CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Molecular Identification and Phylogenetic Studies of some Passerines of Pakistan based on Cytochrome c Oxidase Subunit I

by

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Dedicated

To

My beloved parents

Nazir Ahmad (Late) and Surrya Begum

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CERTIFICATE OF APPROVAL

This is to certify that the research work presented in the dissertation, entitled "**Molecular Identification and Phylogenetic Studies of Some Passerines of Pakistan based on Cytochrome c Oxidase Subunit I**" was conducted under the supervision of **Dr. Sahar Fazal Malik**. No part of this dissertation has been submitted anywhere else for any other degree. This dissertation is submitted to the **Department of Bioinformatics & Biosciences, Capital University of Science and Technology** in partial fulfillment of the requirements for the degree of Doctor in Philosophy in the field of **Bioinformatics.** The open defence of the dissertation was conducted on **October 27, 2023**.

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(Fakhra Nazir)

Abstract

Passeriformes is a species rich and morphologically diverse order among class Aves. It is representing over 60% of the class Aves. It is represented in all terrestrial habitats, distributed all over the world except Antarctica and generally well identified on morphological and molecular bases globally. Species identification and phylogenetic analysis through DNA barcodes using mitochondrial COI gene (cytochrome c oxidase subunit I) was aimed for samples of birds collected from different regions of Pakistan. Mitochondrial DNA was successfully extracted from keel tissue and Folmer region of CO1 gene comprising 650 bps was amplified using universal primers and PCR products were confirmed by 1% agarose gel electrophoresis. Sequencing was carried out by Sanger's method. Sample sequences were analyzed using BLAST at NCBI and BOLD database. Comparison of classical and molecular taxonomy of samples of Passeriformes of Pakistan at family and genus and species level was inferred by referencing the literature by Roberts (1992) for classical taxonomy and BOLD (Barcode of Life Data System) databases for molecular taxonomy. Sequence analysis was performed to establish evolutionary relationship of study data and worldwide species of Passeriformes retrieved from AVIBASE database using MEGAX.

Samples were morphologically identified through available literature as 49 species from 39 genera and 18 family of order Passeriformes Total 43 out of 49 sequences were identified as species of order Passeriformes based on the best matches by comparing their similarity percentage with other sequences in the database. Out of 43 species sequences, 32 were ranging from 97-100% identity with their respective species. Species sequences of 11 were showing similarity $< 97\%$ but with other species of same genus. Further verification at molecular level was performed using the Barcode of Life Data System (BOLD). BINs were assigned to 43 sequences representing 42 BINs. Single BIN was assigned to 2 species (*Prinia buchanani Blyth*, *Prinia hodgsonii Blyth*, BOLD: ACZ2474). Out of these 42 BINs 32 records were taxonomically concordant. Rest of 11 records out of 42 showing unique BINs, as single new record of respective species Passeriformes of Pakistan and have not been submitted from another region of the world in the BOLD database. List of

192 species of order Passeriformes were retrieved from AVIBASE database and phylogenetic relationship was established among 235 barcodes including newly generated 43 sequences of Passeriformes. The comparison revealed changes at different taxonomic levels of 17 species based on our study.

Sequence analysis revealed variation in frequencies of each nucleotide (ATGC) in the barcode region of Passeriformes species of Pakistan at the three codon positions, 1^{st}, 2^{nd}, and 3^{rd}. K2-parameter distances indicates that that average evolutionary divergence among species of Passeriformes was 0.18 the least average evolutionary divergence was 0.019 among *Lanius isabellinus* and *Lanius collurio* in the same way this average evolutionary divergence among other species of genus Lanius was very low as 0.022 among *Lanius tephronotus* and *Lanius cristatus* the highest was 0.263 among *Rhodospiza obsoleta* and *Ptyonoprogne rupestris* and among *Rhodospiza obsoleta* and *Cercotrichas galactotes*.

Evolutionary analyses showed that the differences in COI sequences were usually higher among the species under family as compared to species under genus. Intraspecific hyplotypes were clustered under monophyletic clades with bootstrap support in the NJ tree. The overall phylogeny of Passeriformes remained same as previous studies, forming similar split ups and clades. All 235 species of Passeriformes were first split into two clades. One comprising of the 5 species *Panurus biarmicus, Aegithalos concinnus, Megalurus palustris, Locustella naevia* and rest of the species in other clade which were further splited into sub clusters bases on the similarity and differences in their COI gene sequences. Species under one genus and species under one family were grouped together in each clade showing the similarity in their COI gene sequences and showing their monophyletic nature. DNA barcoding was proved as an effective tool for species molecular identification and their phylogenetic analysis during this study which may help in identification and biogeographic studies of the birds in future.

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Abbreviations

BIN	Barcode Index Number system
BLAST	Basic Local Alignment Search Tool
BOLD	Barcode of Life Datasystem
bp	Base Pairs
CBR	Centre for Bioresource Research
CEMB	Centre of Excellence Molecular Biology, University of Punjab, Lahore
COI	Cytochrome c oxidase subunit I
DDT	Dichloro-diphenyl-tri-chloroethane
DNA	Deoxyribonucleic acid
GDA	Genetics Distance Analysis
ICZN	International Commission on Zoological Nomenclature
IUCN	International Union for Conservation of Nature
KPK	Khyber Pakhtunkhwa
MEGA	Molecular Evolutionary genetic Analysis
ME	Minimum Evolution
MP	Maximum Parsimony
mtDNA	Mitochondrion DNA
nDNA	Nuclear DNA
NCBI	National Centre for Biotechnology Information
N-J	Neighbor Joining
PCR	Polymerase Chain Reaction
RAPD	Randomly Amplified Polymorphic DNA
UPGMA	Unweighted Pair Group Method with Arithmetic Mean

Chapter 1

Introduction

1.1 Theoretical Background

Birds are the most studied group of organisms, especially their origin, biogeography and phylogeny based on molecular data and they act as model for studying phylogeny of animals [1–4].

1.1.1 Worldwide Distribution of Avifauna

According to "Bird Life International" over 10,000 species of birds are distributed across the world with majority in continental regions and remaining on islands. They live in vast variety of habitats and are present at the extremes of land elevations and latitudes, but this distribution is uneven [5–7]. Distribution of known land-bird species is as follows: Neotropical realm holds (c.36%), Indomalayan (c.18%), Afrotropical (c.21%), Palearctic (c.10%), Australasian (c.17%), Oceanic (c.2%) and Nearctic (c.8%) realms. Some countries have richest avian diversity with more than 1,500 species each (Colombia, Peru, Brazil, Ecuador and Indonesia) while others with 1,000 species all around (Bolivia, Argentina, Venezuela, Kenya, China, Tanzania, India, Mexico and Congo). Birds being an important component of ecosystems help understand state of that environment [6].

1.1.2 Taxonomy of Aves (Birds)

The birds belong to class Aves which is composed of 34 recognized orders, out of which 25 are extant, and 9 orders are extinct. Among 9 fossil records of 7 orders are available [5] whereas, according to Sibley and Ahlquist [1], the avian class consists of 29 orders. Due to great number of species and populations of Passeriformes, ornithologists have divided birds into two main categories i.e., Passerines and non-Passerines. Based on their variable behavior, ecology and structure they prefer all types of terrestrial habitats [4].

1.1.3 Modern Avian Taxonomy

Taxonomy has witnessed a number of changes since the Linnaeus period starting from degree of similarities existing between different morphological characters and currently it is based on evolutionary relationships possibly existing between different groups of organisms. The skull was the first morphological trait used for classification of the avian species in 1867, to determine the common inherited features for the avian species [8]. The morphological characteristics based classification has now been generally replaced to molecular based classification with focus on genetic variations. The first DNA based taxonomy was conducted on DNA hybridization method [1, 9–11]. Later, DNA sequencing of particular gene loci was used instead of DNA hybridization technique. The evolutionary relationship among all living organisms is now under study all over the world to reconstruct a tree of life based on DNA sequences of organisms. Recently, this approach has changed the taxonomic classifications concept which tries to predict the possible future taxonomic changes through the reconstruction of phylogenetic tree of organisms. On the bases of differences in the skulls of birds the modern birds (Neornithes) have been divided into two groups, viz., Paleognaths (old jaw: Ostrich, Rheas, Cassowaries, Emus, Kiwis, Tinamous), and Neognaths (new jaw: which includes the rest of the living birds). Figure 1.1 shows the broad avian taxonomy groups recognized by bird taxonomists [12, 13].

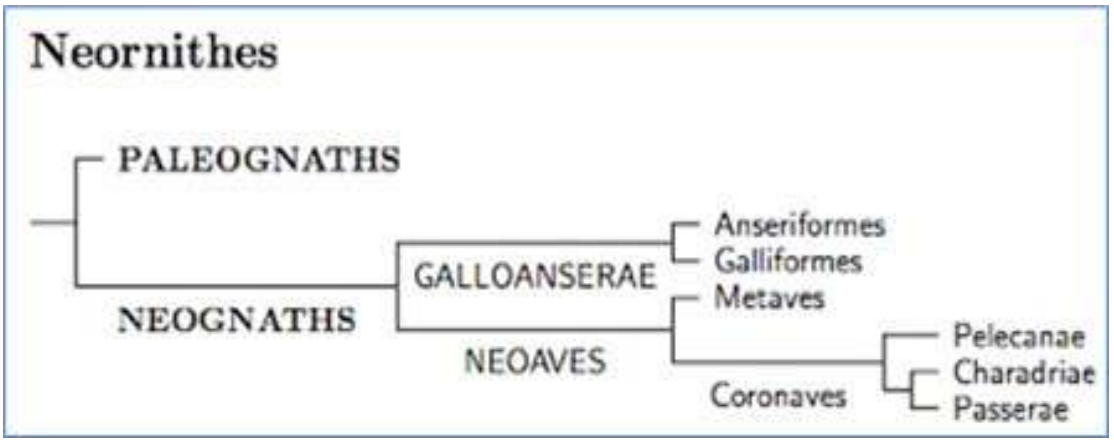


FIGURE 1.1: Avian taxonomy [14, 15]

The Neognaths are further separated into two main groups, i.e., Neoaves and Galloanserae. Galloanserae are further divided into Galliformes (turkeys, chickens, quail, and other land fowls) and Anseriformes (waterfowl). Neoaves are divided into two subgroups, i.e., Metaves and Coroaves. The Metaves include pigeons and doves as well as sand grouse, kagu, sunbittern, grebes, flamingos, mesites, tropicbirds, nightjars and relatives, swifts, and humming birds [16]. This is a large grouping in which some species show a high degree of relatedness, such as grebes and flamingos, whereas some species might be placed in erroneous taxons. Till now phylogenetic trees based on mitochondrial DNA analysis has placed Metaves group in separated clade as its members have close association among themselves instead of showing association with other groups of birds [17–21]. For example, tropic birds were grouped with accipiters, and kagu grouped with woodpeckers and passerines [20]. Coronaves consist of waterbirds (Family Pelecanae), shorebirds (Family Charadriae) and land birds (Family Passerae) [22].

1.1.4 Passeriformes

The order Passeriformes of class Aves has great diversity being the largest clade of birds with almost 5,740 species or 59% of all living bird under 2057 genera and 9672 species globally [9, 10]. The variation in number of orders of class Aves indicates that this grouping keeps on changing with the new studies using newer methods of taxonomy.

According to "Birds of the World" checklist 2021 edition the 3824 species of birds from Asia represents the avifauna diversity in this region [23] Passeriformes have a cosmopolitan distribution and inhabit different terrestrial biotopes being variable in behavior , structure, and ecology, and are present in all continents, except Antarctica, with the greatest diversity in the tropical forests [4, 14]. According to Robert [15] 313 species belonging to 36 families of Passeriformes exist over the areas under Pakistan whereas Avibase [24] database reports 497 species of Passeriformes are reported from Pakistan. Most Passerines generally have a very small body and the smallest weigh from 4 to 6 grams, *Regulus regulus* (Goldcrest) and the *Psaltriparus minimus* (American Bushtit). While some are large, the largest passerines weigh up to 1.5 kilogram, are the *Corvus corax* (Common Raven) and *Menura superba* (Lyrebird). The feed of them mainly consist of fruit, seeds, and the nectar. They also prefer spiders, insects, and annelids as food. Passerines have small body size and relatively easy to observe, collect and study, therefore systematics biologist, behavioral ecologists and evolutionary biologists prefer this group for different descriptive and experimental studies [4, 25].

1.1.5 IUCN Status of Passeriformes

IUCN (International Union for Conservation of Nature), global Red list category claims that Passeriformes 60 species has become extinct, 94 are critically endangered, 216 have become endangered, 374 are found vulnerable, 527 mentioned near threatened and 29 lack sufficient data deficient globally [26]. The main threats indicated to this group of birds include agriculture practices like overgrazing of shrubs destroy their habitats [27]. The deforestation results in natural system modification by construction of dams and roads can also cause habitat loss of these birds. The invasive and other problematic species introduced in the system [28] can create competition for food and shelter [29]. Injudicious use of pesticides on crops like DDT (Dichlorodiphenyltrichloroethane) can affect reproductive success of birds by thinning their egg shell and cause life threat to herbivorous birds [30]. Birds are captured for consumption and sports purpose owing to decline in their local population etc. [31].

Passeriformes normally prefer the terrestrial habitat, as forest, grasslands, shrub lands and rocky hilly areas, along with wetlands. This clade of birds is mostly migratory but some are resident too. "The selection of species for study of diversity of Avian depends on the population trend of Passeriformes species which are mostly stable and population trend $>$ 30% decline over three generations or ten years". This quality of species is helping avoid reaching the vulnerability thresholds (http://www.birdlife.org) and the population trend of Passeriformes species was mostly stable (Table 1.1).

TABLE 1.1: The Classical taxonomy of a range of species

Kingdom	**Animalia**		
Phylum	**Chordate**		
Class	Mammals	**Aves**	
Order	Primates	Galliforms	**Passeriformes**
Family	Hominidae	Phasianidae	Corvidae
Genus	Homo	Gallus	Corvus
Species	*Homo sapiens*	*Gallus gallus*	*Carvous seplendens*

1.1.6 DNA Barcoding

In order to minimize the problems faced by taxonomists in morphology based classification the new approach is introduced by focusing on the sequencing of few loci on mitochondrial genome, generally called as the DNA barcoding. It is relatively rapid genetic method for taxonomic studies that uses standardized genetic markers to identify a species and assesses biodiversity [32]. These markers provide a strong tool for rapid identification and classification of animals and plants without going into morphology-based taxonomic details by just using mitochondrial DNA [33].

The phylogenetic studies of Passeriformes have been observed controversial among different researchers [2]. Phylogenetic relationships among and within families and the genera were reported by multiple studies at molecular level [1, 2, 6, 34–36]. The phylogenetic studies have demanded a detailed analysis of Passeriformes including

the molecular characters, vocalization and morphological traits. In present study we assessed the genetic diversity and phylogenetic relationship among different families of Passeriformes at species level using COI (Cytochrome c oxidase subunit I) genetic marker from mitochondrial DNA (Deoxyribonucleic acid) [34].

Various techniques have been developed and used in the past for analyzing the genome (DNA).The choice of techniques to analyse DNA depends upon the nature of question to addresse, and the material under study. The molecular biologists have extensively utilized COI gene marker for finding the genetic variations among diferrent species of different organism especially birds. DNA barcoding is not only user-friendly, easier, and much convenient but also independent of any previous knowledge of the genome. Hence, very preferable for characterizing those species which are lacking such studies [33].

1.1.7 Mitochondrial Genome

Mitochondrial genome/ DNA (mtDNA) is preferred for DNA barcoding instead of nuclear DNA (nDNA) because it is derived from mother and has a haploid status and shows limited recombination.This makes the track of sequence divergence easier in organisms [37, 38]. The mtDNA has 5-10 time higher chances of mutations than nuclear genome. In past it was thought that mtDNA lack repair activity but multiple repair pathways in mtDNA have been revealed in last four decades research. It includs mismatch repair, break repair in single-strand, excision repair in bases and homologous recombination. These repair pathways function similarly in mtDNA as in nucleus. They are mediated by enzymes and encoded by the nuclear genes and so far in all cases are identified in mammals. Thus mtDNA has greater potential to be used in exploration of phylogenetic and evolutionary relationship of different species [38, 39].

1.1.8 COI gene of Mitochondrial DNA

Different markers in the mitochondrial genome are in use to identify different organisms but COI region has proved to have enough information to classify organisms at species level [32, 33, 40]. Therefore, the main focus of this study was COI gene based molecular characterization of different species of Passeriformes collected from different regions of Pakistan. COI region of mitochondrial genome is a short (∼600-800 bp-base pairs) region of mitochondrial gene, present at 5' end, has been used as DNA barcode to identify species of animals [32, 33, 41, 42]. It is very effective in recovering information from small samples because a very large number of copies are present in each cell. Each eukaryotic cell have over 1,000 mitochondria, each possessing 10 copies of the mitochondrial genome, which suggests that a cell may contain more than 10,000 mitochondrial genome copies compared to thier nuclear whole genome. This makes extraction of sufficient quantities of mtDNA easier even from a smaller quantity of tissues available in non-invasive samples of even dried or decayed material available in the field. Therefore a small quantity of mitochondrial DNA sequence, isolated from some decayed, dead, or denatured specimen is enough to amplify the COI sequence in order to differentiate between species [43]. Further, the COI gene can easily be amplified by PCR (Polymerase Chain Reaction) using universal primers for members of different taxa [44]. In DNA based system of species identification, the standardized techniques of DNA extraction, gene amplification (PCR) and sequencing are used for identification of any unknown organism using a library of all organisms present on earth [40, 45].

The mtDNA has been extensively used in phylogenetic studies of the animals as it evolves comparatively at much rapid pace than the nuclear DNA. It results in the rapid sequence change and accumule the differences among closely related species and between populations [8]. First time in the history John Avise has sequenced the mtDNA and identified their sequence divergences and evolutionary history record was provided within species. This has helped find linkage between systematics and population genetics and established the field of phylogeography. Avise along with others researchers found prominent mtDNA divergences among sister species [40, 46].

1.1.9 Molecular Phylogenetic

Phylogenetic is an approach to know the relationships among different taxa by keeping their formal biological classification persistent in order to work out evolutionary lineage of a species [47]. The molecular phylogenetic focuses on studying hereditary differences at genetic level, especially in DNA sequence. It finds evolutionary relationships among different species of organisms based on genetic diversity which can be expressed in a phylogenetic tree [48]. Molecular phylogenetic correlates the molecular evolution as it is the process of highlighting mutations (selective changes) at molecular level (genes or proteins, etc.) which are indicated through various branches of the tree of life [49] developed based on the levels of similarities in the mocular structures.

Comparison of mtDNA trees with the species trees shows general concordance which implies the use of analysis of DNA for specimen idendification insead of morphological based specimen idendification. In the past DNA-based identification has been applied for reconstruction of food webs by identifying food fragments from the stomachs [50] and for recognition of products which were prepared from the protected species of animals [51]. After that Hebert [52] suggested COI region (648-bp) of mtDNA which was consistently differentiating species of multiple group of organism as a standard "DNA barcode" to help in making library of sequences of differen organisms which can be linked to thier vouchered specimens. He further stated that this gene region can easily be recovered and provided sequence divergences as evidence between 13,000 species of animals which were closely related [37]. He also worked on binomials of birds which has been intensively studied and extends the previous investigations of species boundaries now by COI barcodes and created a very firm binomials system [40].

1.2 Problem in Focus

The ICZN (International Commission on Zoological Nomenclature) rules have enabled zoologists to name some 1.7 million species, out of total 10 to15 million

species expected to be present on this earth. Hebert [33] estimated that as many as 15,000 taxonomists would be required to routinely identify organisms if our dependence on morphological diagnosis is sustained and still they would probably not be able to cover all taxa [42]. The insufficient expertise and knowledge may lead to misidentification of specimens [53]. It is tenuous and time-consuming job to correctly assign a particular species to a taxonomic group [54–56]. Phenotypic plasticity and limited distinguishing morphological characteristics may lead to wrong identification of species. Moreover, morphological identification methods may overlook the existence of cryptic taxa; because such species have no qualitative morphological and/or morphometric data available that help in their identification [57] As an alternate to the morphology-based taxonomic and systematic studies, recent advancement in molecular techniques can facilitate rapid and more reliable taxonomic identification of the species and their systematic organization in the phylogenetic tree [53, 58]. These molecular genetic techniques have been used to address the unresolved controversies regarding phylogenetic relationships arising because of insufficient and conflicting classical taxonomy data about these groups [59]. Keeping to the existing situation regarding status of molecular taxonomy and systematics of the fauna and flora of Pakistan it was planned to employ DNA barcoding to reveal the evolutionary and genetic relationship among different species of Passeriformes of Pakistan.

1.3 Gap Analysis

The high success rate of DNA barcodes in characterization of animals in general and the birds in particular, and the major phases of diversification among different species and phylogenetic relationships of order Passeriformes of Pakistan have not yet been extensively studied except only one family Corvidae of this order. Passeriformes, being the largest order of avian fanna and among the most diverse clades of terrestrial vertebrates need to be studied, but the baseline data to monitor changes in the species of this diverse group of birds and to find thier genetic diversity over time is also missing from Pakistan. Limited studies about genetic

diversity based on different genetic markers has been reported and only at family level.

