016	0.0000	0.0000	0.000	0.0016	0.0016	0.0016	0.0000	0.0016	0.0000	0.0032	0.0016	0.0000	0.0000	

Figure 3

Evolutionary Divergence based on p-distance computed through COX1Sequences of Corvus splendens collected from Pakistani localities and compared with other representative taxa of the world. The number of base differences per site from between sequences is shown. This analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated (complete deletion option). There were a total of 618 positions in the final dataset.