1.4 Problem Statement

The outstanding performance of the DNA barcodes to characterize bird species has validated it as a powerful tool to identify the cases which need a deeper scrutiny. Interesting evolutionary characterizations i.e., hybridization, genetic introgression, genetic diversification, recent radiations and cryptic divergence have been represented by the inconsistencies between taxonomy and DNA barcodes. Passeriformes act as a good model for performing biogeographic, phylogenetic, and phylogeographic studies for gaining detailed insights in the process of speciation being the largest order of birds. The extensive analyses of avian evolutionary patterns at a large-scale are also promoted for the growth of a standardized COI sequences based library.

1.5 Objectives of Study

The following objectives were planned for this study to settle taxonomic status of Passerines of Pakistan based on molecular genetics:

- To perform a molecular characterization of species of Passeriformes of Pakistan by developing barcode through amplification of Cytochrome c oxidase subunit I (COI).

- To compare the genetic diversity and to establish evolutionary relationship based on COI gene of the different species of Passeriformes from Pakistan and the world.

1.6 Research Philosophy

Despite of many years of work in taxonomy, the working biologists still face problem in proper identification of species of different organisms. DNA sequencing has been suggested as a key technology that can make species identification fast and accurate. COI gene of the mitochondrial genome (mtDNA) has already been recognized as an effective marker which can be exploited in molecular identification at species level. Its efficacy has been proved through a number of studies on different taxa. These studies have demonstrated that taxonomic controversies in most species can be resolved by DNA barcoding. DNA barcoding has also been provided ways of identifying cryptic and extinct species and provided support to match adults with immature specimens in recent studies. In future, DNA barcoding may act as a standard tool to identify species. This research will help us to develop a molecular based identification of different Passerine species. It will also help to define the genetic status of different Passerine species and their phylogenetic relationship. The information thus collected can be used in planning a strategy for the future research based management for Passerine conservation, specially controlling illegal trade. The study will also provide basis for future research on the Passerines in Pakistan to maintain a predator-prey balance and thence the smooth flow of the ecosystem in the area. Furthermore, trans-boundary collaboration in research and management of Passerines could be initiated and strengthened.

1.7 Research Methodology

In this study 49 species of Passeriformes an order of class Aves were studied using morphological and molecular techniques. Samples were collected and analyzed with focus on COI region of mitochondrial genome used as standard for identifying species of organisms. These steps were followed to achieve study objectives.

- **Collection of samples**

  - Sample collection
  - Recording of GPS coordinates
  - Preservation of samples
- Morphological characterization of samples
- DNA Extraction
  - Reagent preparation
  - DNA extraction
  - Gel electrophoresis
  - Quality and quantity assessment
- PCR
  - Primer selection
  - Preparation of reaction mixture
  - Confirmation of PCR product
- DNA Sequencing
  - Preparation of sequence reagent
  - Sequencing
  - Purification of sequence reaction
- In Silico Analysis
  - Sequence BLAST Analysis
  - Barcode Index Numbers (BINs) Assignment from BOLD Database
  - Accession Numbers Assignment from NCBI Database
  - Passeriformes of Pakistan List Retrieval from AVIBASE Database
  - Sequence Retrieval from NCBI Database
- Sequence Analysis by MEGAX
  - Multiple Sequence Alignment

- Nucleotide Composition
- Codon Usage Bias
- Variation at Amino Acid Level
- Estimation of Transition / Transversion Bias
- Tajima's Neutrality Test
- Estimation of Evolutionary Divergence
- Phylogenetic Analysis

Chapter 2

Literature Review

2.1 Motivation of Current Research

DNA barcodes of 41% of known bird species from different geographic areas are available which provide data rich enough for answering the questions about evolution and also offer high quality data which can help in species identification at molecular level [60]. DNA barcoding of birds from Pakistan has not yet attempted at extensive level therefore this study was planned to cover this area of research from Pakistan.

2.2 Theory and Background Information

2.2.1 Origin of Passerines Based on Morphological Studies

Ricklefs [61] studied that there are more frequent clades at tribe to family level with five or few species among group of passerine birds than expected by speciation process or extinction. It was suggested through previous analysis that normally clades marginalized ecologically or geographically. The hypothesis was tested for small clades which normally prefer peripheral positions morphologically. The relationship of morphological characters was noticed for seeking some hard substance

and feeding in dense vegetation and from perched positions. These findings revealed that clades can protect from extinction by adopting the peripheral space in morphology and ecology.

Managold [62] found phylogenetic affinities among species of family Vangidae on morphological basis. This was first cladistic analysis on morphological basis who excluded several taxa from Vangidae as Calicalicus madagascariensis. It supported the hypothesis that vangas sensu stricto and Australo Papuan are sister taxons forming monophyletic group. DNA based analysis conflict this hypothesis that, bush shrikes and helmet shrikes are close relatives of genus vangas. Therefore, it opens new windows for DNA based studies to cross check the morphological taxonomy of birds.

King [63] studied the passerine birds from four different biogeographic regions based on their morphology to find factors influencing their colonization history. Multivariate analysis was conducted to find answers to the questions of geographical variations effecting species richness and the composition of biota affecting linage with time. The results of the study support the concept that new clades fill the morphological space regarding diversification. Therfore, more studies should be conducted to find geographical variations affecting species richness in particular geographical areas.

Jønsson [64] also analyzed the passerine assemblages in temporally and spatially for their coexistence and conflict the idea of early evolution on the morphological basis in any particular region except two out of four regions. Results indicated that passerine clades are continuously replacing their position in clades without predicting the range of diversification in any region or group. In future, each clade of continental avifauna will occupy morphological space of any region for evolutionary conservatism to fill the ecological niche.

2.2.2 Origin of Passerines based on Molecular Studies

Sibley [65] conducted the first study on classification and reconstruction of phylogeny/molecular taxonomy of living birds by DNA- DNA hybridization method.

The UPGMA (Un Weighted Pair Group Method Using Arithmatic Averages) method was used to construct dendrograms from DNAs for 1058 species of living birds with 310 as radiolabeled tracers. The order Passeriformes were classified as Suborder Tyranni (Suboscine) and Suborder Passeri (oscine) whereas the ocines, were further clustered into the corvida and the passerida clads. This study is still used as reference for classification and reconstruction of phylogeny/molecular taxonomy of living birds.

Latter studies by Barker [66] strongly supported phylogenetic tree derived from DNA–DNA hybridization, using sequences of nuclear gene for passerine birds. It revealed the dispersal of passerine to different regions of the world (Eurasia, Australasia, Africa, and New World). However, comparative analysis of passerine diversification and adaptation needs reassessment based on molecular studies.

Oscine super-tree was reconstructed by Jønsson [67] by compiling phylogeny data from 99 published studies. Almost half of all avian diversity is made up oscine passerine birds, but its relationships and classification has been controversial for long. Study on 1723 species of passerine was conducted to estimate their phylogenetic relationships among them. Overall resolution of 83% was observed which fully bi-furcated the tree. However, larger gaps were observed in taxa from Australian region and deep branching was found in Sylvioidea but nine South American oscines remained poorly resolved. It recommends further studies on South American oscines to resolve the issue of their evolutionary relationship. Chaves [68] identified and discriminated Tyrannidae species, focusing on occurring in Cerrado biomes of Brazil and the Atlantic Forest using of DNA barcodes method. Tyrannidae belongs to suboscine family and their diversity has created taxonomic issues based on morphological similarities, although these species were phylogenetically distant. During a study 71 flycatcher species were analysed by sequencing 542 bp of COI gene collected from geographically different locations. The analysis showed that majority of species have exclusive haplotypes, and displayed intraspecific diversity ¡ interspecific divergence. Among Elaenia obscura individuals, high intraspecific diversity. There is need to find same taxonomic issues based on morphological similarities for oscines too based on DNA barcoding method.

Alstrom [69] estimated the phylogeny of the avian genus Emberiza and other monotypic genera. Results of study support monophyly of Emberizini (Old World), but conflict the sister relationship of Emberizini (New World). Plectrophenax and Calcarius formed a separated clade from other Emberizini. Most of the clades followed the traditional classification. Phylogeny of genus Emberiza should be estimated from other parts of the world too to reconstruct complete evolutionary relationship among species of whole genus. Jønsson [70] have constructed the molecular phylogeny (Oscine family). Oriolidae shoed wide distribution (Australia to the Old World). This study helps in assessment of direction and time of the dispersal for family Oriolidae. The museum specimens (fresh tissue, toe pads) have been processed using nuclear introns and mitochondrial genes from 29 species out of 33 species of Oriolidae. This phylogenetic analysis supports the classical concept of systematics and showed sequence of dispersal. The Oriolidae are poorly adapted in Pacific island life in contrast to other families of birds living in that region.

Ohlson [71] used 219 taxa to find phylogenetic relationship for the New World suboscine using nuclear markers (5) with 6300 bp to estimate their ages. Tyrannida and Furnariida have showed a great divergence while Tityridae, Melanopareiidae and Conopophagidae followed the previous relationships. Further studies needed for other groups of birds to estimate their ages. Ericson [72] conducted studies which proved that order Passeriformes originated from Gondwana. A time-scale was used to find the evolution of passerines major clades and was determined by using fossils record of five passerine with nuclear markers and which updated the status of New Zealand wrens which splited 85–52 Million years ago from Antarctica. Mitchell [73] studied that New Zealand wrens are the sister group of Passeriformes. Pavenksky [4] presented an overview of order Passeriformes and current state of their evolution and phylogeny based on molecular and morphological studies data. It has summarized the results of such studies in the last 25 years. It is being used as reference for future studies on order Passeriformes. Jonsson [74] analyzed the phylogeny of Corvids using multiple nuclear loci. The Corvids are comprised of 800 species, forming a morphologically diverse clade of passerine birds. Baseline phylogeny was established covering biogeographical, macroecologica and macroevolutionary analysis including 667 species. This data helped find

the temporal consistency of families as higher than genera in present taxonomy.

Selvatti [75] have studied the diversification of crown passerines at family-level, based on the phylogenetic hypothesis of unmatched multi calibrated, relaxed clock inference. In a detailed taxon sampling of passerines, the phylogenetic tree has shown that Acanthisittia diverge in the early Paleo-gene which is comparable to the other avian orders by representing a more intense adaptive radiation from all other passerines. This study has shown that Miocene has occupied niches which were left by Tyrannides before they were occupied by any other Oscines. Gibb [76] studied the complete mitochondrial genomes of over 100 passerine species of New Zealand in a phylogenetic context. Dating analyses of this vigorous phylogeny which is supported by the passerine fossil record, have suggested the early Paleocene origin of oscines which were escaped from Australasia. This study has provided data about origin of oscines. Mitchell [73] studied that New Zealand wrens are the sister group of Passeriformes and their study help understand their evolution. Only morphological analyses are present for Acanthisittidae and no genetic analysis available therefore, is a strong need for study on genetic analysis of Acanthisittidae. Marki [77] studied 300 species of Meliphagides the largest Australasian bird group lacking phylogenetic studies. 286 species were analyzed using four nuclear and five mitochondrial markers and novel relationships were established among 60 sequenced species. It shows the importance of genetic studies for phylogenetic analysis to reestablish the evolutionary relationships among species of birds.

2.3 Diversity of Passerines of Pakistan

The avian diversity of Passerines in Pakistan was highlighted by following studies in different regions of Pakistan and it was observed that majority of the species under study were belonging to the order Passeriformes.

2.3.1 Punjab

Fazal [78] made a comparative study of passerine population with special reference to flora at three old and new city locations in Lahore city during 2008. Decline in passerine avian diversity was recorded due to dense human population affecting breeding sites especially change in trend of plantation of ornamental plants forced the birds to migrate. Relative abundance of native trees in old city has facilitated the increase in passerine population as compared to new parks where exotic plant varieties were introduced. The studies suggest the plantation of native flora in Lahore parks to facilitate the increase in avian population to conserve avian biodiversity.

2.3.2 Sindh

A baseline study was conducted in selected sites of Dhingano-Lakhat Riverine Forests, Sindh, Pakistan to find species diversity and distribution of birds. The field observation and review of literature about avian fauna revealed that 223 species of birds belonging to 19 orders use this area as permanent / seasonal home including both passerine and non-passerines birds and they equally contribute to the avian diversity of the area. Order Passeriformes had dominated the diversity with largest number of species recorded from different location of the study area. This baseline data will help in exploring the diversity of different species of bird and identification of management priorities [79].

2.3.2.1 Avian diversity at Taunsa Barrage

Bibi [80] carried a study at Taunsa Barrage Wildlife Sanctuary during 2009 to 2011 to identify the avian diversity of the area. Direct census method was used to collect, 58,598 bird species from 53 families belonging to 171 species. Five dominant species of the area were *Corvus splendens, Fulica atra, Egretta garzetta, Bubulcus ibis, and Aythya ferina.* A regression analysis showed a decreasing population trend in 14 species. Habitat degradation, illegal hunting and use of pesticide were

identified as main threats to these bird species. Therefore, protection of natural habitat of the area was recommended as an urgent need to protect avian diversity for stable functioning of the ecosystem. Dauda [81] conducted a study at Uchali Wetland, Pakistan to model bird species abundance and evaluated bird species diversity. Data was subjected to Shannon evenness index, Simpson diversity, rank abundance curve to model a diversity analysis using statistics. A total of 25,361 individuals from 47 species of birds, less than observed in the year 1991. The annual species decrease (6.59%) was reported from encountered 192 bird species. There is a need to take actions to conserve avifana of the area.

Ali [82] conducted a study at Keti Bunder, to reassess the avian diversity and the threats to them. For this purpose direct and indirect methods were used to record information about the avian diversity from November, 2015 to February, 2016. About 4280 birds belonging to 49 species were observed and recorded during this survey. The most abundant species of study area were Common coot, Little egret, greater flamingo, cattle egret, and greater egret. It was concluded that major role in decline of avian population in the area under study was the anthropogenic activities especially pollution. Another study was conducted at Langh lake, Sindh, during January-2018 to December-2018 to observe bird species by using binocular (8×30) and camera. Identification of birds was made by Helam Field Guide, Birds of Pakistan [83] and relative abundance and diversity of birds were recorded. During this study 131 bird species were observed for their response to disturbance in order to control their conservation activities. Out of 14 orders under study Passeriformes was recorded as highest in number and fifty percent of Passeriformes has shown medium response towards disturbance [84]. This shows their cosmopolitan nature and adaptaion in the continuously changing environments.

2.3.3 Khyber Pakhatunkhawa (KPK)

Pathan [85] conducted a survey at Swat valley during 2013 to study the avian fauna and the major threats to their populations. Data was collected by interview from local habitants and by observation methods. They recorded 138 species belonging

to 13 orders and 48 families with highest population density of 31 species of order Passeriformes. The area being rich with flora and less hunting trends resulted into high population level of migratory and resident avian species in the area.

2.3.4 Gilgit Baltistan

In a study 83 bird species were recorded in year 2010-2012. Species from Passeriformes were dominated. There were more residents than winter visitors and summer visitors. The feeding habit of the birds revealed that there were more omnivorous, followed by carnivorous, insectivorous, granivorous, herbivorous and frugivorous respectively. This effort recommends that the government should declare the key bird areas like the Qurumbar Lake, Qurumbar river and its tributaries as no-hunting zones [86].

2.3.5 Azad Jammu and Kashmir (AJK)

A study was conducted from March 2009 - October 2009 to find the avian diversity in Azad Jammu and Kashmir (AJK). Predetermined sites were surveyed in the mornings and evenings to observe birds by using Fixed Point Count and Line-transect methods. Highest number of Passeriformes was recorde out of all 70 bird species recorded [87].

2.4 Methods of Molecular Based Studies on Passeriformes

For this purpose a large number of dataset has been generated using molecular sequences for 1,119 genera from 127 families of Passerines to find their genetic diversity and to establish family-level phylogenetic relationship to complement previous limited information on passerines [66, 88–91].

2.4.1 DNA-DNA Hybridization

Earlier [92] Johansson has made first study on passerine to find family-level relationships by DNA hybridization. Comparison between passerine ecology and behavior were highlighted by these studies. The nucleotide sequence variations from 69 passerine taxa were analyzed by using the c-mos genes and nuclear RAG-1. In another study on Passerida, the phylogenetic relationships were found by DNA–DNA hybridization technique using 3 nuclear introns. This molecular level study has helped in identifying previously un known relationships.

2.4.2 Nuclear and Mitochondrial DNA Sequences

Chesser [93] studied sub-oscine birds based on mitochondrial as well as nuclear DNA sequences to find their phylogenetic relationships. The current classifications of sub-oscines was separated by comparing the relationships among families and their subfamilies. This study has supported novel features in this phylogeny of sub-oscine. It also revealed some key differences, especially regarding relationships among sub-oscine families and sub-families. The study indicates behavioral evolution of many sub-oscine groups and shown great impact on their reconstruction of character based evolution.

Cebois [94] assessed the passerine "Tapestry"and investigated phylogenetic relationships among the Muscicapoidea a superfamily, a diverse group of songbirds using nuclear RAG-1 gene sequences. The study presented new comparative sequence data for taxon sample. The sequences of Nuclear DNA confirmed the monophyly of muscicapoid group and the sister relationship of Mimidae, and Sturnidae was also supported by this study. Johnson, [95] studied the Passerida and identified first time by DNA–DNA Hybridization data [96]. Phylogenetic relationships among Passerida were found using nuclear introns and monophyly of the group was established.

2.4.3 DNA Barcoding

According to Meyer [97] DNA barcoding provides a few sampled datasets to test its promise in species identification. Therefore, a lot of work in DNA barcoding is taking place all over the world to identify different organisms at species level. The following are few examples of barcoding of passerine birds.

According to Wogh [98] majority of species are unidentified because of rise in the rate of extinction and increased monitoring in spite of 250 years of systematics. New proposed technology of DNA sequencing and COI mtDNA gene being barcode for identification of samples has increased the speed of identification of organisms in the last few years. Aliabiden [95] performed DNA barcoding of birds using different markers. It has been proved successful for identification of different samples of organisms at species level based on COI geneof the mitochondrial DNA but sometime fail to delimit the boundaries of closely related sister species. A study was conducted on birds for CO1 gene and they targeted the species that occur hybridized on a Holarctic scale. It was concluded that DNA barcoding some time fail to identify hybridizing parapatric species pairs.

2.5 DNA Barcoding of Birds at Global Level

Hebert [40] found that sequence of 648-bp of COI gene of mitochondria might serve as a standardized region for DNA barcoding to identify different species of animal. This tool can help in compiling a public library by a fast and a cheap sequencing method. Barcoding of the bird species from the different areas of the world has taken place to help compile this library. Year wise study details are mentioned below.

2.5.1 Korean Birds

A study was conducted on Korean birds in 2006 for 92 bird species to find their COI barcods and average 25 times higher differences were observed within species

than among closely related species. The COI barcodes was proved effective for identification as only one was misidentified out of 239 specimens. A DNA microarray (19-24 bps) has identified 17 species of birds on the nucleotide diversity basis through a high-throughput and sensitive method [99]. This has proved the effectiveness of DNA barcods for identification of previously misidentified species of birds.

2.5.2 Argentina and North American Birds

Five hundred Argentinian birds were barcoded to find patterns of genetic diversity among them. Most of the bird species (southern Neotropical) showed deep divergence in their sequence from their nearest- neighbor. There was high diversity in Neotropical avifauna and genetic splits were recorded 21 species. Shared polymorphisms was lacking in species [100, 101].

2.5.3 Scandinavian Birds

Johnsen [96] conducted a comprehensive DNA barcode survey by sequencing COI mitochondrial gene of Scandinavian birds. The samples of 296 bird species were collected and genetic divergences in the sequences of 78 bird species from Scandinavia and the bird species of North America were compared among whose breed range include both. The Scandinavian bird species have shown (94%) unique clusters and the 6% bird species shown overlapped barcodes. The effectiveness of standardized DNA barcoding approach has been highlighted through this study and this will help merge this data with the established data about the avian in the barcode library. Results have showed genetic divergences of unexpected patterns among North American birds through DNA barcoding of COI.

2.5.4 Birds of Turkey

Bilgin [102] used DNA barcoding technique to evaluate the genetic diversity of bird species of Turkey. The total 73 birds have been collected from 33 species

which belong to 26 genera. The sequences of COI gene were generated and then compared with COI gene sequences of 301 sequences retrieved from the BOLD Database. Global phylo-geographic comparisons of theses sequences were made to find their taxonomy and intraspecific divergence. The findings of this analysis has highlighted the suitability of this technique for identification of species and compared local genetic variation with the global network.

2.5.5 Birds of Netherlands

Aliabadian [103] has sequenced the COI gene of mitochondria and used it as a marker for species identification of 387 specimens of 147 bird's species from Netherlands. The interspecific divergences average was 9.54% and intraspecific divergences average noted was 0.29%. Unique barcode were represented in 95% of species with 6 species of gulls and shared at least one barcode. *Sylvia curruca* has shown a deep divergence with an average of 5.76% - 8.68% between individuals. In haplotype network analysis two taxa were clearly separated i.e., *S. curruca* and *S. blythi*.

2.5.6 North American Birds

Hebert [40] conducted a study on the birds of North American and differentiated 260 bird species based on variations in their sequences of COI gene. It was found that all these species have a different COI sequence, and this difference is eighteen times greater within species as compared to the species which are closely related. The results indicated the identification of 4 new bird species highlighting the importance of DNA barcoding and helped in compiling the library for the birds of the North American based on the sequences of COI gene.

2.5.7 USA, Canada and North American Birds

Kerr [104] has provided a comprehensive DNA barcode analysis for 643 species of the USA and Canada from North American birds, representing 93% of avi-fauna.

The distinct barcode clusters were obtained for 94% of the species and 2% species were representing the cryptic bird species. The results of the study, indicates the constrained mitochondrial intraspecific variation which supports the new concept of limited mitochondrial diversity.

For the birds of North American Kerr [104] primarily used the BirdR1, and BirdF1 primers but FalcoFa, BirdR2, or VertebrateR1 were also used as additional primers if the amplification was not successful. The reason for the BirdF1/R1 primers not always successfully amplify the DNA was the prevented annealing for sequence significant difference between the DNA sequence and primer.

COI sequences of three avian families (Phasianidae, Strigidae, and Accipitridae) were barcoded for 171 individuals and deep genetic splits, distinct geographica clusters, and threshold levels were observed and suggested COI gene as significant evolutionary unit [105].

New Zealand birds of 928 specimens from 180 species of birds along with 1416 sequences from Genbank used to analyse to find taxonomy and evolution. DNA barcodes were 88.5% successfully identified, 13 groups were recently diverged. Character-based identification method was proved more successful than phylogenetic tree and distance based methods [106].

2.6 DNA Barcoding of Birds in Asia

2.6.1 Road Vehicle Collision Killed Birds in India

Rawankar [107] studied barcoded COI gene of 3 species of Strigiformes reported from Indian subcontinent killed by road vehicle collision. Technique was successful and identified bird species as *Otus bakkamoena, Tyto alba*, and *Athene brama* sequences were generated and submitted to the GenBank database.

2.7 DNA Barcoding of Birds in Pakistan

Rawankar, [108] studied the unresolved taxonomy of Corvus macrorhynchos using complete mitogenome reported from India and Pakistan. A circular mitochondrial genome of 16,927 bp with nucleotide content of T (24.8%), A (30.6%), C (29.8%), and G (14.8%) was used. Results supported that Corvus macrorhynchos culminates subspecies is genetically distinct and act as separate biospecies.This is a study from Pakistan showing genetic analysis of Corvus macrorhynchos from Corvidae family to find evolutionary relationship with other members of the family. Huang [109] conducted a study on Corvidae family of birds by analyzing the COI gene sequences of 39 species belonging to 12 genera and phylogenetic relationships were identified. K2-parameter distance for these species calculated and 22 times higher average value of genetic distance was observed between the species as compared to within species. The phylogenetic tree was constructed on the bases of Maximum likely-hood. The monophyly nature of the Corvidae was proved through COI gene sequence data.

Khan [110] conducted a study on three different species of crows, jungle crow, house crow, and jackdaw (*C. macrorhynchos, C. splendens, C. monedula*) from Mansehra, Pakistan. An inadequate knowledge about the evolutionary patterns and phylogenetic relationships of corvids is available in Pakistan. The genetic variation among the taxa can be measured by counting morphological and biochemical diversity but polymorphism is at the height in the DNA level studies. The RAPD (Randomly Amplified Polymorphic DNA) analysis was used in this study to estimate the genetic diversity and to establish DNA based phylogenetic relationship among these three species of crow. This is a study from Pakistan in which evolutionary patterns and phylogenetic relationship was established among 3 species of family Corvidae. On the other hand Raza [65] used DNA barcoding for taxonomy studies of Pakistani collared dove at molecular level first time. Phylogenetic tree analysis revealed that Pakistani collared dove, African collared dove, Eurasian collared dove (*Streptopelia roseogrisea, Streptopelia decaocto*) shared a common clade of genus Streptopelia.

2.8 Bioinformatics Tools for Sequence Analysis

The following softwares are normally used for phylogenetic trees reconstruction. Phylogenies are estimated through neighbor-joining, UPGMA, maximum parsimony, Bayesian phylogenetic inference, maximum likelihood and distance matrix methods.

For sequence alignment normally ClustalW and MUSCLE is used. ClustalW perform multiple sequence alignment by Distance matrix or nearest neighbour method. MEGA stands for Molecular Evolutionary Genetics Analysis. It uses Distance, Parsimony and Maximum Composite Likelihood Methods. Bayes Phylogenie uses Markov chain Monte Carlo methods to find Bayesian inference, multiple models and mixture model by auto-partitioning. BEAST stands for Bayesian Evolutionary Analysis Sampling Trees. It draws Bayesian inference, relaxed molecular clock and demographic history.

BioNumerics is a Universal platform for the management, storage and analysis of all types of biological data, including tree and network inference of sequence data. It uses Neighbor-joining, maximum parsimony, UPGMA, maximum likelihood, distance matrix methods. It Calculate the reliability of trees/branches using bootstrapping, permutation resampling or error resampling.

MOLPHY finds Molecular phylogenetics of protein or nucleotide by Maximum likelihood method. MrBayes finds Posterior probability estimation through Bayesian inference. PAML stands for Phylogenetic analysis by maximum likelihood. It uses Maximum likelihood and Bayesian inference methods. PAUP performs Phylogenetic analysis using parsimony, distance matrix and maximum likelihood methods. PhyML performs fast and accurate estimation of phylogenies using maximum likelihood [111]. DNA barcoding of birds have been carried out in different parts of world as mentioned above and most of the species in these studies belong to order Passeriformes.

2.9 Applications of Study

DNA barcoding studies can help in quick, precise and more reliable identification of different species of organisms of known and unknown specimens against conventional morphological taxonomy. The morphologically identical but reproductively isolated species are called sibling species, and species identification process helps on segregation of sibling species which are difficult to be differentiated using classical taxonomy. Phylogenetic studies are used in undestanding the evolutionary lineage of the species/ evolutionary relationship between species which help in determining the origin and distribution pattern of various species. This study will provide data about molecular characterization of Passeriformes and the phylogenetic relationship among its species collected from different area of Pakistan. This is baseline data and will be used in geographical studies in future and will contribute in construction of worldwide DNA Barcode reference library.

2.10 Ongoing and Near Future Trends of DNA Barcoding and Gap Analysis

The concept of DNA barcoding was conceived by Besansky [52] for construction of a library of sequences of all living species after their identification through COI gene as a standardized genetic fragment [42] This helped in the exponential growth of reaching 5 million sequences in the library by 2015. There are more than 47,000 sequences of birds from around 6,000 species all over the word, according to the Barcode of life Database systems (BOLD) accessed in 2016 out of total l0, 473 species [60]. The classical classification of birds is mainly based on their morphological features but their uniform body structure is a great hurdle in this process, therefore DNA structure and biochemical methods have recently been employed to reassess their species status and phylogenetic relationships [112]. The phylogenetic affinities and taxonomic status of group of birds are better understood than other organisms, which make them an ideal group for analysis DNA barcoding as standardized genetic method for species identification [113]. Birds were selected

as first group of animals for testing the efficacy of DNA barcoding because of their well-developed classical taxonomy that helped in the estimation of genetic variation and species delineation set by Linnaeus classification [40]. Therefore, a large-scale barcoding study was performed for the first time in 2005 on birds as a taxonomic group [113]. Extensive studies now have been conducted on birds to test the applicability of DNA barcoding [114]. The majority of DNA barcoding birds have been conducted on the Northern Hemisphere species [40, 111]. Therefore, there is a need to identify the worth of DNA barcoding in identifying species accurately from other non-continental regions too where mutation rates, speciation and biogeographic patterns, may be quite different [114].

Investigation of evolutionary process in birds through DNA barcoding covers a set of diverse approaches. The events of recent radiations have been linked to the null or low genetic divergences in the COI regions where noticeable phenotypic differentiations were observed in different species [115]. In some cases, having deep genetic divergence in DNA barcodes within a species further investigation was taken up with other phylogenetic and molecular markers and it was observed that some of these cases deserved new species status as they were showing isolated evolutionary lineage [116]. Moreover, research on DNA barcoding of bird has covered large geographical areas and highlighted the diversifying factors affecting the genetic divergence among them in different biogeographic regions by comparing the patterns of genetic divergence [117–121]. DNA barcoding was also employed to study the evolutionary patterns and mechanisms in mitochondrial gene [96, 116, 119, 122] along with other applications. DNA barcoding of birds have been carried out in different areas of world has been taking place as discussed above but in Pakistan only few species of birds are barcoded. The whole genome of one species of genus corvus i.e., *Corvus macrorhynchos* (jungle crow) has been studied and three species of crow, *C. monedula* (jackdaw), *C. macrorhynchos* (jungle crow), and house crow (*C. splendens*) based on RAPD (Randomly Amplified Polymorphic DNA) analysis from Mansehra. Phylogenetic studies based Cyto b gene of *Pavo cristatus* (Indian peafowl) has also been studied from Pakistan. Molecular characterization of the Pakistani collared dove was performed through COI gene based DNA barcoding. But still a lot of work in needed to be done on

DNA barcoding of birds of Pakistan. Molecular Identification and Phylogenetic analysis of Passeriformes fauna of Pakistan through DNA Barcoding has not yet been attempted [65, 108–110][123–127][128].

Chapter 3

Methodology and Techniques

3.1 Methodology

A flow diagram to explain the steps of methodology for study is provided below:

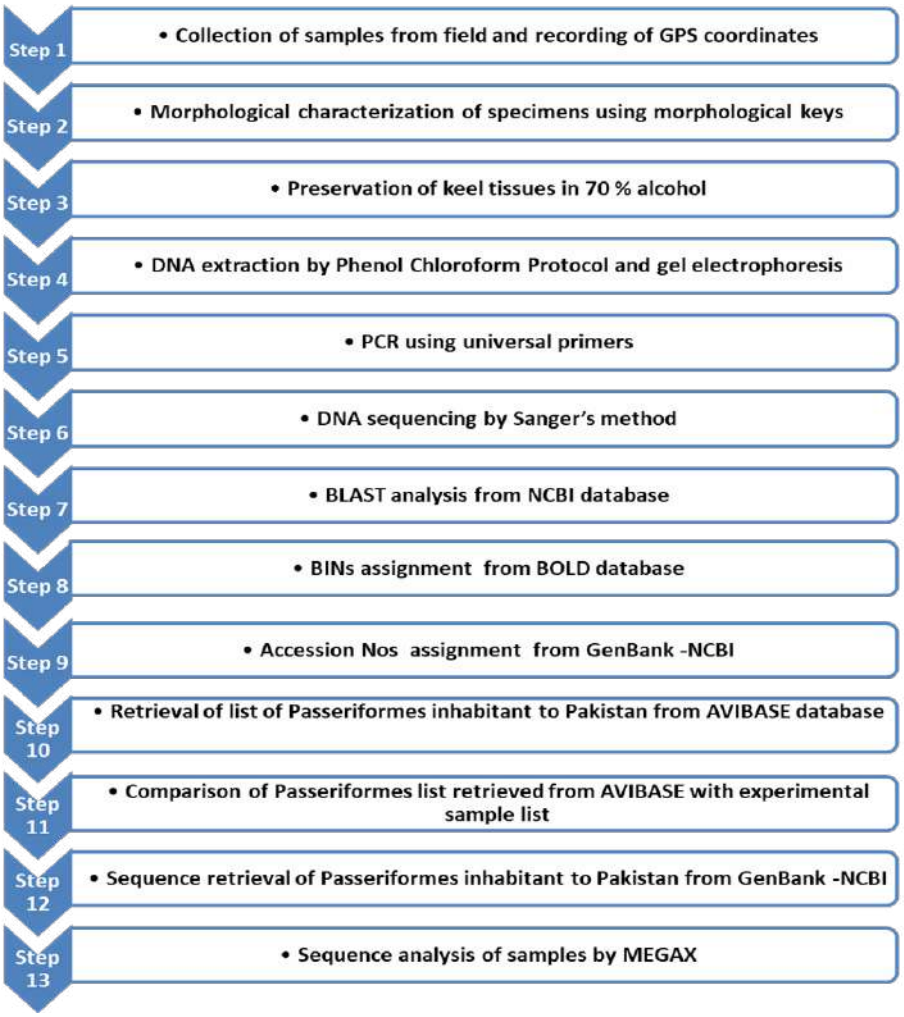


FIGURE 3.1: Flow diagram of proposed methodology

The present study was planned to conduct species characterization at molecular level and to find phylogenetic relationship among different species of Passeriformes collected and processed and sequenced from Pakistan and the sequences of species of Passeriformes retrived from NCBI (National Centre for Biotechnology Information) based on cytochrome c oxidase subunit I (COI) region of their mitochondrial DNA.

3.2 Sample Collection

A random sampling method was used for collection of the passerine bird specimens of 49 species from different locations of Pakistan based on their availability. Samples were collected from taxidermists and hunters to avoid killing of birds. Each sample labeled properly along with sample ID and GPS coordinates recorded by GPS meter (Garmin eTrex 30x, Kansas, US) details are mentioned in Table 3.1. The collected samples were carried to Molecular Biology and Molecular Ecology Laboratory, Centre for Bioresource Research (CBR), Islamabad, Pakistan for further processing. The fresh tissue samples of size ranging from 0.2-0.5 inches were taken with sharp scalp from keel tissues. These samples were preserved in plastic bottles of size 100 ml containing 70% ethanol and stored at -20^o Celsius.

3.3 Morphological Characterization

Collected samples were identified bases on their morphological characteristics by consulting morphological keys [15, 129, 130]. Weight, body color & length, wing color & length, tail color & length, bill color & length, eye color (iris), legs & feet color, and nape color were observed. The morphometric data is presented in Appendix A. Specimens were identified as 49 species under 20 families of Passeriformes referencing the T. J. Roberts [15]. The population trend of these 49 species was retrieved from the red list of threatened species from IUCN (https://www.iucnredlist.org/) [131] and their conservation status was noted as "least concerned" according to this list (Table 3.1). The specimens were deposited

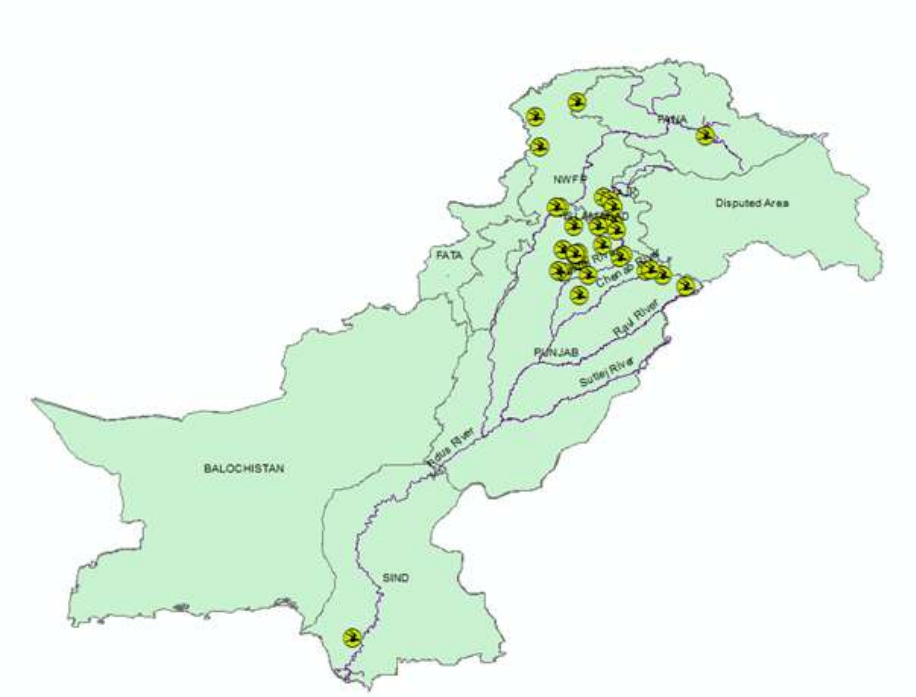


FIGURE 3.2: Map showing collection localities of Passerines of Pakistan (GPS-based map on Google Earth)

as preserved voucher at the Museum of Centre for Bioresource Research (CBR), Islamabad, Pakistan.

TABLE 3.1: Sampling details of Passeriformes species used in this study, including GPS Co-ordinates, location, and population trend

S. No.	**Species**	**Common Name**	**Location, District**	**Population Trend***	**GPS Coordinates**	
					Latitude (N)	**Longitude (E)**
1.	*Acridotheres ginginianus*	Bank Myna	New Satellite Town, Sargodha	Increasing	32.054	72.722
2.	*Alauda arvensis*	intermedia	Katha Masral, Khushab	Decreasing	32.551	72.384
3.	*Anthus richardi*	Richard's Pipit	Fatowal, Sialkot	Stable	32.241	75.046
4.	*Anthus trivialis*	Tree Pipit	Bhakhrevali, Gujrat	Decreasing	32.583	74.286
5.	*Calandrella brachydactyla*	Greater Short-toed Lark	Pipli, Chakwal	No data	32.919	72.647
6.	*Carpodacus erythrinus*	Common Rosefinch	Abbotabad	Decreasing	34.148	73.262
7.	*Chrsomma sinense*	Yellow -eyed Babbler	Hastal, Attock	Stable	33.521	72.622

S. No.	Species	Common Name	Location, District	Population Trend*	GPS Coordinates	
					Latitude (N)	Longitude (E)
8.	*Cisticola juncidis*	streaked fantail warbler	Dharab Dam, Chakwal	Increasing	32.893	72.662
9.	*Copsychus fulicatus*	Oriental Magpie-robin	Kot Sarang, Chakwal		33.028	72.365
10.	*Copsychus saularis saularis*	Common Raven	Bagga, Jehlum	Stable	32.897	73.689
11.	*Corvus corax corax*		Bawarian Wala, Gujrat	Increasing	32.573	74.19
12.	*Corvus macrorhynchos*	House Crow	Bigardo, Skardu	Stable	35.441	75.466
13.	*Corvus splendens splendens*	Rufous	Miana Thub, Rawalpindi	Stable	33.557	73.272
14.	*Dendrocitta vagabunda saturatior*	Tree pie	Rahman A Bad, Rawalpindi	Stable	33.591	73.446
15.	*Dicrurus leucophaeus*		Sanghoi, Jehlum	No Data	32.85	73.621
16.	*Emberiza buchanani*		Dharab, Chakwal	Stable	32.916	72.651
17.	*Emberiza lathami*	Crested Bunting	Karsal, Chakwal	Stable	33.105	73.221
18.	*Eremopterix griseus*		Pipli, Chakwal	Stable	32.915	72.646
19.	*Galerida cristata arenicola*	Crested Lark	Sodhi Jay Wali, Khushab	Decreasing	32.572	72.291
20.	*Gymnoris xanthocollis xanthocollis*	Chestnut-Shouldered-Bush Sparrow	Pipli, Chakwal	Stable	32.919	72.654
21.	*Hypsipetes leucocephalus*	Black Bulbul	Abasin River Kund Park, Nowshehra	Stable	33.932	72.244
22.	*Lanius schach schach*	Long-tailed Shrike	Pipli, Chakwal	No Data	32.914	72.645
23.	*Lanius vittatus*	Bay-backed Shrike	Nekapura, Sialkot	Stable	32.484	74.545
24.	*Melanocorypha bimaculata*		Katheel Houn, Rawalpindi	Stable	33.582	73.491
25.	*Oriolus oriolus*	Eurasian Golden Oriole	Chitral (Kunar River)	Stable	35.845	71.791
26.	*Orthotomus sutorius*	Common Tailor bird	Numbal, Rawalpindi	Stable	33.432	73.534
27.	*Parus major*	Great Tit	Nathigali, Rawalpindi	Increasing	34.072	73.39

S. No.	Species	Common Name	Location, District	Population Trend*	GPS Coordinates	
					Latitude (N)	Longitude (E)
28.	*Passer rutilans rutilans*	Russet Sparrow	Pipli, Chakwal	Stable	32.918	72.654
29.	*Passer hispaniolensis*	Spanish Sparrow	Dir	Decreasing	35.209	71.873
30.	*Sturnus roseus*	Rosy Starling	Gujjo, Thatta	No data	24.744	67.811
31.	*Pericrocotus cinnamomeus*	Small Minivet	Indus River, Attock	Stable	33.923	72.308
32.	*Phoenicurus ochruros*	Black/ Indian Redstart	Dharab Dam, Chakwal	Increasing	32.91	72.676
33.	*Phylloscopus affinis*	Tickell's Leaf-Warbler	Kahuta, Rawalpindi	Stable	33.593	73.46
34.	*Seicercus xanthoschistos*	Grey hooded warbler	Pipli, Chakwal	Stable	32.919	72.649
35.	*Prinia buchanani*	Blyth	Thoa Bahadur, Chakwal	No data	32.944	72.702
36.	*Prinia hodgsonii Blyth*	Grey-breasted Prinia	Pipli, Chakwal	Stable	32.918	72.655
37.	*Prinia socialis socialis*	Ashy Prinia	Dharab Dam, Chakwal	Stable	32.913	72.677
38.	*Pycnonotus leucogenys*	Himalayan Bulbul	Pipli, Chakwal	Increasing	32.919	72.653
39.	*Stachyris pyrrhops*	Black-chinned babbler	DHA, Islamabad	Stable	33.51	73.135
40.	*Sturnus vulgaris*	Common starling	Pind Dadan Khan,	Jehlum	32.482	72.923
41.	*Sylvia curruca curruca*	Lasser Whitethroat	Chumbi, Chakwal	Stable	32.79	72.716
42.	*Tephrodornis pondicerianus*	Common Woodshrike	Chumbi, Chakwal	Stable	32.81	72.728
43.	*Terpsiphone paradisi paradise*	Asian/Indian Paradise-flycatcher	Murree, Rawalpindi	Stable	33.921	73.461
44.	*Garrulax lineatus lineatus*	Streaked Laughing thrush	Barsat, Gilgit	Stable	36.156	72.676
45.	*Urocissa flavirostris flavirostris*	Yellow-billed Blue Magpie	Kahuta, Rawalpindi	Stable	33.593	73.462
46.	*Zosterops palpebrosus palpebrosus*	Oriental White –eye	Kahuta, Rawalpindi	Decreasing	33.592	73.46
47.	*Alauda gulgula australis*	Oriental Skylark	New Satellite Town, Sargodha	Decreasing	32.06	72.723

S. No.	Species	Common Name	Location, District	Population Trend*	GPS Coordinates	
					Latitude (N)	Longitude (E)
48.	*Myophonus caeruleus*	Blue Whistling-Thrush	Pipli, Chakwal	Decreasing	32.918	72.653
49.	*Oenanthe picata*	Variable Wheatear	Pipli, Chakwal	Stable	32.918	72.649

* Population trend of species under study according to IUCN red list of threatened species (www.iucnredlist.org) and GPS coordinates were recorded by GPS meter(Garmin eTrex 30x, Kansas, US).

3.4 Genomic DNA (gDNA) Extraction

The phenol-chloroform protocol for Genomic DNA extraction was used for DNA extraction of each tissue sample with some modifications [132, 133] according to the sample and lab conditions for getting the gene sequence for subsequent sequence analysis [128]. Working area was decontaminated prior to DNA extraction with 10% Clorox solution (Bleach) to minimize decontamination and all scissors, scalps and forceps were washed with 70% alcohol and sterilized by flaming till red hot.

Following procedure was carried out for DNA extraction.

1. Tissues samples of 0.2-0.5 inches size were washed with distilled water to remove the contaminants.

2. Then samples were placed in 300 μL lysis solution (10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, pH 8.0), 40 μL of 10% SDS (Appendix B).

3. A volume of 37ul of 1M DTT and 37 μl proteinase K (20 mg/ml) were also added to this solution and incubated the samples at 56 0C for 4-5 hours for protein digestion.

4. The upper aqueous solution was transferred to clean Eppendorf tubes following centrifugation at 13000 rpm for 10 minutes and an equal phenol volume was added to it.

5. The solution was gently mixed by hands and again centrifuged at 10000 for 8 minutes.

6. The upper aqueous solution was transferred to clean Eppendorf tubes and chloroform: isoamyl: alcohol (25:24:1) in equal volume was added to this solution.

7. Again mixture was centrifuged for next 08 minutes at 10,000 xg after proper mixing.

8. Supernatant of solution was carefully drawn off to new tubes.

9. Ice chilled isopropanol (99%) to the tubes was added and kept for precipitation at 40C for next 4 hours.

10. Samples were centrifuged again for 10-15 minutes at 10-14,000 xg to separate the precipitated DNA.

11. Then precipitated DNA was washed with 70

12. DNA in 30 μl of TE buffer (pH8.0) was re-suspended with 20 μg/ml of RNase and stored at -200C until further processing.

3.5 Gel Electrophoresis

For extracted gDNA confirmation, agarose gel (1%) was prepared and electrophoresis was done on gel electrophoresis apparatus (Labnet. International, USA). Gel was prepared by adding 1g agarose powder in 100mL of 0.5X Tris Borate EDTA (TBE) buffer and then boiling on hot plate (IKA-Combimag RCT, Janke & Kunkel GmbH & Co.KG) to completely dissolve the gel. After boiling, cooling of gel was allowed for few minutes and then 30μL of Ethidium Bromide was added for florescence. Gel was allowed then into casting tray to solidify.

After solidification of gel, comb allowed to be removed and placed in a gel tank already filled with 0.5X TBE buffer. Then 3μL genomic DNA was mixed with 3μL bromophenol blue as a tracking dye and loaded in each well to run at 100

voltage electric current for a time limit of 25-30 min. Gel was then visualized by using Gel Documentation System (Alpha Innotech, Taiwan) and gel images were saved as DNA bands on computer with labeled propelyr for future use.

3.6 Quantity/Quality Assessment of gDNA

DNA quantification was carried out through spectrophotometer (584A Diod Array Spectrophotometer, Hewlett-Packard, USA). The extracted DNA solution absorbance was recorded at the value having a wavelength of 260nm and 280nm. For this purpose 10μL of DNA sample was mixed with 990μL TE buffer and absorbance was recorded on spectrophotometer. The OD288 value was measured on spectrophotometer [134]. The quantity of gDNA was measured in "ng/μL" using following formula;

$$DNAconcentration(\mu g/\mu L) = \frac{A260 \times DF}{1000}$$

In the above formula A260 is absorbance at the wavelength of 260 nm, the dilution factor (DF) with 50 constant as a coefficient for extinction. Quality (purity) ratio of the extracted DNA was obtained by dividing absorbance of samples at 260 nm by absorbance at 280 nm.

3.7 Polymerase Chain Reaction (PCR)

After DNA extraction, selected regions of mitochondrial genome, i.e. folmer region of CO1 gene comprising ~650 bp from the first half of the mitochondrial DNA was selected for amplification as adopted in many subsequent studies [40, 44, 135].

3.7.1 Primer Selection

A pair of Universal primers (BirdF1/BirdR1) was selected for the amplification of COI region of DNA [136]. Primers were supplied by (Oligo, MACRO GEN

(Seoul, Republic of Korea). Universal primer BirdF1 was used as forward primer and BirdR1 was used as reverse primer for COI gene amplification of 650 bp and in case of problem Falco1 was used as forward primer and Bird R2 and Vertebrate R1 were used as reverse primers. The product size along other primers is listed in the Table 3.2.

TABLE 3.2: Primers and amplicon size (bp) used for amplification of COI gene

Primer Name	**Sequence (5' to 3')**	**Target Gene**	**Amplicon Size (bp)**
BirdF1	TTCTCCAACCACAAAGACATTGGCAC		
BirdR1	ACGTGGGAGATAATTCCAAATCCTG		
FalcoF	ATCAACAAACCACAAAGACATCGGCAC	CO1	~650
BirdR2	ACTACATGTGAGATGATTCCGAATCCAG		
VertebrateR1	TAGACTTCTGGGTGGCCAAAGAATCA		

3.7.2 Primer Dilution

PCR water was added to primers obtained in lyophilized form to get 100μL of each primer of the pair. This stock solution was mixed properly by repeated up and down movement of tubes. The primer dilutions were then stored as -20^{o}C.

3.7.3 Reaction Mixture

PCR reaction mixture of 25μL was prepared by mixing 1x reaction buffer, 1.5mM $MgCl_2$, 0.2mM dNTPs, 5pmol Primers (both forward and reverse) and1.5U/μL Taq DNA polymerase (Thermoscientific), deionized water (Serva Electrophoresis, Germany) and 5μL of DNA sample was used. The mixture was short spinned in centrifuged for proper mixing of all ingredients (Table 3.3).

TABLE 3.3: Reaction mixture used for gene amplification during PCR

Reaction Mixture Components	**Stock Solution Concentration**	**Concentration in 25μL Reaction Mixture**	**Volume in 25μL Reaction Mixture(μL)**
Taq Buffer	10X	1X	2.5

TABLE 3.3: Reaction mixture used for gene amplification during PCR

Reaction Mixture Components	**Stock Solution Concentration**	**Concentration in 25μL Reaction Mixture**	**Volume in 25μL Reaction Mixture(μL)**
$MgCl_2$	25mM	2.5Mm	3.0
dNTPS	10mM	0.2mM	0.5
Taq pol.	5U/μL	0.1U/μL	0.5
Primer (F)	100pmol	0.25pmol	1.25
Primer (R)	100pmol	0.25pmol	1.25
DMSO			2.0
Template			5
PCR H_2O			9
Total			25.0

3.7.4 PCR Cycle Profile

Polymerase chain reaction was carried out in PCR sprint thermal cycler (Thermoelectron Corporation, USA). Thermo cycler undergoes three stages: at first denaturation of the DNA was done at 94^{o}C for 5mins. Second stage consists of 35 cycles in which further denaturation was done at 94^{o}C for 1 min, primers annealing with their respective region of DNA at 48^{o}C for 1 min and the process of elongation was done at 72^{o}C for 2 min. Stage three, the extension process consisted of 1 cycle in which further extension of the target region was done at 72^{o}C for 5min. The PCR profile is listed in Table 3.4.

3.7.5 Confirmation of PCR Product

For confirmation of the PCR amplified product the process of gel electrophoresis was done by using, 1% agarose gel which was prepared by following the above gel preparation steps. For size estimation of amplified product the 100 base pairs (bp) DNA ladder (Solis Bio Dyne Estonia; Figure 3.3) was also loaded into the first well along with the samples to analyze the amplicon size. The gel was allowed to run for 30-40min at the given voltage of 100 volts. Gel was visualized under the

Gel Documentation System. The remaining PCR product was stored at -20^{o}C in freezer until further processing.

TABLE 3.4: Details of PCR profile used for DNA amplification

Stages	**Cycle conditions**	**Temperature (^{o}C)**	**Time (min)**	**No. of cycles**
1	Initial denaturation	94	5	1
	Denaturation	94	1	
2	Primer Annealing	48	1	2
	Elongation	72	2	
3	Extension	72	5	1

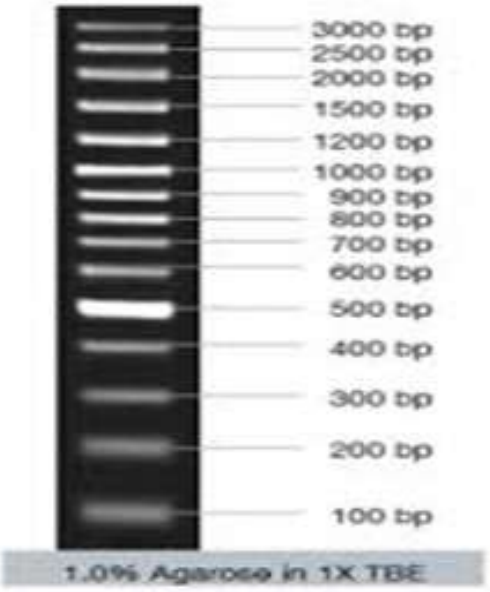


FIGURE 3.3: The DNA ladder having twelve fragments with sizes ranging from 100bp to 3000bp.

3.8 DNA Sequencing

After confirmation of amplification, PCR products were sent to CEMB (Centre of Excellence Molecular Biology, University of Punjab, Lahore) for DNA sequencing. Sequencing was done by using Sanger's method [137].

3.9 In Silico Analysis

3.9.1 Bioinformatics Tools and Biological Databases

The following software and databases were used for data retrieval and phylogenetic sequence analysis of data. Details of these tools and databases are given below.

3.9.1.1 Bio Edit Software Version 7.0.2

It is software with tools used for creating and editing biological sequences. This tool was used to trim the nucleotide sequences of species of Passeriformes under study and sequences were converted into FASTA format.

3.9.1.2 BLAST Program

BLAST (The Basic Local Alignment Search Tool) help in finding the homologous regions between sequences at local level (https://blast.ncbi.nlm.nih.gov/Blast.cgi) [138]. The protein or nucleotide sequences were compared through this program to the other sequence from different databases and the statistical significance was calculated for matches. BLAST also helps in inferring evolutionary relationships between sequences. Blast analysis was performed for sequences under study to find their best matches with other submitted sequences from different areas of world in different databases to identify them.

3.9.1.3 NCBI (The National Centre for Biotechnology Information)

The NCBI (www.ncbi.nlm.nih.gov) is a branch of UNSNLM (the United States National Library of Medicine) a part of NIH (National Institutes of Health). NCBI database provide access to the genomic and biomedical information to advance health and science knowledge. BLAST option was used at NCBI database to find the homology of sequences under study.

3.9.1.4 GenBank Database

The GenBank is a part of INSD (International Nucleotide Sequence Database) collaboration and provide up-to-date information about DNA sequences. The three organizations DDBJ (DNA DataBank of Japan), ENA (the European Nucleotide Archive), and GenBank at NCBI exchange their data on daily basis. During Blast analysis the sequences under study were aligned with the sequences present in these databases. Worldwide data of nucleotide sequences of Passeriformes was also retrieved from this database for phylogenetic analysis.

3.9.1.5 BOLD Systems

BOLD (Barcode of life Data Systems) (www.Boldsystems.org) [139] is a database designed and devoted specifically for generation of sequences and DNA barcode data application. It also provides an online platform for analysing DNA sequences [140, 141]. BOLD includes more than 542,000 species and their 5.9 million data of DNA sequences (DNA barcode). Sequences under study were then submitted to BOLD Systems and Barcode Index numbers (BINs) were assigned to them.

3.9.1.6 AVIBASE- The World Bird Database

Avibase (avibase.bsc-eoc.org) [142] is an information system about databasing of birds of the world. It maintains records of more than 10000 species along with 22000 subspecies. It organizes bird distribution and taxonomic global data online from the publishers of avian taxonomic. The website offers many checklists for different regions of the world having more than 20,000, species. Data about all species of order Passeriformes reported from Pakistan was collected for further analysis from this website.

3.9.1.7 CLUSTALW

Clustal www.genome.jp/tools/clustalw [143] is a program widely used in Bioinformatics in multiple versions based on the type of algorithm used to perform multiple

sequence alignment [144]. The improved version of ClustalW was released in 1994, including allowing individual sequences to be weighted down or up according to or respectively in a partial alignment. It applies various ways to improve progressive alignment algorithm based on divergence or similarity in sequences during alignment [145]. In this study ClustalW was used for sequence alignment of both amino acid and nucleotide sequences before further analysis of sequences.

3.9.1.8 MEGA Software

MEGA (Molecular Evolutionary Genetics Analysis) software is computer based and conducting molecular evolution analysis statistically and phylogenetic trees are constructed. Updated versions of MEGA are available on the MEGA website (www.megasoftware.net) [146]. Being widely used and cited the software was selected for phylogenetic analysis of sequences under study.In this study MEGAX was used for multiple sequence alignment, computing nucleotide compositions, computing the genetics distance between species (GDA) and phylogenetic tree reconstruction. The methods for constructing phylogeny are available as Minimum Evolution (ME), Neighbour-Joining (NJ), UPGMA, and Maximum Parsimony (MP). These methods can be used for finding the true tree using 11 different models. Each method to generate a phylogenetic tree was compared to determine which model can make a tree with fewer anomalies. The method selected for constructing phylogeny was Neighbour-Joining (NJ) method with p-distance model for sequences under study.

3.10 Sequence BLAST Analysis

The sequence analysis was done by Bio Edit software Version 7.0.2 (bioedit.informer .com) [147] for converting sequences into the FASTA format. The fined peak sequences were selected for the analysis while with noise one sequenced again. The sequences peaks after proof reading were subjected to BLAST (blast.ncbi.nlm.gov) [138] at NCBI GenBank portal to identify the specimens of these sequences under

study [148]. The sequences were identified as species of Passeriformes using the nucleotide Blast program (Table 4.5) on the bases of similarity percentage (%) among sequences.

3.11 Barcode Index Numbers (BINs) Assignment from BOLD Database

It is an online framework for generating a BIN web page of each cluster of a barcode sequence after applying well established algorithms to form OTU (operational taxonomic units) that are closely related to species. It forms a curated registry which is integrated with an online database of taxonomic data and specimens (http://v3.boldsystems.org/) [139, 141] which assign unique BINs to each cluster and index in such regimented way that genetically identical taxa from different studies reside under shared identifiers registered in the Barcode of Life Data System (BOLD). The barcode index number system clusters COI sequence data into operational taxonomic units (OTUs) called BINs (Barcode Index Numbers), independent of prior taxonomic assignment [144]. As such it provided a confirmation of the concordance between different clusters of barcode sequences for species designations [149].

In this analysis 49 records of COI region of mitDNA samples of Passeriformes were selected and 43 were with BINs representing 42 BINs. These BINs were further categorized into taxonomically concordant and singletons. Concordant Taxonomy means multiple samples showing same taxonomy therefore same BIN was assigned to multiple samples submitted from different regions of the world to BOLD database. Singletons were the ones whose only one species sample was submitted.

3.12 Accession Numbers Assignment from NCBI Database

The sequences of 43 species of Passeriformes were submitted to NCBI database and accession numbers were assigned to these sequences (Table 4.2).

3.13 Passeriformes of Pakistan List Retrieval from AVIBASE Database

The AVIBASE database was searched for species of Passeriformes reported from Pakistan. The list of Passeriformes was downloaded. The 49 species were sequenced during this study on the bases of their availability during study period. But other reported species were not sequenced in laboratory therefore the sequences of these species of Passeriformes were searched on the NCBI database.

3.14 Sequence Retrieval from NCBI Database

The NCBI data bank for gene sequences was mined for available mitochondrial gene sequences of Passeriformes species for COI, regions. The database was searched with keywords "species name", "mitochondria" and "COI". The sequences of COI were downloaded in FASTA format. In NCBI web portal database, nucleotide option was selected, where species name along with required mitochondrial gene or accession number of sequence (if known) was entered then entered the gene bank option to obtain the required gene sequence. Then these sequences were uploaded at MEGAX for further analysis.

Sequences of COI gene of the Passeriformes species from areas of the world were retrieved from NCBI database for the purpose of phylogenetic analysis of these species of Passeriformes. Out of 474 species (https://avibase.bsc-eoc.org) of Passeriformes reported from Pakistan, the sequences of only 192 species from different

areas of the world were available and retrieved from NCBI. Details of these species are shown in Table 3.5 accession no. were retrieved from NCBI and conservation status was retrieved from AVIBASE database (https://avibase.bsc-eoc.org/) and red list of the threatened species at IUCN (https://www.iucnredlist.org/) [131].

TABLE 3.5: Details of species of Passeriformes whose sequences retrieved from NCBI database

S. No.	**Scientific Name**	**Common Name**	**Accession no.**	**Conservation status**
1	*Pitta brachyura*	Indian Pitta	AB843703	Least Concerned
2	*Aegithina tiphia*	Common Iora	JQ173906	Least Concerned
3	*Pericrocotus cinnamomeus*	Small Minivet	EU541461.1	Least Concerned
4	*Lanius collurio*	Red - backed Shrike	MH938034	Least Concerned
5	*Lanius isabellinus*	Isabelline Shrike	GQ482020	Least Concerned
6	*Lanius cristatus*	Brown Shrike	GQ482013	Least Concerned
7	*Lanius tephronotus*	Gray - backed Shrike	EF621598	Least Concerned
8	*Lanius excubitor*	Great Gray Shrike	JF498786	Least Concerned
9	*Lanius minor*	Lesser Gray Shrike	KX283124	Least Concerned
10	*Oriolus chinensis*	Black - naped Oriole	GQ482278	Least Concerned
11	*Dicrurus macrocercus*	Black Drongo	JQ174696	Least Concerned
12	*Dicrurus hottentottus*	Hair - crested Drongo	JQ174691	Least Concerned
13	*Rhipidura aureola*	White - browed Fantail	JQ176131	Least Concerned
14	*Hypothymis azurea*	Black - naped Monarch	KC354929	Least Concerned
15	*Garrulus glandarius*	Eurasian Jay	GQ481963	Least Concerned
16	*Urocissa erythroryncha*	Red - billed Blue - Magpie	JQ176603	Least Concerned
17	*Pica pica*	Eurasian Magpie	GQ482478	Least Concerned
18	*Nucifraga caryocatactes*	Eurasian Nutcracker	GU571501	Least Concerned
19	*Pyrrhocorax pyrrhocorax*	Red - billed Chough	GQ482576	Least Concerned
20	*Pyrrhocorax graculus*	Yellow - billed Chough	GQ482571	Least Concerned
21	*Corvus monedula*	Eurasian Jackdaw	GQ481647	Least Concerned
22	*Corvus frugilegus*	Rook	GQ481640	Least Concerned
23	*Panurus biarmicus*	Bearded Reedling	GU571521	Least Concerned
24	*Ammomanes deserti*	Desert Lark	FJ465300	Least Concerned
25	*Eremopterix nigriceps*	Black - crowned Sparrow - Lark	MF580208	Least Concerned
26	*Eremophila alpestris*	Horned Lark	GQ481854	Least Concerned

S. No.	Scientific Name	Common Name	Accession no.	Conservation status
27	*Calandrella acutirostris*	Hume's Lark	GQ481412	Least Concerned
28	*Calandrella rufescens*	Lesser Short - toed Lark	GQ481420	Least Concerned
29	*Riparia riparia*	Bank Swallow	GQ482616	Least Concerned
30	*Riparia diluta*	Pale Sand Martin	GQ482609	Least Concerned
31	*Ptyonoprogne rupestris*	Eurasian Crag - Martin	GQ482570	Least Concerned
32	*Ptyonoprogne fuligula*	Rock Martin	MF580225	Least Concerned
33	*Hirundo rustica*	Barn Swallow	KY754510	Least Concerned
34	*Hirundo smithii*	Wire - tailed Swallow	GU460335	Least Concerned
35	*Cecropis daurica*	Red - rumped Swallow	GQ481533	Least Concerned
36	*Delichon urbicum*	Common House - Martin	GQ481695	Least Concerned
37	*Delichon dasypus*	Asian House - Martin	GQ481692	Least Concerned
38	*Culicicapa ceylonensis*	Gray - headed Canary - Flycatcher	JQ174591	Least Concerned
39	*Cephalopyrus flammiceps*	Fire - capped Tit	JX221707	Least Concerned
40	*Periparus ater*	Coal Tit	JX508791	Least Concerned
41	*Periparus rubidiventris*	Rufous - vented Tit	HQ228194	Least Concerned
42	*Lophophanes dichrous*	Gray - crested Tit	HM185314	Least Concerned
43	*Cyanistes cyanus*	Azure Tit	GQ481683	Least Concerned
44	*Parus monticolus*	Green - backed Tit	JX849735	Least Concerned
45	*Remiz pendulinus*	Eurasian Penduline - Tit	GU572079	Least Concerned
46	*Aegithalos concinnus*	Black - throated Tit	HQ605288	Least Concerned
47	*Sitta cashmirensis*	Kashmir Nuthatch	KJ467143	Least Concerned
48	*Sitta tephronota*	Eastern Rock Nuthatch	KJ467157	Least Concerned
49	*Sitta frontalis*	Velvet - fronted Nuthatch	KJ467146	Least Concerned
50	*Chondroma muraria*	Wallcreeper	GQ482777	Least Concerned
51	*Certhia hodgsoni*	Hodgson's Treecreeper	KP282529	Least Concerned
52	*Troglodytes troglodytes*	Eurasian Wren	KP772837	Least Concerned
53	*Cinclus cinclus*	White - throated Dipper	GU571819	Least Concerned
54	*Cinclus pallasi*	Brown Dipper	GQ481589	Least Concerned
55	*Pycnonotus cafer*	Red - vented Bulbul	JF498897	Least Concerned

S. No.	Scientific Name	Common Name	Accession no.	Conservation status
56	*Pycnonotus jocosus*	Red - whiskered Bulbul	KX529958	Least Concerned
57	*Regulus regulus*	Goldcrest	GQ482599	Least Concerned
58	*Cettia cetti*	Cetti's Warbler	GQ481547	Least Concerned
59	*Phylloscopus humei*	Hume's Warbler	GU572031	Least Concerned
60	*Phylloscopus griseolus*	Sulphur - bellied Warbler	MK360480	Least Concerned
61	*Phylloscopus collybita*	Common Chiffchaff	KU870799	Least Concerned
62	*Phylloscopus trochiloides*	Greenish Warbler	GQ482461	Least Concerned
63	*Phylloscopus magnirostris*	Large - billed Leaf Warbler	HQ608867	Least Concerned
64	*Phylloscopus reguloides*	Blyth's Leaf Warbler	HQ608865	Least Concerned
65	*Iduna caligata*	Booted Warbler	GQ481975	Least Concerned
66	*Iduna rama*	Sykes's Warbler	KJ453177	Least Concerned
67	*Hippolais languida*	Upcher's Warbler	KJ453167	Least Concerned
68	*Acrocephalus melanopogon*	Moustached Warbler	GQ481267	Least Concerned
69	*Acrocephalus agricola*	Paddyfield Warbler	GU571212	Least Concerned
70	*Acrocephalus concinens*	Blunt - winged Warbler	KJ453132	Least Concerned
71	*Acrocephalus dumetorum*	Blyth's Reed Warbler	AB893942	Least Concerned
72	*Acrocephalus scirpaceus*	Eurasian Reed Warbler	GQ481283	Least Concerned
73	*Acrocephalus arundinaceus*	Great Reed Warbler	FR847226	Least Concerned
74	*Acrocephalus stentoreus*	Clamorous Reed Warbler	KJ453157	Least Concerned
75	*Megalurus palustris*	Striated Grassbird	JF957023	Least Concerned
76	*Locustella naevia*	Common Grasshopper - Warbler	GU571955	Least Concerned
77	*Prinia crinigera*	Striated Prinia	HQ608884	Least Concerned
78	*Prinia gracilis*	Graceful Prinia	KU722459	Least Concerned
79	*Prinia flaviventris*	Yellow - bellied Prinia	HQ608883	Least Concerned
80	*Prinia inornata*	Plain Prinia	KT240052	Least Concerned
81	*Sylvia nana*	Asian Desert Warbler	JQ176322	Least Concerned
82	*Sylvia nisoria*	Barred Warbler	GU572123	Rare/Accidental
83	*Sylvia crassirostris*	Eastern Orphean Warbler	GU571644	Least Concerned

S. No.	Scientific Name	Common Name	Accession no.	Conservation status
84	*Sylvia mystacea*	Menetries's Warbler	JQ176321	Least Concerned
85	*Sylvia communis*	Greater Whitethroat	GU572120	Least Concerned
86	*Pomatorhinus erythrogenys*	Rusty - cheeked Scimitar - Babbler	JQ175951	Least Concerned
87	*Laticilla burnesii*	Rufous - vented Prinia	MK069051	Near-threatened
88	*Pellorneum ruficeps*	Puff - throated Babbler	JQ175707	Least Concerned
89	*Alcippe poioicephala*	Brown - cheeked Fulvetta	JQ173957	Least Concerned
90	*Argya earlei earlei*	Striated Babbler	MH265887	Least Concerned
91	*Turdoides malcolmi*	Large Gray Babbler	KC439341	Rare/Accidental
92	*Trochalopteron erythrocephalum*	Chestnut - crowned Laughingthrush	MH265810	Least Concerned
93	*Heterophasia capistrata*	Rufous Sibia	EU447047	Least Concerned
94	*Leiothrix lutea*	Red - billed Leiothrix	JF498868	Least Concerned
95	*Muscicapa sibirica*	Dark - sided Flycatcher	GQ482226	Least Concerned
96	*Muscicapa dauurica*	Asian Brown Flycatcher	GQ482216	Least Concerned
97	*Muscicapa striata*	Spotted Flycatcher	GU571987	Least Concerned
98	*Cercotrichas galactotes*	Rufous - tailed Scrub - Robin	MF580191	Least Concerned
99	*Copsychus malabaricus*	White - rumped Shama	JF498845	Least Concerned
100	*Cyornis rubeculoides*	Blue - throated Flycatcher	JQ174627	Least Concerned
101	*Niltava sundara*	Rufous - bellied Niltava	JQ175559	Least Concerned
102	*Muscicapa thalassina*	Verditer flycatcher	EF422241	Least Concerned
103	*Luscinia megarhynchos*	Common Nightingale	JQ175293	Rare/Accidental
104	*Luscinia svecica*	Bluethroat	KC789641	Least Concerned
105	*Calliope pectoralis*	Himalayan Rubythroat	KU973748	Least Concerned
106	*Tarsiger cyanurus*	Red - flanked Bluetail	GQ482758	Rare/Accidental
107	*Tarsiger chrysaeus*	Golden Bush - Robin	JQ176404	Least Concerned
108	*Ficedula strophiata*	Rufous - gorgeted Flycatcher	JQ174846	Least Concerned

S. No.	Scientific Name	Common Name	Accession no.	Conservation status
109	*Ficedula albicilla*	Taiga Flycatcher	GQ481891	Rare/Accidental
110	*Ficedula parva*	Red - breasted Flycatcher	GU571893	Least Concerned
111	*Phoenicurus frontalis*	Blue - fronted Redstart	JX970703	Least Concerned
112	*Phoenicurus erythronotus*	Rufous - backed Redstart	GQ482382	Least Concerned
113	*Phoenicurus phoenicurus*	Common Redstart	GU572026	Rare/Accidental
114	*Phoenicurus erythrogastrus*	White - winged Redstart	GQ482376	Least Concerned
115	*Monticola saxatilis*	Rufous - tailed Rock - Thrush	GQ482171	Least Concerned
116	*Monticola solitarius*	Blue Rock - Thrush	JQ175415	Least Concerned
117	*Saxicola maurus*	Siberian Stonechat	GQ482623	Least Concerned
118	*Saxicola caprata*	Pied Bushchat	JQ176180	Least Concerned
119	*Saxicola ferreus*	Gray Bushchat	JQ176181	Least Concerned
120	*Oenanthe albonigra*	Hume's Wheatear	DQ683479	Least Concerned
121	*Oenanthe oenanthe*	Northern Wheatear	GU571994	Least Concerned
122	*Oenanthe lugens*	Mourning Wheatear	HM046870	Least Concerned
123	*Oenanthe finschii*	Finsch's Wheatear	MF795487	Least Concerned
124	*Oenanthe pleschanka*	Pied Wheatear	GU571995	Least Concerned
125	*Oenanthe xanthoprymna*	Red - tailed Wheatear	JX255949	Least Concerned
126	*Oenanthe deserti*	Desert Wheatear	KP252229	Least Concerned
127	*Oenanthe isabellina*	Isabelline Wheatear	JF498802	Least Concerned
128	*Zoothera dauma*	Scaly Thrush	EF515802	Least Concerned
129	*Turdus viscivorus*	Mistle Thrush	GQ482883	Least Concerned
130	*Turdus iliacus*	Redwing	GU572145	Near-threatened
131	*Turdus merula*	Eurasian Blackbird	MK262687	Least Concerned
132	*Turdus ruficollis*	Red - throated Thrush	GQ482870	Least Concerned
133	*Sturnus contra*	Asian Pied Starling	KC439338	Least Concerned
134	*Sturnia pagodarum*	Brahminy Starling	EU525542	Least Concerned
135	*Sturnia malabarica*	Chestnut - tailed Starling	JQ176301	Least Concerned
136	*Acridotheres tristis*	Common Myna	AY666196	Least Concerned
137	*Acridotheres fuscus*	Jungle Myna	EF484196	Least Concerned
138	*Dicaeum erythrorhynchos*	Pale - billed Flowerpecker	KJ442637	Least Concerned
139	*Aethopyga siparaja*	Crimson Sunbird	MH929095	Least Concerned

S. No.	Scientific Name	Common Name	Accession no.	Conservation status
140	*Prunella collaris*	Alpine Accentor	AB843102	Least Concerned
141	*Prunella himalayana*	Himalayan Accentor	GQ482565	Least Concerned
142	*Prunella fulvescens*	Brown Accentor	GQ482563	Rare/Accidental
143	*Prunella atrogularis*	Black - throated Accentor	GQ482560	Least Concerned
144	*Motacilla cinerea*	Gray Wagtail	JF957025	Least Concerned
145	*Motacilla flava*	Western Yellow Wagtail	GU571985	Least Concerned
146	*Motacilla citreola*	Citrine Wagtail	GQ482203	Least Concerned
147	*Motacilla alba*	White Wagtail	KY754516	Least Concerned
148	*Anthus godlewskii*	Blyth's Pipit	GQ481341	Least Concerned
149	*Anthus campestris*	Tawny Pipit	KP252167	
150	*Anthus pratensis*	Meadow Pipit	GU571732	Near-threatened
151	*Anthus hodgsoni*	Olive - backed Pipit	GQ481349	Rare/Accidental
152	*Anthus cervinus*	Red - throated Pipit	GU571250	Rare/Accidental
153	*Anthus spinoletta*	Water Pipit	GQ481365	
154	*Anthus rubescens*	American Pipit	GQ481358	
155	*Bombycilla garrulus*	Bohemian Waxwing	GU571754	Rare/Accidental
156	*Hypocolius ampelinus*	Hypocolius	KP252196	Rare/Accidental
157	*Fringilla coelebs*	Common Chaffinch	MK262511	Least Concerned
158	*Fringilla montifringilla*	Brambling	GU571404	Least Concerned
159	*Coccothraustes coccothraustes*	Hawfinch	GU571829	Least Concerned
160	*Carpodacus rubicilla*	Great Rosefinch	GQ481529	Least Concerned
161	*Carpodacus thura*	Himalayan White - browed Rosefinch	EU847701	Least Concerned
162	*Leucosticte nemoricola*	Plain Mountain - Finch	GQ482053	Least Concerned
163	*Leucosticte brandti*	Black - headed Mountain - Finch	GQ482049	Least Concerned
164	*Rhodospiza obsoleta*	Desert Finch	FJ465359	Least Concerned
165	*Linaria flavirostris*	Twite	GQ481479	Least Concerned
166	*Linaria cannabina*	Eurasian Linnet	GQ481455	Least Concerned
167	*Loxia curvirostra*	Red Crossbill	GU571958	Least Concerned
168	*Carduelis carduelis*	European Goldfinch	MK262089	Least Concerned
169	*Serinus pusillus*	Fire - fronted Serin	GQ482639	Least Concerned
170	*Spinus spinus*	Eurasian Siskin	GQ481495	Rare/Accidental

S. No.	Scientific Name	Common Name	Accession no.	Conservation status
171	*Emberiza melanocephala*	Black - headed Bunting	JQ174776	Least Concerned
172	*Emberiza bruniceps*	Red - headed Bunting	KC439313	Least Concerned
173	*Emberiza calandra*	Corn Bunting	GU571867	Rare/Accidental
174	*Emberiza fucata*	Chestnut - eared Bunting	JF499130	Least Concerned
175	*Emberiza cia*	Rock Bunting	GQ481747	Least Concerned
176	*Emberiza godlewskii*	Godlewski's Bunting	GQ481766	Least Concerned
177	*Emberiza stewarti*	Chestnut - breasted Bunting	KP877676	Least Concerned
178	*Emberiza leucocephalos*	Pine Bunting	GQ481772	Least Concerned
179	*Emberiza hortulana*	Ortolan Bunting	KX283114	Rare/Accidental
180	*Emberiza striolata*	Striolated Bunting	MF580175	Least Concerned
181	*Emberiza schoeniclus*	Reed Bunting	GU571874	Least Concerned
182	*Emberiza aureola*	Yellow - breasted Bunting	EF515786	Critically endangered
183	*Emberiza pusilla*	Little Bunting	GU571872	Least Concerned
184	*Emberiza rutila*	Chestnut Bunting	GQ481805	Least Concerned
185	*Passer domesticus*	House Sparrow	MK262682	Least Concerned
186	*Passer moabiticus*	Dead Sea Sparrow	MF767304	Least Concerned
187	*Passer montanus*	Eurasian Tree Sparrow	JF957028	Least Concerned
188	*Petronia petronia*	Rock Sparrow	GQ482355	Least Concerned
189	*Carpospiza brachydactyla*	Pale Rockfinch	FJ465315	Least Concerned
190	*Montifringilla nivalis*	White - winged Snowfinch	GQ482176	Least Concerned
191	*Lonchura punctulata*	Scaly - breasted Munia	JF498874	Least Concerned
192	*Lonchura malabarica*	Indian silverbill	MF580167	Least Concerned

3.15 Sequence Analysis by MEGAX

Sequence data was used for phylogenetic analysis using MEGAX software [150]. CLUSTALW software [145] was used for the multiple sequence alignment of 43 samples sequenced in laboratory for species of Passeriformes and 192 sequences of species of Passeriformes retrieved from NCBI database. The program MEGAX

was used to find nucleotide composition, Codon Usage Bias, Variations at Amino acid level, Nucleotide transition / transversion bias, Tajima's Neutrality Test, Evolutionary Divergences and inference phylogenetic relationships among different species by the neighbor-joining method. Kimura-2-parameter was used for the estimation of nucleotide distances between the sequence pairs calculated the diversities by the nucleotide substitution model [151, 152]. The phylogenetic analysis was performed of 235 sequences of COI gene of mitDNA of Passeriformes at both nucleotide and amino acid level. The following different analyses were performed for nucleotide sequences of COI gene of mitDNA of Passeriformes samples using different bioinformatics tools.

3.15.1 Multiple Sequence Alignment

ClustalW (www.custal.org/omega/) [145] a bioinformatics tool was used for sequence alignment before reconstruction of Phylogenetic tree. Collectively 235 nucleotide sequences were aligned using 43 sequences of Passeriformes species collected from different areas of Pakistan and 192 sequences of worldwide sequence data of Passeriformes species retrieved from NCBI database, and phylogenetic tree was reconstructed to find closely related species. All these sequences were aligned with default parameters i.e., Gap opening Penalty 15 and Gap extension Penalty 6.66 for Pair wise and Multiple Alignment by ClastalW. DNA weight Matrix was selected 1.6, transition weight was 0.5 and delay divergent cut off (%) was 30 whereas negative matrix was off. Then file was exported as MEGA format and phylogeny option was selected for further analysis as shown in Appendix C.

3.15.2 Nucleotide Composition

MEGAX was used to calculate the nucleotide composition of each Thymine (T), Adenine (A), Guanine (G) and Cytosine (C)) for each available selected region of the COI gene, of order Passeriformes, the average frequency of nucleotide was calculated. The nucleotide frequency at codon 1^{st}, 2^{nd}, 3^{rd} positions was also calculated. Nucleotide composition of an organism reveals its genome. GC content

varies among species and has several implications. It determines the nucleotide frequencies, codon and their amino acid usage. Nucleotide composition is similar among the species living in the same environments therefore help in determining the association among close groups in their phylogeny [153, 154].

3.15.3 Codon Usage Bias

Codon usage bias for COI genes was estimated using MEGAX software. It provides the average frequency of relative synonymous usage of each codon in the gene.

3.15.4 Variations at Amino Acid Level

The sequence alignment of protein of the COI gene loci of mitDNA was performed which have been widely used in species identification and taxonomic studies. The protein sequence analysis of this gene was done by using translation option in MEGAX software. Total 235 Nucleotide sequences of Passeriformes species were included in analysis. Regions of greater or lesser variations in the sequence were examined at each amino acid position to identify variable and conserve sequences within gene. The translated sequences were aligned using ClustalW from the online program (http://www.genome.jp/tools/clustalw/) [145]. All of the protein sequences were converted from nucleotide sequences using MEGAX program. The quality of COI gene of each sequence was checked by selection of only the coding sequences and was further proved to minimize sequence errors. Same procedure was followed for 235 nucleotide sequences of species of Passeriformes to find phylogenetic relationship among them (Appendix F).

The sequences composition of amino acid of the COI loci from 235 species of Passeriformes including 43 sequenced and 192 downloaded from NCBI Gen Bank were selected for calculation of conserved and variable sites through MEGAX software. A variable site can be parsimony-informative or singleton because it contains two different types of amino acids on the other hand a site that is not variable is referred as a conserve/constant site.

The Variation and the conservation in the protein sequences are proved useful for identification of species and help in designing the primers either universal or species specific. COI genes of 43 species of order Passeriformes were sequenced and uploaded in MEGAX (Molecular Evolutionary Genetics Analysis) version 10.0 [150] and their amino acid sequences were obtained by using option of translated protein sequences on MEGAX to find the total conserved and variable sites in each sequence.

MEGAX software was used to compute the synonymous and non-synonymous mutations in the sequences. For this purpose conservative, variable, parsive informative and singleton sites were computed for 43 amino acid sequences. Same procedure was followed for 235 amino acid sequences of species of Passeriformes to compute synonymous and non-synonymous mutations among them.

3.15.5 Estimation of Transition / Transversion Bias

Transition means one purine exchanges with another or one pyrimidine with another while, transversion means a purine exchanges to one pyrimidine or vice versa. The nucleotide transition/transversion bias of COI gene in Passeriformes was estimated using MEGAX. It involved 235 nucleotide sequences including 43 newly generated COI sequences of Passeriformes of Pakistan and 192 COI sequences of other Passeriformes retrieved from NCBI GenBank. All the gaps and missing data from the sequences were eliminated during the analysis. The average nucleotide transition/transversion bias (R) in COI gene was 2.26 and 51.3383% sites were found to be evolutionary invariable.

3.15.6 Tajima's Neutrality Test

Tajima's Neutrality Test was created by a Japanese scientist Fumio Tajima [155]. It discriminates between DNA sequences that are evolving randomly and those evolving under the influence of any environmental factor (e.g. genetic drift). For a sequence to be evolving randomly, the value of D must be zero. In the analysis 235

nucleotide sequencesof Passeriformes involved. 1^{st}, 2^{nd}, 3^{rd} codon positions were included in analysis. All positions which were ambiguous each sequence pair were removed by using pairwise deletion option. There were a total of 992 positions in the final dataset. Evolutionary analyses were conducted in MEGAX [150].

3.15.7 Estimation of Evolutionary Divergence

Evolutionary divergences are the differences accumulated in the sequences of organisms over time. To calculate these divergences MEGAX was used and 235 sequences of COI gene from different Passeriformes were selected for analysis.

Genetic distance was calculated by the MEGAX [150] software by entering the aligned 235 sequences of Passeriformes into Distance option in MEGAX. The p-distance was calculated using this model to estimate the genetic distance between different DNA sequences calculating the number of differences between two sequences divided by the sequence length. The steps to enter the data into MEGA included: click on the distance tab, calculate overall mean of a standard comparison (p value) and add 1000 bootstrap repetitions to evaluate the variance.

3.15.8 Phylogenetic Aanlysis

Evolutionary analyses were conducted in MEGAX Neibour joining method. This involved 235 nucleotide sequences in analysis from COI gene including 43 newly generated sequences of Passeriformes of Pakistan and 192 sequences of other Passeriformes downloaded from NCBI GenBank along with out-group to predict their effective ancestry. The available 235 nucleotide sequences of Passeriformes were aligned and entered in the phylogeny option of the MEGAX by which options of phylogenetic trees were available. The Neighbour joining tree method was used to analyze the phylogenetic relations among different species of Passeriformes. Then file was exported as MEGA format and phylogeny option was selected for analysis and neighbor joining method was used to reconstruct the phylogenetic

tree. For this purpose default parameters were selected and original and consensus tree were saved as PDF file.

Chapter 4

Results and Analysis

The COI region of mitochondrial genome has been reported to be capable of identifying unknown sample of birds to species level. Similar work has been reported by many researchers [40, 55, 56, 83] they found that this mitochondrial marker is an accurate and a rapid genetic method for identification of species and for determining evolutionary relationships among different avian species. It was also found during this study that the same region of DNA was proficient in reconstructing the phylogenetic relationship among different species of Passeriformes. The 49 samples of Passeriformes species were collected from different area of Pakistan based on their availability to carry out this study both on morphological as well as molecular basis to accomplish the designed objectives of the study. For this purpose, the morphological features as weight, body color & length, wing color & length, tail color & length, bill color & length, eye color (iris), legs & feet color, and nape color of all forty nine specimens were thoroughly studied. The morphological characteristics of an organism help in determining the taxonomy and phylogeny of a particular species [156]. Collected samples identified based on morphological features using standard morphological keys and reference books "The Birds of Pakistan" by Robert and Animal Life Encycloped by Grzimek's and "Birds of Pakistan" by Richard Grimmett [15, 83, 130]. The morphometric data is presented in Appendix A. Preserved vouchers of the specimens deposited at Centre for Bioresource Research (CBR), Museum at Islamabad, Pakistan.

4.1 Morphological Characterization of Specimens

Specimens were identified as 49 species, belonging to 39 genera and 18 families of order Passeriformes of class Aves and phylum Chordate morphologically based on their characteristics. Morphological identification based on morphological characters is available in Table 4.1 and characters are provided in Appendix A.

TABLE 4.1: Morphological based classification of samples of order Passeriformes

S.No.	Family	Genus	Species
1	Alaudidae	Alauda	*Alauda arvensis*
			Alauda gulgula
		Galerida	*Galerida cristata*
		Melanocorypha	*Melanocorypha bimaculata*
		Eremopterix	*Eremopterix griseus*
		Calandrella	*Calandrella brachydactyla*
2	Campephagidae	Tephrodornis	*Tephrodornis pondicerianus*
		Pericrocotus	*Pericrocotus cinnamomeus*
3	Corvidae	Dendrocitta	*Dendrocitta vagabunda*
		Corvus	*Corvus splendens*
			Corvus corax
			Corvus machorynchus
		Urocissa	*Urocissa flavirostris*
4	Dicruridae	Dicrurus	*Dicrurus leucophaeus*
5	Emberizidae	Emberiza	*Emberiza buchanani*
			Emberiza lathami
6	Fringillidae	Carpodacus	*Carpodacus erythrinus*
7	Laniidae	Lanius	*Lanius vittatus*
			Lanius schach
8	Monarchidae	Terpsiphone	*Terpsiphone paradisi*
9	Motacillidae	Anthus	*Anthus richardi*
			Anthus trivialis
10	Oriolidae	Oriolus	*Oriolus oriolus*

TABLE 4.1: Morphological based classification of samples of order Passeriformes

S.No.	**Family**	**Genus**	**Species**
11	Passeridae	Passer	*Passer hispaniolensis*
			Passer rutilans
		Petronia	*Petronia xanthocollis*
12	Paridae	Parus	*Parus major*
13	Pycnonotidae	Pycnonotus	*Pycnonotus leucogenys*
		Hypsipetes	*Hypsipetes leucocephalus*
14	Sturnidae	Acridotheres	*Acridotheres ginginianus*
		Sturnus	*Sturnus roseus*
			Sturnus vulgaris
15	Sylviidae	Prinia	*Prinia buchanani*
			Prinia socialis
			Prinia hodgsonii
		Phylloscopus	*Phylloscopus affinis*
		Sylvia	*Sylvia curruca*
		Seicercus	*Seicercus xanthoschistos*
		Orthotomus	*Orthotomus sutorius*
		Cisticola	*Cisticola juncidis*
16	Timaliidae	Chrysomma	*Chrysomma sinense*
		Garrulax	*Garrulax lineatus*
		Stachyris	*Stachyris pyrrhops*
17	Turdidae	Saxicoloides	*Saxicoloides fulicatus*
		Phoenicurus	*Phoenicurus ochruros*
		Copsychus	*Copsychus saularis*
		Oenanthe	*Oenanthe picata*
		Myophonus	*Myophonus caeruleus*
18	Zosteropidae	Zosterops	*Zosterops palpebrosus*

Specimens were identified as 49 species, belonging to 39 genera and 18 families of order Passeriformes of class Aves and phylum Chordate.

4.2 Genomic DNA (gDNA) Extraction

Genomic DNA (gDNA) was successfully extracted from keel tissues of 49 samples of Passeriformes of Pakistan using the phenol-chloroform protocol [132, 133]. From keel tissues of specimens, a portion (COI gene comprising 650 bp-base pairs) of the mitochondrial genome from its first half was amplified and using universal primer BirdF1 and BirdR1 was sequenced [44, 157]. The same primers have been used to amplify COI region in different mammals, mentioned by many researchers [40, 135–137, 158–160]. Extracted gDNA was confirmed using agarose gel (1%) on gel electrophoresis apparatus.

4.3 DNA Quantification

The DNA quality by measuring A260/A280 ratio was assessed which ranges from 1.7 to 2.0 ratios for genomic DNA of all extracted from three replicates for each samples. The wavelength ratio A260 was measured for purified DNA quantification. For genomic DNA the quantity ratio ranges from 500 to 3000 ng /μL of extracted genomic DNA samples.

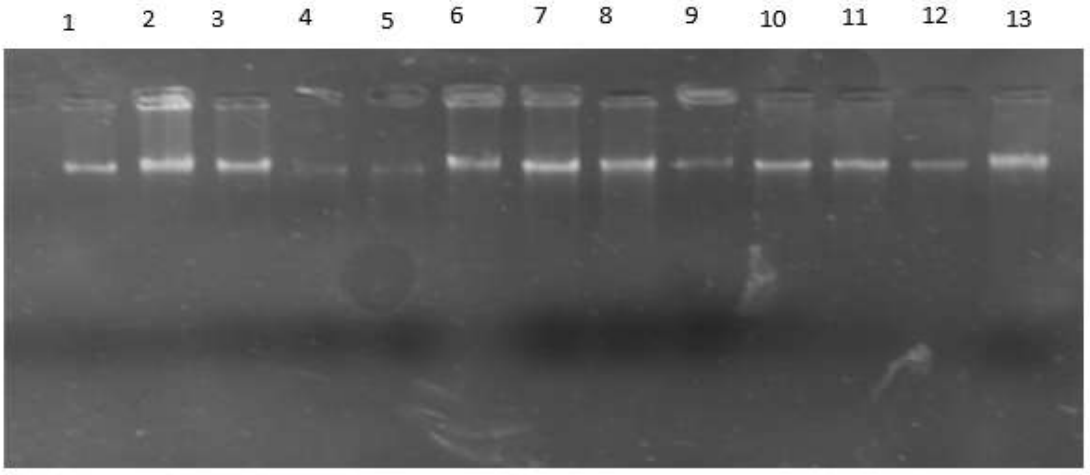


FIGURE 4.1: Qualitative analysis of genomic DNA using agarose (1%) gel electrophoresis for Passeriformes samples: *Eremopterix griseus, Prinia buchanani, Passer hispaniolensis, Corvus splendens, Galerida cristata, Oenanthe picata, Prinia hodgsonii, Copsychus fulicatus, Lanius schach, Myophonus caeruleus, Garrulax lineatus, Parus major* respectively.

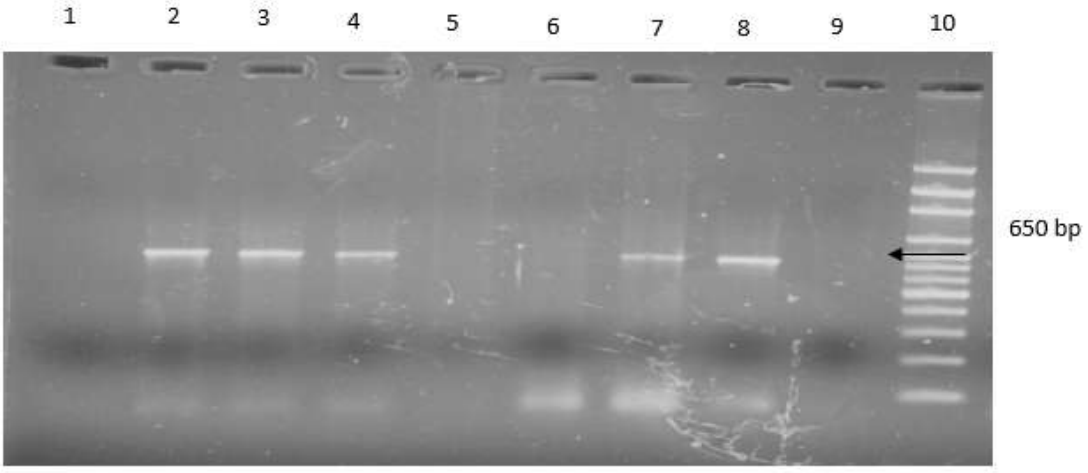


FIGURE 4.2: Amplified PCR product of COI (650 bp) gene of Passeriformes samples using universal Primer R1 Lane 1 to 9 represent: *Eremopterix griseus, Prinia buchanani, Passer hispaniolensis, Corvus splendens, Galerida cristata, Oenanthe picata, Prinia hodgsonii, Copsychus fulicatus, Lanius schach,* respectively. Lane 10: Negative control.

4.4 PCR Amplification

Genomic DNA (gDNA) was successfully extracted from keel tissues of 49 samples of Passeriformes of Pakistan. Photographs of gels with good quality DNA bands were saved with proper reference to be amplified by PCR (Figure 4.1). The PCR reaction for the extracted DNA of samples was carried out by using universal primer (Table 3.1). The optimum annealing temperature was found to be 60°C to amplify CO1 gene. In analysis of samples, 650 bp of CO1 gene was amplified. The representative gel results of 650bp segment are shown in the Figure 4.2.

4.5 DNA Sequencing

The Sanger's sequencing of the PCR products was carried from the Centre of Excellence Molecular Biology, University of Punjab, Lahore (CEMB), and sequences were then converted into FASTA format. These sequences of samples were further analyzed for the molecular identification along with phylogenetic analysis.

4.6 Molecular Characterization of Sequences

The sequences obtained after Sanger's sequencing were further analyzed by performing BLAST analysis on NCBI (http: www.ncbi.nlm.nih.gov). Total 43 out of 49 sequences were identified as species of order Passeriformes based on the best matches by comparing their similarity percentage with other sequences in the database (Table 4.2). Normally species with query sequence $>$ 97 % similarity are assigned to same species. Most of the species i.e., 32 of the submitted sequences (S# 1 to S# 32) were ranging from 97-100 % identity with their respective species and thus confirmed molecular characterization as well. The sequences of 10 species (S# 33 to S#43) were showing similarity $<$ 97 % but with other species of same genus. Total 49 samples were sequenced but when BLAST analysis was performed 6 samples (S#2, 3, 4, 20, 34, 40 of Table 3.1) showed similarity with species of diff erent genera from order other than passeriformes with 98-100% similarity. Therefore they were excluded from further analyses. Further verification with BOLD database also revealed that sequences of these species were not the same as morphologically characterized. Nucleotide sequences of 43 species of Passeriformes were then submitted to NCBI database and the accession numbers were assigned to them as mentioned in last column of Table 4.2.

TABLE 4.2: Sequence similarity of BLAST analysis and Accession No. of Passeriformes samples

S#	Species (Query)	Query Cover	Identity	Best Match	Species (Subject)	Accession No.
1.	*Passer hispaniolensis*	100%	100%	GQ482315.1	*Passer hispaniolensis*	OR234858
2.	*Corvus corax corax*	100%	100%	GQ481624.1	*Corvus corax corax*	OR244265
3.	*Copsychus saularis saularis*	100%	100%	KJ442642.1	*Copsychus saularis saularis*	OR244278
4.	*Sturnus roseus*	100%	100%	EF484209.1	*Sturnus roseus*	OR250785
5.	*Melanocorypha bimaculata*	89%	100%	FJ465342.1	*Melanocorypha bimaculata*	OR244254
6.	*Sylvia curruca curruca*	100%	99.86%	KF946881.1	*Sylvia curruca curruca*	OR238387

S#	Species (Query)	Query Cover	Identity	Best Match	Species (Subject)	Accession No.
7.	*Emberiza buchanani*	100%	99.86%	GQ481741.1	*Emberiza buchanani*	OR244256
8.	*Calandrella brachydactyla*	100%	99.86%	GU571293.1	*Calandrella brachydactyla*	OR260047
9.	*Galerida cristata arenicola*	100%	99.85%	GQ481940.1	*Galerida cristata arenicola*	OR234857
10.	*Zosterops palpebrosus palpebrosus*	94%	99.83%	EU447060.1	*Zosterops palpebrosus palpebrosus*	OR244267
11.	*Carpodacus erythrinus*	100%	99.71%	GU571323.1	*Carpodacus erythrinus*	OR244257
12.	*Oenanthe picata*	96%	99.70%	DQ683509.1	*Oenanthe picata*	OR260093
13.	*Prinia socialis socialis*	92%	99.69%	KT240058.1	*Prinia socialis socialis*	OR240108
14.	*Acridotheres ginginianus*	100%	99.56%	EF484197.1	*Acridotheres ginginianus*	OR244255
15.	*Corvus splendens splendens*	100%	99.56%	GU326327.1	*Corvus splendens splendens*	OR238478
16.	*Dendrocitta vagabunda saturatior*	94%	99.53%	KJ442640.1	*Dendrocitta vagabunda saturatior*	OR238486
17.	*Pycnonotus leucogenys leucogenys*	98%	99.42%	HQ168045.1	*Pycnonotus leucogenys leucogenys*	OR244270
18.	*Passer cinnamomeus rutilans*	100%	99.28%	GQ482325.1	*Passer cinnamomeus rutilans*	OR259988
19.	*Eremopterix griseus*	85%	99.16%	KP975223.1	*Eremopterix griseus*	OR244260
20.	*Phoenicurus ochruros*	100%	98.95%	GQ482384.1	*Phoenicurus ochruros*	OR240090
21.	*Pericrocotus cinnamomeus*	94%	98.78%	EU541461.1	*Pericrocotus cinnamomeus*	OR244264
22.	*Chrysomma sinense*	95%	98.60%	JQ174444.1	*Chrysomma sinense*	OR240092

S#	Species (Query)	Query Cover	Identity	Best Match	Species (Subject)	Accession No.
23.	*Terpsiphone paradisi paradise*	94%	98.62%	KJ442633.1	*Terpsiphone paradisi paradise*	OR250915
24.	*Dicrurus leucophaeus*	93%	98.31%	JQ174693.1	*Dicrurus leucophaeus*	OR251001
25.	*Trochalopteron lineatum lineatum*	88%	98.05%	EU447035.1	*Trochalopteron lineatum lineatum*	OR250794
26.	*Corvus macrorhynchos*	100%	97.98%	AB842677.1	*Corvus macrorhynchos*	OR244274
27.	*Tephrodornis pondicerianus*	93%	97.85%	JQ176406.1	*Tephrodornis pondicerianus*	OR238389
28.	*Lanius schach schach*	100%	97.84%	JF957014.1	*Lanius schach schach*	OR240091
29.	*Parus major*	99%	97.26%	HQ833096.1	*Parus major*	OR250798
30.	*Orthotomus sutorius*	100%	96.97%	HQ608882.1	*Orthotomus sutorius*	OR260092
31.	*Cisticola juncidis*	100%	96.77%	AB843427.1	*Cisticola juncidis*	OR260094
32.	*Oriolus oriolus*	99%	96.68%	KC354938.1	*Oriolus oriolus*	OR244259
33.	*Hypsipetes leucocephalus*	93%	96.47%	JQ175126.1	*Hypsipetes madagascarien*	OR250917
34.	*Urocissa flavirostris flavirostris*	93%	96.01%	JQ176603.1	*Urocissa erythrorhyncha*	OR250808
35.	*Phylloscopus affinis*	93%	95.85%	JQ175813.1	*Phylloscopus trivirgatus*	OR244271
36.	*Lanius vittatus Valenciennes*	100%	95.82%	JF957014.1	*Lanius chach*	OR234819
37.	*Stachyrdopsis pyrrhops*	99%	95.52%	HQ917502.1	*Stachyris ruficeps*	OR250809
38.	*Emberiza lathami*	99%	91.91%	GQ481822.	*Emberiza schoeniclus*	OR260283
39.	*Prinia hodgsonii*	91%	91.79%	MH265925.1	*Prinia bairdii*	OR244266

S#	Species (Query)	Query Cover	Identity	Best Match	Species (Subject)	Accession No.
40.	*Copsychus fulicatus*	99%	90.29%	KC354898.1	*Copsychus saularis*	OR234860
41.	*Prinia buchanani*	100%	90.17%	HQ608884.1	*Prinia crinigera*	OR234851
42.	*Myophonus caeruleus*	100%	90.01%	KF946849.1	*Saxicola rubetra*	OR250811
43.	*Alauda gulgula australis*	100%	89.95%	GQ481305.1	*Alauda gulgula australis*	OR260282

4.7 Assignment of Barcode Index Numbers (BINs) from BOLD Database

Sequences of 49 species of Passeriformes were submitted to BOLD database and BINs were assigned to 43 species. The identification of sequences of 6 species (S#2, 3, 4, 20, 34, 40 of Table 3.1) have shown discrepancies at GenBank NCBI and BOLD database therefore, these species sequences were not included in further analysis.

4.7.1 Barcode Index Numbers (BINs)

The BINs system tries to clusters COI gene sequence data into their operational taxonomic units (OTUs) called BINs (Barcode Index Numbers). It provides a mean of confirming the similarity (concordance) between barcode sequences clusters for species designations. This analysis offered validation on the bases of input records against all others in the same BINs including those submitted by other users by comparing their taxonomy. This analysis was performed at BOLD systems database.

Sequence identification through the BOLD identification engine revealed that the sequences had maximum homology to 43 species of Passeriformes and specific

barcode index numbers were assigned to 43 samples. These 43 samples of order Passeriformes were classified into 21 families and 35 genera on the bases of molecular studies at BOLD database as elaborated in Table 4.3.

TABLE 4.3: Confirmation of sequences and BINs assigned to samples of Passeriformes at BOLD database

S. No.	**Family**	**Genus**	**Species/Subspecies**	**BINs**
1	Alaudidae	Alauda	*Alauda arvensis intermedia*	BOLD:AAF5733
			Alauda gulgula australis	BOLD:ACZ2564
		Galerida	*Galerida cristata arenicola*	BOLD:AAD9870
		Melanocorypha	*Melanocorypha bimaculata*	BOLD:AAX4494
		Eremopterix	*Eremopterix griseus*	BOLD:ACH8855
		Calandrella	*Calandrella brachydactyla*	BOLD:AAF5733
2	Campephagidae	Pericrocotus	*Pericrocotus cinnamomeus*	BOLD:AAL2706
3	Cisticolidae	Prinia	*Prinia buchanani*	BOLD:ACZ2474
			Prinia socialis socialis	BOLD:ACZ2475
			Prinia hodgsonii	BOLD:ACZ2474
		Orthotomus	*Orthotomus sutorius*	BOLD:ACH8281
		Cisticola	*Cisticola juncidis*	BOLD:ACS3101
4	Corvidae	Dendrocitta	*Dendrocitta vagabunda saturatior*	BOLD:ACH6125
		Corvus	*Corvus splendens splendens*	BOLD:AAR9140
			Corvus corax corax	BOLD:AAB5621
			Corvus machorynchus	BOLD:ABW5160
		Urocissa	*Urocissa flavirostris flavirostris*	BOLD:ACZ2846
5	Dicruridae	Dicrurus	*Dicrurus leucophaeus*	BOLD:AAI9717
6	Emberizidae	Emberiza	*Emberiza buchanani*	BOLD:AAF0356
			Emberiza lathami	BOLD:ACZ3094
7	Fringillidae	Carpodacus	*Carpodacus erythrinus*	BOLD:AAB3874
8	Laniidae	Lanius	*Lanius vittatus*	BOLD:ACZ1830
			Lanius schach schach	BOLD:ABZ8019
9	Leiothrichidae*	Trochalopteron	*Trochalopteron lineatum*	BOLD:AAY1381
10	Monarchidae	Terpsiphone	*Terpsiphone paradisi para*	BOLD:ACS4664
11	Muscicapidae*	Myophonus	*Myophonus caeruleus*	BOLD:AAC5713
		Phoenicurus	*Phoenicurus ochruros*	BOLD:AAC1536
		Copsychus	*Copsychus saularis saularis*	BOLD:ACE7266

S. No.	Family	Genus	Species/Subspecies	BINs
			Copsychus fulicatus	BOLD:ACZ2562
		Oenanthe	*Oenanthe picata*	BOLD:AAW9936
12	Oriolidae	Oriolus	*Oriolus oriolus*	BOLD:ACZ2757
13	Paridae	Parus	*Parus major*	BOLD:ACZ3140
14	Passeridae	Passer	*Passer hispaniolensis*	BOLD:ABX5008
			Passer cinnamomeus rutilans	BOLD:AAC1399
15	Phylloscopidae*	Phylloscopus	*Phylloscopus affinis*	BOLD:ACH0131
16	Pycnonotidae	Pycnonotus	*Pycnonotus leucogenys*	BOLD:AAN3860
		Hypsipetes	*Hypsipetes leucocephalus*	BOLD:ACZ2808
17	Sturnidae	Acridotheres	*Acridotheres ginginianus*	BOLD:ACE4748
		Pastor	*Pastor roseus*	BOLD:AAE0119
18	Sylviidae	Sylvia	*Sylvia curruca curruca*	BOLD:AAC0536
19	Timaliidae	Chrysomma	*Chrysomma sinense*	BOLD:AAV9282
		Stachyriodopsis	*Stachyriodopsis pyrrhops*	BOLD:ACZ2835
20	Vangidae*	Tephrodornis	*Tephrodornis pondicerianus*	BOLD:AAU3934
21	Zosteropidae	Zosterops	*Zosterops palpebrosus palpebrosus*	BOLD:AAB2042

Note: (*) New families as compared to classical Taxonomy.

In this analysis 42 BINs were assigned to 43 species sequences and among these 1 BIN was assigned to 2 species (*Prinia buchanani Blyth, Prinia hodgsonii Blyth*, BOLD: ACZ2474, Table 4.5). Out of these 42 BINs 32 records were taxonomically concordant mean already present in the BOLD database with non- unique BINs. Rest of 11 records out of 42 showing unique BINs, as single record of respective species of these 11 has been submitted by our study in the BOLD database. The sequences of 11 species were not present in the BOLD database from any other part of the world and have been submitted as a singleton specimen from Pakistan. This contributes as diversity of Passeriformes of Pakistan in BOLD database.

4.7.2 Non Unique BINs with Taxonomic Concordance from BOLD Database

Concordant taxonomy means multiple samples showing same taxonomy therefore same BIN was assigned to many samples. The Table 4.4 shows 32 samples showing non unique BINs that were shared by multiple samples submitted from diferent parts of the world in the BOLD database. The highest BIN similarity was shown by *Passer hispolansis* i.e., 44 including our sample. The least BIN similarity was 2 (*Chrysomma sinense, Prinia socialisis socialisis, Zosterops palpebrosus palpebrosus, Phylloscopus affinis, Trochalopteron lineatum lineatum Terpsiphone paradisi paradisi*) including our sample. These non-unique BIN assigned to 32 samples validated the taxonomy of the species of Passeriformes of Pakistan.

TABLE 4.4: Non unique BINs of samples of Passeriformes with taxonomic concordant estimated by BOLD database

S. #	Identification	BINs	Total Members
1.	*Passer hispaniolensis*	BOLD:ABX5008	44
2.	*Sylvia curruca curruca*	BOLD:AAC0536	27
3.	*Tephrodornis pondicerianus*	BOLD:AAU3934	4
4.	*Dendrocitta vagabunda saturatior*	BOLD:ACH6125	4
5.	*Corvus splendens splendens*	BOLD:AAR9140	16
6.	*Chrysomma sinense*	BOLD:AAV9282	2
7.	*Lanius schach schach*	BOLD:ABZ8019	22
8.	*Prinia socialis socialis*	BOLD:ACZ2475	2
9.	*Melanocorypha bimaculata*	BOLD:AAX4494	5
10.	*Acridotheres ginginianus*	BOLD:ACE4748	5
11.	*Pastor roseus*	BOLD:AAE0119	20
12.	*Carpodacus erythrinus*	BOLD:AAB3874	31
13.	*Eremopterix griseus*	BOLD:ACH8855	3
14.	*Corvus corax corax*	BOLD:AAB5621	40
15.	*Pericrocotus cinnamomeus*	BOLD:AAL2706	3
16.	*Zosterops palpebrosus palpebrosus*	BOLD:AAB2042	2

S. #	Identification	BINs	Total Members
17.	*Pycnonotus leucogenys*	BOLD:AAN3860	12
18.	*Phylloscopus affinis*	BOLD:ACH0131	2
19.	*Copsychus saularis saularis*	BOLD:ACE7266	31
20.	*Trochalopteron lineatum lineatum*	BOLD:AAY1381	2
21.	*Myophonus caeruleus*	BOLD:AAC5713	5
22.	*Terpsiphone paradisi paradisi*	BOLD:ACS4664	2
23.	*Emberiza lathami*	BOLD:ACZ3094	3
24.	*Passer cinnamomeus rutilans*	BOLD:AAC1399	17
25.	*Orthotomus sutorius*	BOLD:ACH8281	3
26.	*Oenanthe picata*	BOLD:AAW9936	12
27.	*Emberiza buchanani*	BOLD:AAF0356	6
28.	*Calandrella brachydactyla*	BOLD:AAF5733	18
29.	*Galerida cristata arenicola*	BOLD:AAD9870	44
30.	*Phoenicurus ochruros*	BOLD:AAC1536	17
31.	*Corvus macrorhynchos*	BOLD:ABW5160	4
32.	*Dicrurus leucophaeus*	BOLD:AAI9717	4

4.7.3 Unique BINs with Single Specimen from BOLD Data base

The 11 species shown in Table 4.5 are the single specimens of each species which were submitted to BOLD database by our study and no record of these species from other parts of the world was already available in BOLD database during study time. These 11 species were identified by BOLD database and new BINs were assigned to these species as a new representative of respective species. This indicates that the sequence data of these 11 species was not available in BOLD database.

TABLE 4.5: Unique BINs of sequences of Passeriformes estimated by BOLD database

S.#	Species	BINs
1.	*Lanius vittatus*	BOLD:ACZ1830
2.	*Copsychus fulicatus*	BOLD:ACZ2562
3.	*Oriolus oriolus*	BOLD:ACZ2757
4.	*Parus major*	BOLD:ACZ3140
5.	*Stachyridopsis pyrrhops*	BOLD:ACZ2835
6.	*Hypsipetes leucocephalus*	BOLD:ACZ2808
7.	*Urocissa flavirostris flavirostris*	BOLD:ACZ2846
8.	*Alauda gulgula australi*	BOLD:ACZ2564
9.	*Prinia buchanani Blyth*	BOLD:ACZ2474
10.	*Prinia hodgsonii Blyth*	BOLD:ACZ2474
11.	*Cisticola juncidis*	BOLD:ACS3101

Comparison of the Table 4.1 and Table 4.5 indicate that unique BINs (BOLD: ACZ2757, BOLD: ACZ3140, BOLD: ACS3101) assigbed to 3 species sequences (*Oriolus oriolus, Parus major, Cisticola juncidis*) respectively were confirmed through GenBank NCBI based on percentage similarity about 97% as well as from the BOLD engine. BOLD engine provided the identification of the rest of the 8 specimens where as GenBank NCBI showed variation at the species level only with % similarity less than 96%. Further more our dataset discovers 8 unique BINs out of 43 sequences which indicate the need for further sampling and identification in order to add more species into these databases to increase their accuracy.

BOLD engine provided the identification of the rest of the 8 specimens where as GenBank NCBI showed variation at the species level only with % similarity less than 96%. Further more our dataset discovers 8 unique BINs out of 43 sequences which indicate the need for further sampling and identification in order to add more species into these databases to increase their accuracy [161].

4.8 Comparison of Classical and Molecular Taxonomy of Samples of Passeriformes of Pakistan

Comparison of molecular and classical taxonomy of samples of Passeriformes of Pakistan at family, genus and species level was inferred by referencing the T. J. Roberts [15] for classical taxonomy and BOLD (Barcode of Life Data System) databases (www.boldsystems.org) [139] and was further confirmed through data from other databases like Avibase database (https://avibase.bsc-eoc.org) [142] for molecular taxonomy as shown in Table 4.6. The purpose was to compare the classical taxonomy at molecular level.

The comparison revealed changes at different taxonomic levels of 17 species out of 43 samples. Family of 12 species has been changed from one to another, both family and genus were changed for 2 species and only genus was changed for 2 species whereas species level change was observed for only 1 species.

The family of *Tephrodornis pondicerianus* was changed from Campephagidae to Vangidae, *Chrysomma sinense* from Timaliidae to Sylviidae, *Garrulax lineatus* from Timaliidae to Leiothrichidae, *Phylloscopus affinis, Cisticola juncidis, Orthotomus sutorius, Prinia buchanani, Prinia socialis, Prinia hodgsoniifrom Sylviidae to Phylloscopidae,* and *Saxicoloides fulicatus, Myophonus caeruleus, Phoenicurus ochruros, Copsychus saularis saularis, Oenanthe picata* from Turdidae to Muscicapidae (Table 4.6).

At genus level 4 species have been moved from one genus to another genus. *Garrulax lineatus* moved from Garrulax genus to Trochalopteron genus (*Trochalopteron lineatum*) *Cyanoderma pyrrhops* moved from genus Cyanoderma to Stachyriodopsis (*Stachyriodopsis pyrrhops*). *Sturnus roseus* moved from Sturnus genus to Pastor (*Pastor roseus*). In the same way *Saxicoloides fulicatus* placed from Saxicoloides to Copsychus genus (*Copsychus fulicatus*). Only one species name was changed i.e., *Passer rutilans rutilans* and renamed to *Passer cinnamomeus rutilans* (Table 4.6).

TABLE 4.6: Comparison of classical versus molecular taxonomy of samples of Passeriformes of Pakistan at species, genus and family level

S. #	Classical taxonomy based on Literature [15]			Molecular taxonomy based on BOLD Database [129]		
	Species	**Genus**	**Family**	**Species**	**Genus**	**Family**
1	*Tephrodornis pondicerianus*	Tephrodornis	Campephagidae	-	-	Vangidae
2	*Chrysomma sinense*	Chrysomma	Timaliidae	-	-	Sylviidae
3	*Garrulax lineatus*	Garrulax	Timaliidae	*Trochalopteron lineatum*	Trochalopteron	Leiothrichidae
4	*Cyanoderma pyrrhops*	Cyanoderma	Timaliidae	*Stachyriodopsis pyrrhops*	Stachyriodopsis	-
5	*Sturnus roseus*	Sturnus	Sturnidae	*Pastor roseus*	Pastor	-
6	*Phylloscopus affinis*	Phylloscopus	Sylviida	-	Phylloscopus	Phylloscopidae
7	*Cisticola juncidis*	Cisticola	Sylviida	-	-	Cisticolidae
8	*Orthotomus sutorius*	Orthotomus		-	-	
9	*Prinia buchanani*	Prinia		-	-	
10	*Prinia socialis*			-	-	
11	*Prinia hodgsonii*			-	-	
12	*Saxicoloides fulicatus*	Saxicoloides	Turdidae	*Copsychus fulicatus*	Copsychus	Muscicapidae
13	*Myophonus caeruleus*	Myophonus		-	-	
14	*Phoenicurus ochruros*	Phoenicurus		-	-	
15	*Copsychus saularis saularis*	Copsychus		-	-	
16	*Oenanthe picata*	Oenanthe		-	-	
17	*Passer rutilans rutilans*	Passer	Passeridae	*Passer cinnamomeus rutilans*	-	-

Among the 11 unique BINs assigned by the BOLD database there were 5 BINs that showed change at taxonomic level (Table 4.5). There were 3 species (*Cisticola juncidis*, *Prinia buchanani*, Prinia hodgsonii) with the family changed, 1 species (*Saxicoloides fulicatus*) with the family and genus and I speices (*Cyanoderma pyrrhops*) with genus changed were observed. Further more changes at taxonomic level from our dataset for 17 species out of 43 sequences indicate the need for extensive sampling for identification purpose in order to add more species into these databases to increase their accuracy for identification and verification [161].

The genus tephrodornis have birds with ashy colour. The species *Tephrodornis pondicerianus* named as Common Woodshrike was first described as subspecies to locality of Madras. Gmelin has placed this subspecies in the genus Muscicapa along with the flycatchers. In the past this species included *Tephrodornis affinis* named (Sri Lanka Woodshrike) as a subspecies and now treated as a species with distinct plumage as well as calls and is restricted within Sri Lanka. Several subspecies are named under this species [162].

Tephrodornis affinis and *T. pondicerianus* previously were grouped as *T. pondicerianus* [6, 163]. Our results prove the same concept of placing *Tephrodornis pondicerianus* in genus tephrodornis. According to bird life international [164] *Tephrodornis pondicerianus* is found across Asia in scrub habitats and thin forest. In the past it has been placed in the cuckoshrike group of birds belonging to family Campephagidae and the helmetshrike birds from Prionopidae family but is counted under family Tephrodornithidae (ITIS). According to Robert [15] *Tephrodornis pondicerianus* (Common Woodshrike), belongs to genus Tephrodornis and family Campephagidae But according to bird life international, Avibase and IUCN Red List accessed in 2021 [24, 26, 131], it is now placed under another family i-e Vangidae. Our results have supported the idea of *Tephrodornis pondicerianus* placed in family Campephagidae according to literature [15] and shifted to family Vangidae according to identification of our sample by BOLD database.

Chrysomma sinense (Yellow-eyed Babbler) from genus Chrysomma was studied and according to classical taxonomy it was placed under family Timaliidae but molecular studies separated it into family Sylviida (Old World warblers) [26] and

according to BOLD database (www.boldsystems.org) [139] but into Paradoxornithidae family according to Avibase database., this was earlier grouped into two families i-e Turdidae and Timaliidae and outsize in Muscicapidae . It is native to the Southeast and South Asia inhabiting wetland, shrubland, and grassland habitats [165]. In IUCN Red List, it is mentioned as Least Concern (LC) because it has stable population and wide distribution. Its traditional placement was in family Timaliidae (Old World babbler family) although Chrysomma genus forms a clade with the other genera like parrotbills and Sylvia warblers belonging to the family Sylviidae [63]. Our study sample *Chrysomma sinense* belongs to genus Chrysomma and was placed under family Timaliidae according to classical taxonomy [15] and molecular studies separated it into family Sylviida according to BOLD database (www.boldsystems.org) [139] identification system in our study.

Molecular studies has also replaced family Timaliidae to family Leiothrichidae and traditionally subsumed genus Garrulax [162] to genus Trochalopteron for *Garrulax lineatus* species and which is now named as *Trochalopteron lineatum* (Streaked Laughingthrush). Recently it has been split to from a broader family Timaliidae. Now the traditionally recognized some genera of this family have been rediscovered and now placed as separate clade family Timaliidae while other genera are considered polyphyletic according to genetic data availability [166]. Many species are dependent on molecular data to be screened as yet not tested much of taxa [167]. Results of our study are supported by this evidence and species name of *Garrulax lineatus* has been changed to *Trochalopteron lineatum* because of the change in genus name from Garrulax [15] to genus Trochalopteron according to BOLD database (www.boldsystems.org) [139].

The genus Cyanoderma [15] has also been replaced by genus Stachyridopsis (BOLD database 2022) [139] and species name from *Cyanoderma pyrrhops* to *Stachyridopsis pyrrhops* accordingly but family Timaliidae remained same as earlier according to our comparison of taxonomy. The *Stachyridopsis pyrrhops* (Black-chinned Babbler) is normally found in the Indian Subcontinent preferring temperate forests and tropical lowland forests habitats. Geographical range of *Cyanoderma pyrrhops* is Himalayas (Kashmir to central Nepal). According to Avibase taxonomic concepts

its name has been changed from *Cyanoderma pyrrhops* to *Stachyris pyrrhops* onward (https://avibase.bsc-eoc.org retrieved in 2020) [141].

Our study show that the *Pastor roseus* belongs to genus Pastor according to modern taxonomy BOLD database (www.boldsystems.org retrieved in 2022) [139] but in classical taxonomy [15] it was named as genus Sturnus and species *Sturnus roseus* acoordingly. The *Sturnus roseus* (Rosy Starling) belongs to the family Sturnidae but sometimes named under monotypic genus Pastor. Recent studies are supporting this split but still their closest living relatives are not known. Avibase taxonomic concepts v.3 has published *Sturnus roseus* (Rosy Starling) as *Pastor roseus* (database (https://avibase.bsc-eoc.org retrieved in 2020) [141]. All this data is supporting our findings of results.

Phylloscopus affinis (Tickell's Leaf Warbler) has a yellowish underside and supercilium and commonly found in Asia and a common target for bird watchers'of this region and now belongs to Phylloscopidae family according to BOLD database (www.boldsystems.org retrieved in 2022) [139] instead of family Sylviida [15] Same concept is supported by the Avibase database (https://avibase.bsc-eoc.org retrieved in 2020) [141], which we found by comparison of classical and molecular taxonomy of *Phylloscopus affinis*.

The Passeriformes species *Cisticola juncidis* and *Orthotomus sutorius* belongs to family Sylviidae according to classical taxonomy [15] but now after molecular level analysis they are in family Cisticolidae according to BOLD database (www.boldsystems.org retrieved in 2022) [139]. The *Cisticola juncidis* (Streaked Fantail Warbler) has wide distribution as Old World warbler with breeding range from southern Europe to Africa, This small bird can be identified by its rufous rump, brownish tail tipped white, lacks of any gold on collar found in grasslands. The *Orthotomus sutorius*, (Common Tailorbird) is found across tropical Asia common resident of urban gardens. Findings of our results are well supported by other databases (https://avibase.bsc-eoc.org retrieved in 2020) [141].

The samples of these three species i-e *Prinia buchanani*, *Prinia socialis socialis*, *Prinia hodgsonii* of genus Prinia of family Sylviidae [15] were replaced by family

Cisticolidae after molecular studies according to BOLD database (boldsystems.org retrieved in 2022) [139] during comparison of our samples for identification. The *Prinia buchanani* (Rufous-fronted Prinia) belongs to Cisticolidae family and is normally found in India and Pakistan preferring tropical or subtropical dry forests habitats. The *Prinia socialis* is a resident breeder in the Subcontinent. The distinctive colours along with upright tail make its identification easy in farmland and urban gardens [168]. These studies are supporting our findings of change in family of 3 species of Prinia genus.

Findings of our taxonomic comparison of species *Saxicoloides fulicatus* has placed it under genus Saxicoloides and under family Turdidae according to previous morphological based classification system [15] but now after genetic analysis the molecular based classification system has replaced its species name to *Copsychus fulicatus* and genus Copsychus and family Muscicapidae. The *Saxicoloides fulicatus* (Indian Robin) now new name *Copsychus fulicatus* is widespread species of birds of family Muscicapidae well distributed in the Indian Subcontinent (Bhutan, Nepal, India, Pakistan, Sri Lanka and Bangladesh). They are commonly observed perching on rocks, open scrub areas and low thorny shrubs [162]. The Indian robin after molecular studies followed by phylogenetic analysis has been moved to Copsychus genus of family Muscicapidae but formerly it was placed in Saxicoloides the monotypic genus according to recent studies [169–171]. Results of our findings are supported by all this literature.

In the same way *Myophonus caeruleus*, *Phoenicurus ochruros*, *Copsychus saularis saularis*, and *Oenanthe picata* were ranked under family Turdidae according to traditional system of classification [15] but in modern system of classification now they are ranked under family Muscicapidae according to BOLD database system identification (www.boldsystems.org retrieved in 2022) [139] during our comparison of taxonomy. The *Myophonus caeruleus* (blue whistling thrush is common in Southeast Asia, Central Asia, and China. At dawn and the dusk, it whistles loud song like human and is widely distributed with variations in their size and plumage. The *Phoenicurus ochruros* (Black Redstart) belongs to genus Phoenicurus but was formerly classified under Thrush family (Turdidae), but is now placed under family

Muscicapidae as an Old World flycatcher BOLD database (www.boldsystems.org retrieved in 2022) [139] and Avibase database (https://avibase.bsc-eoc.org retrieved in 2020) [141]. The *Copsychus saularis* (Oriental Magpie-Robin, was formerly classified as under the family Turdidae [15], but considered now as flycatcher of the Old World. These birds have white and black colour and upright long tail. They are common in urban gardens and forests of the Indian Subcontinent and Southeast Asia. And is national bird of Bangladesh.

4.9 Sequence Analysis by MEGAX

The following analysis were performed on sequences of COI gene locus of mitochondrial genome (DNA) of Passeriformes to find variations in these sequences and find whether this locus of sequence is capable of reconstructing the phylogenetic relationship as claimed by different currently taxonomic studies using MEGAX software.

4.9.1 Multiple Sequence Alignment

It was carried out for the barcode region (COI) of 235 species of Passeriformes out of which 43 were sequenced in the laboratory and 192 sequences of Passeriformes were retrived from GenBank of NCBI database. The analysis was separately performed for nucleotides and amino acids sequences by means of ClustalW [145] integrated in MEGAX (Appendix C).

Multiple sequence alignment is pre requirement for phylogenetic analysis to find the evolutionary relationships among multiple sequences and shows the common patterns of genes and align the sequences based on the similarity. In our study they aligned successfully indicating variations and similarities among all included sequences.

4.9.2 Nucleotide Composition

The nucleotide composition of COI gene of different species of Passeriformes shows how different or similar ATGC content is in the same gene of different species. MEGAX was used to compute the frequencies of each nucleotide (ATGC) in the barcode region of Passeriformes species of Pakistan as shown in Table 4.7 The analysis involved 43 newly generated sequences of Passeriformes and 193 retrieved sequences from GenBank of NCBI database of Pakistan.

Cytosine showing highest average amount 32.5% followed by Adenine 25.9%, Thamine 24.5% and least Guanine 17.1% of nucleotides and total average amount of nucleotides was 670.7 in all sequences. It shows a clear variations in the frequencies of nucleotides Thyamine T(U) ranging from 21.1% (*Lophophanes dichrous*) to 28.4% (*Cercotrichas galactotes*) deviating from aveage values. Highest Cytosine value was 38.0% (*Remiz pendulinus*) and lowest was 27.0% (*Rhipidura aureola*). Highest value of Adinine 29.1% (*Cisticola juncidis*) and lowest 22.4% (*Rhipidura hypoxantha*) was observed. Guanine value was highest 18.9% (*Hippolais languida*) and lowest 15.5% (*Prinia buchanani*) showing deviation range from average value of Guanine in the all 235 sequences [Appendix D].

TABLE 4.7: Average nucleotide composition of COI gene at 1^{st}, 2^{nd} and 3^{rd} position of codon of Passeriformes of Pakistan

Position	**T(U)**	**C**	**A**	**G**	**Total**
Nucleotide composition	24.5	32.5	25.9	17.1	670.7
1^{st} position of codon	**T-1**	**C-1**	**A-1**	**G-1**	**Total**
Average	16.8	28.0	23.4	31.8	223.5
2^{nd} position of codon	**T-2**	**C-2**	**A-2**	**G-2**	**Total**
Average	42.1	28.2	15.4	14.3	223.5
3^{rd} position of codon	**T-3**	**C-3**	**A-3**	**G-3**	**Total**
Average	14.6	41.4	38.9	5.1	223.7

Nucleotide composition of an organism reveals its genome. GC content varies among species and has several implications. It determines the nucleotide frequencies, codon and their amino acid usage. Nucleotide composition is similar among

the species living in the same environments therefore help in determining the association among close groups in their phylogeny [153, 154]. In prokaryotes, the GC content in any organism strongly correlated with mean values at the three codon positions, 1^{st}, 2^{nd} and 3^{rd} and also relate with global nucleotide frequencies of organisms and amino acid usage [172]. The variability in GC content in prokaryotes is high showing homogenicity [173]. On contrary to this GC content in vertebrate like mammals and birds shows highly similar GC content [174] and show similar pattern of codon usage [175]. However, intragenic heterogeneity has been observed in birds [176]. This nucleotide composition help understand pattern of substitutions and relative effect of selective forces resulting these changes [177].

The average frequency of nucleotide Thymine at the 1^{st} position of codon in 16.8%, Cytosine 28.0%, Adenine 23.4% and for Guanine is 31.8%. The frequency of Guanine is highest at this position while the lowest for Thymine. 1^{st} position of codon in all species shows the most variation in nucleotide frequencies (Table 4.7). Highest value of Thyamine at the 1^{st} position of codon was observed in the species *Parus monticolus* (19.9) followed by the species of genus Prinia, *Prinia buchanani* (19.0), *Prinia socialis socialis* (19.0) *Prinia hodgsonii* (18.2). While a pattern of similarity can be seen for Thyamine (17.7) within genus Lanius for *Lanius collurio*, *Lanius isabellinus*, *Lanius cristatus*, *anius tephronotus*, *Lanius excubitor*.

Clear variations can be observed in the Thymine nucleotide frequencies of species of genera Phylloscopus, *Phylloscopus humei* (15.7), *Phylloscopus griseolus* (16.5), *Phylloscopus collybita* (18.3), P*hylloscopus trochiloides* (16.5), *Phylloscopus magnirostris* (16.0), *Phylloscopus reguloides* (16.0). This shows that *Phylloscopus collybita* has more adaptaion in the available environment as compared to other members of genus.

At 1^{st} condon position highest value of Cytosine was 29.9 for *Emberiza bruniceps* but there was variation in the Cytosine values among 13 other members of the genus Emberiza with lowest value 26.8 for Emberiza rutila. This variation shows their better ability to survive in changing environments. A pattern of similarity was observed in the 7 species of genus Anthus studied ranging from 28.1-29.0.

At 1^{st} codon position highest value of Adnine was 26.0 for *Hirundo smithii* but there was a wide difference in the value of Adnine i.e., 22.9 for *Hirundo rustica* the member of the same genus Hirundo which indicates the better adaptation of these species in the changing environments. Less variations in the amount of Adnine at 1^{st} codon position was observed in the genus Lanius i.e., 23.8, 23.8, 23.8, 23.4, 23.8 for *Lanius collurio*, *Lanius isabellinus*, *Lanius cristatus*, *Lanius tephronotus*, *Lanius excubitor* respectively. This similarity in the amount of Adenine for species of genus Lanius indicates their fewer tendencies to adapt the changing environments.

At 1^{st} codon position highest value of Guanine was 32.9 observed for *Emberiza schoeniclus* along with other species of different genera. There was variation observed in 13 other species of genus Emberiza with minimum value of Guanine 31.0 for *Emberiza fucata*. This variation in the nucleotide composition is indicating their best adaptions for survival in continuously changing climates. A less variation was observed in the value of Guanine in the 7 species of genus Acrocephalus ranging from 31.2 to 32.5, which indicates their less adaptability in the changing environments.

The average frequency of nucleotide Thymine at the 2^{nd} position of codon in 42.1% , for nucleotide Cytosine it is 28.2%, for Adenine it is 15.4% and for Guanine it is 14.3%. The frequency of Thymine is highest at this position while the lowest for Guanine. The average frequency of nucleotides at 2^{nd} position of codon in mitochondrial COI gene of Passeriformes species of Pakistan is shown in Table 4.7. The analysis involved total 235 sequences including 43 newly generated mtCOI sequences and 192 retrievd sequences of Passeriformes of Pakistan.

At 2^{nd} position of codon 6 species of genus Phylloscopus has shown the variation in Thyamine nucleotide frequencies (Table 4.7). The lowest value was 40.7 for *Phylloscopus reguloides* and highest 43.3 for *Phylloscopus trochiloides*. In the same way species of other genera has also shown variation in the value of Thyamine nucleotide as species of genus Acrocephalus values ranging from 42.4 to 40.7 among 7 species. Genus Prinia with 4 species values ranging from 40.7 to 42.6. The highest frequency of Cytosine 30.0 was observed at 2^{nd} codon position for *Carpospiza brachydactyla* and the lowest 27.3 for multiple species.

Variations were observed in 13 species of genus Emberiza ranging from 27.3 to 28.8. In the same way variations were also observed in the values of Cytosine at 2^{nd} codon position for 6 species of genus Anthus ranging from 27.3 to 29.0. Variations were also observed among species of other genera under study this indicates their tendency to adapt the changes in the changed environments. The highest frequency observed for Adenine at the 2^{nd} position of codon was 16.5 for *Phoenicurus frontalis* and lowest was 14.1 for *Alauda gulgula australis*. These ranges show the variations in the frequency of Adenine among the species of Passeriformes, which indicate their tendency to survive in the continuously changing environments. Within genus comparatively fewer variations were observed in the 4 species under a genus Lanius ranging 15.6-16.4. A high variation was observed in 7 species of genus Acrocephalus ranging from 14.9-16.1 indicating their better survival for fittest.

At 2^{nd} position of codon the frequency of Guanine was ranging from 12.2 to 15.1 for *Prinia buchanani* and *Lonchura punctulata* respectively but overall variation among different species of a genus was very less. For 5 species under genus Phylloscopus were ranging 14.2 to 14.7, for 7 species of Acrocephalus values were ranging from 14.3 to 14.7 as well.

At 3^{rd} position of codon the average frequency of nucleotides is as follows 14.6% Thyamine, 41.4% for Cytosine, 38.9% for Adenine and 5.1% Guanine. They are almost similar for maximum species involved in this analysis. The frequency of nucleotides at 3^{rd} position of codon in mitochondrial COI gene of Passeriformes species of Pakistan is shown in Table 4.7. The analysis involved total 235 sequences including 43 newly generated mtCOI sequences and 192 retrievd sequences of Passeriformes of Pakistan.

There was great variations observesd for the values of Thyamine at 3^{rd} codon poition ranging from 25.0 *Cercotrichas galactotes* and 7.4 for *Parus major*. Even with genra species have shown variations in Thyamine frequencies for eample the 7 species of Phylloscopus genus shownas value range as 13.4 to 16.9. Among 8 species of genus Oenanthe it ranges from 12.1 to 16.6. In the same way for 6 speies of genus Anthus it ranges from 17.4 to 9.7 *Anthus campestris, Anthus*

pratensis repectively. The great variations in the frequencies of Thyamine at 3^{rd} codon poition indicat, that these species of Passeriformes have great potential for survival in the changing climates. There was great variations observesd for the values of Cytosine at 3^{rd} codon poition ranging from 28.1 *Lanius schach schach* and 51.9 for *Oenanthe alboniger*. Variation among 7 species under Acrocephalus genus were ranging 38.1 to 44.2 which is comparitevly less varied as compared to overall variation among all species under study. The frequency of Adenine at 3^{rd} codon poition was ranging from 47.1 for *Lonchura punctulata* and 28.4 for *Rhipidura hypoxantha*.This range also indicates variations in frequencies of Adenine among different species of Passeriformes under study and shows their capacity to adapt changes in their habits.

At 3^{rd} position of codon the the frequency of Guanine was ranging from 13.3 to 1.3 for *Rhipidura hypoxantha* and *Pitta brachyuran* respectively. This is indicating a lot of variations in the frequency of Guanine among different species of Passeriformes under study. It is indicating their great potential for survival in the changing environments. Within genus these variations in the values of the Adenine were also observed as in the 7 species of genus Acrocephalus were ranging from 39.4-34.6. Within genus variations were also observed as in 5 species of genus Sylvia values ranging from 2.8 to 5.2 for *Sylvia nana*, *Sylvia communis* respectively.

Nucleotide composition is a tool which provids valuable information about genetic variations in the species of a polulation and among populations. It helps in finding genetic diversity at different taxonomic levels which increase the understanding of genetic make up, adaptive strategies and evolutionary history of different organisms and their populations. Nucleotide composition patterns refer to the charateristics of a certain group of organisms therefore help in distinguishing certain species or populations.

4.9.3 Codon Usage Bias

Codon usage bias for COI genes is given below (Table 4.8). It was estimated using MEGAX software. It provides the average frequency of relative synonymous usage of each codon in the gene. From all 223 codons a few are showing negative value like UUG(L), AAG (K), UGU(C), UGC(C) while stop codons UAA (*), UAG(*), AGA (*), AGG(*) also showing zero value. While rest of codons have shown positive values ranging from 0.00-11.3 for AAG (K) and AUC (I) respectively.

Codon usage means frequency of a codon to be coded into particular amino acid in the specific genetic code to act as start or stop signal for protein synthesis process. Analysing the codon usage can help understand the evolutionary relationships, genetic diversity along with adaptations, gene expressions among different species of a population and even the origion of that particular geen. It helps understand complex processes of shaping the genetic diversity in the world of biology. Codon usage values range from 0-61 representing the frequency with which a particular code is coded into a specific amino acid. A zero value indicates a strong selective pressure against its use, the values close to zero suggest that codon is rarely used while a value 61 represnts high preference of codon to be coded into specific amino acid. Codon usage is influenced by factors as mutation rates and natural selection. The results of our study indicate low preferences of the codons to be coded into specific amino acids indicating less genetic variations among species of Passeriformes.

Table 4.8: Codon usage bias for COI gene sequences of Passeriformes

S #	Codon	Count	RSCU	S #	Codon	Count	RSCU
1.	UUU(F)	2.3	0.31	33.	UAU(Y)	0.9	0.34
2.	UUC(F)	12.6	1.69	34.	UAC(Y)	4.3	1.66
3.	UUA(L)	1.7	0.29	35.	UAA(*)	0.0	0.0
4.	UUG(L)	0.0	0.01	36.	UAG(*)	0.0	0.0
5.	CUU(L)	4.3	0.71	37.	CAU(H)	1.5	0.67
6.	CUC(L)	9.1	1.51	38.	CAC(H)	3.1	1.33
7.	CUA(L)	17.	2.92	39.	CAA(Q)	4.5	1.84
8.	CUG(L)	3.3	0.56	40.	CAG(Q)	0.4	0.16
9.	AUU(I)	4.5	0.57	41.	AAU(N)	1.2	0.27

S #	Codon	Count	RSCU	S #	Codon	Count	RSCU
10.	AUC(I)	11.3	1.43	42.	AAC(N)	7.8	1.73
11.	AUA(M)	8.6	1.57	43.	AAA(K)	1.1	1.96
12.	AUG(M)	2.3	0.43	44.	AAG(K)	0.0	0.04
13.	GUU(V)	2.7	0.68	45.	GAU(D)	0.8	0.22
14.	GUC(V)	5.3	1.33	46.	GAC(D)	6.3	1.78
15.	GUA(V)	6.6	1.64	47.	GAA(E)	2.3	1.78
16.	GUG(V)	1.4	0.36	48.	GAG(E)	0.3	0.22
17.	UCU(S)	1.5	0.72	49.	UGU(C)	0.0	0.0
18.	UCC(S)	4.4	2.20	50.	UGC(C)	0.0	0.0
19.	UCA(S)	4.1	2.03	51.	UGA(W)	5.4	1.96
20.	UCG(S)	0.1	0.07	52.	UGG(W)	0.1	0.04
21.	CCU(P)	2.8	0.71	53.	CGU(R)	0.3	0.45
22.	CCC(P)	5.5	1.41	54.	CGC(R)	0.6	0.77
23.	CCA(P)	6.9	1.78	55.	CGA(R)	2.0	2.64
24.	CCG(P)	0.4	0.09	56.	CGG(R)	0.1	0.15
25.	ACU(T)	2.2	0.66	57.	AGU(S)	0.2	0.08
26.	ACC(T)	4.9	1.49	58.	AGC(S)	1.8	0.90
27.	ACA(T)	5.8	1.75	59.	AGA(*)	0.0	0.0
28.	ACG(T)	0.3	0.10	60.	AGG(*)	0.0	0.0
29.	GCU(A)	3.9	0.64	61.	GGU(G)	3.4	0.63
30.	GCC(A)	10.5	1.75	62.	GGC(G)	4.8	0.89
31.	GCA(A)	9.3	1.55	63.	GGA(G)	11.1	2.08
32.	GCG(A)	0.4	0.06	64.	GGG(G)	2.1	0.40

4.9.4 Variations at Amino Acid Level

The composition of amino acid sequences of the COI loci from 235 species of Passeriformes including 43 sequenced and 192 downloaded sequences from NCBI GenBank were selected for calculation of conserved and variable sites through MEGAX software. A variable site can be parsimony-informative or singleton for having at least two different types of amino acids as compared to a conserve / constant site in Table 4.9 (Annexure F).

TABLE 4.9: Amino acid sequence variations in Passeriformes samples

Conserved sites (C)	**Variable sites (V)**	**Parsimony-informative (Pi)**	**Singleton (S)**
225/330	37/330	20/330	17/330

From all the data set of 330 selected sites from submitted sequences 225 sites were conserved which is the highest value proves that COI gene region is the most conserved region of a genome. Variable sited were only 37 out of 330, Parsimony informative sited were 20 out of 330 and single sites in each sequence of submitted sequences were 17 only which show the mutation in genes with time as they were present in only few sequences and not all (Appendex F).

In phylogenetic studies, (Psi) are calculated which indicate a site with at least two types of amino acids occure and each atleast two times. It was calculated through MEGAX software and 17 different parsimony informative sites (Psi) were [167] found. Researchers observed the parsimony informative sites (Psi) for avian species of Bucerotidae family using COI gene sequence and found no Singleton sites in the data. Singleton site works for association relationship. It would have to be one to one relationships [178].

The results shown in Appendex F may help in the identification of variable and conserved regions of COI gene loci. The structure and function of the encoded proteins show correspondence with each other therefore, there should be areas of conserved and variability domains in the sequence. The conserved regions may be ideal for species identification and helpful in designing the universal primers for amplification of the internally variable regions. In addition, the specific primers for each species can be designed by using unique variable sites which are particular to each species [30].

The process of evolution can be significantly affected by the change in percentage composition of nucleotides within a codon position. It can affect evolution by different means.

Firstly it can be due to genetic variations within a population resulted by the changes in nucleotide composition at some specific codon position. This variation acts as a raw material for the process of natural selection. If the change in nucleotide composition is positive means create some beneficial trait in the organism will result in higher fitness of that organism in the specific environment. It will increase their survival by reproducing that particular allele in more frequently in that population [30].

Protein structure and functions can alter by change in nucleotide composition at specific codon followed by amino acid composition causing change in protein during translation. Amino acids have their specific chemical properties therefore changes in the amino acid composition can direct to changes in protein structure and functions. This will have significant impact on phenotype which further will affect their reproduction, and evolutionary success.

The nucleotide percentage at different codon positions can affect codon usage bias. It can be due to uneven distribution of some synonymous codons which may have encoded the same amino acids. Organisms prefer to choose some specific codons over the others. The percentage changes in the nucleotide composition among the codon can affect the codon usage bias. The codon usage bias can further affect the protein folding, translational efficiency and even regulation of genes and ultimately can influence the fitness and survival of the organism through competition within population [147].

The changes in nucleotide composition at different codon positions can be due to selective pressure of the environment. Over time resulting alleles may become prevalent due to better adaptation in the new environment. It means that change in percentage nucleotide composition within different codon positions can cause introduction of genetic variations, alteration in the protein functions along with structures. This is ultimatly showing impact on codon usage bias and directing towards adaptation in the new environments. Over time, these changes affect the overall gentic make up of that population resulting in phenotypic changes in that population [147].

In the investigation of animal species and population genetics the variations in mitochondrial DNA are proved as a significant tool. Recently, reserchers interest has increased in the structure, function and evolution of 13 encodes polypeptides of mtDNA with recognition of relationship between mutations in mtDNA and human disease [157].The amino acid sequence of the COI loci were selected from 235 species of Passeriformes. The COI locus is proved as highly conserved sequence as compared to cyt b, ND2 and ND5 genes as cyt b and COI loci are frequently used for the testing of avian species and their phylogenetic studies in current years [8].

4.9.5 Estimation of Transition/Transversion Bias

Transition means one purine exchange with another or pyrimidine with another while, transversio exchange a purine to a pyrimidine and vice versa. The nucleotide transition/transversion bias of COI gene in Passeriformes was estimated using MEGAX. It involved 235 nucleotide sequences including 43 newly generated COI sequences of Passeriformes of Pakistan and 192 COI sequences of other Passeriformes retrieved from NCBI GenBank. All the missing data and gaps were eliminated from the sequences during the analysis. The average nucleotide transition/transversion bias (R) in COI gene was 2.31.

The transition bias was observed constant (17.4572) in the pair C→T, pair A→G, pair T→C and G→A suggesting that in COI gene the ratio of pyrimidine (C→T) conversions was same as purine (A→G) conversions. On the other hand, the transversion bias for COI gene was same for (C→G) to (A→T) [4].

The probability of converting one purine with another or one pyrimidine with another according to our results show 17.4572 value which is same for all nucleotides and bases. The value of probability is 3.7714 for converting one purine to one pyrimidine and the values are same for all nucleotides.

TABLE 4.10: The estimated transition / transversion bias of COI gene in Passeriformes

	A	**T**	**C**	**G**
A	-	3.7714	3.7714	17.4572
T	3.7714	-	17.4572	3.7714
C	3.7714	17.4572	-	3.7714
G	17.4572	3.7714	3.7714	-

The rate of transition and transversion in a DNA sequence can act as a valuable tool and can provide information in various evolutionary and biological contexts. The rate of substitutiions helps in understaning the evolutionary relationships, infering functional implications, assessing genetic diversity, and calculating the consequences of the mutational proceses. It provides the detailed insight into the above mentioned processes that have helped in shaping the genetic variations over the period of time.

The transitions are more common and rapidly accumulate over time where as transversions are less common indicationg fewer genetic variations among populations. A higher rate of transition indicates a poplation's recent exapansion while a higher rate of transversion indicates a long term or ancient genetic diversity. A lower rate of of transversion indicates a higher degree of conservation means similarity in their DNA sequences showing close evolutionary relationships among them. The ratio of transition to transversion is used to find evoltionary relationships among different species of a population or among populations.The higher ratio indicates a close genetic relationship means a more recent common ancestry whereas lower ratio indicates greater evolutionary distance means more genetic divergence.

The results of our study are supporting the concept of transitions being common phenomenon in nature with high values and transversions baing less common with low values indicationg fewer genetic variations among populations.

4.9.6 Tajima's Neutrality Test

Tajima's Neutrality Test was created by a Japanese scientist Fumio Tajima [155]. It discriminates between DNA sequences that are evolving randomly and those evolving under the influence of any environmental factor (e.g. genetic drift). For a sequence to be evolving randomly, the value of D must be zero. In the analysis 235 nucleotide sequencesof Passeriformes involved. 1^{st}, 2^{nd}, 3^{rd} codon positions were included in analysis. All positions which were ambiguous each sequence pair were removed by using pairwise deletion option. There were a total of 992 positions in the final dataset. Evolutionary analyses were conducted in MEGAX [146].

TABLE 4.11: Tajima's Neutrality Test for COI gene sequences of Passeriformes

	m	S	Ps	θ	π	D
Values	235	317	0.319556	0.052953	0.093349	2.405111

Abbreviations: m = number of sequences, n = total number of sites, S = Number of segregating sites, ps = S/n, θ = ps/a1, π = nucleotide diversity, and D is the Tajima test statistic.

Average evolutionary rate was scaled and set 1 for all sites and mean rate of evolution is shown against site number. This means that sites showing a rate < 1 showing average slow evolution than those with a rate > 1. The ML estimate of the gamma shape parameter is 0.3458. The maximum Log likelihood for this computation was -47798.134. In the analysis 235 nucleotide sequencesof Passeriformes involved. 1^{st}, 2^{nd}, 3^{rd} codon positions were included in analysis. All positions which were ambiguous each sequence pair were removed by using pairwise deletion option. There were a total of 992 positions in the final dataset. Evolutionary analyses were conducted in MEGAX [146]. The value of mean (relative) evolutionary rate is calculated here as 0.319556 which is < 1 showing that sited are evolving slowing. The presence of excessive rare allelles in genes shows a $<$ -2 D value during Tajima's test which indicates thier selective sweep or positive selection in nature. Whereas, the presence of excessive common allelles in genes

shows a ¿ 2 D value during Tajima's test which indicates their balancing or suggestive selection in nature. Our results have shown D value $>$ 2 hich indicates their balancing or suggestive selection in nature.

Variations in COI gene can be used to distinguish the closely related species at genus and family level in almost all taxa of different animals [153]. It suggest the diversity of 648bp of COI gene of mitochondria to act as potential barcode for different species of animals [44] as each species has its own unique nucleotide sequence of COI gene [94]. Modern Molecular technology help identify the genetic variation at DNA level.

It is proved by recent studies that out of the four loci i-e COI, cyt *b*, ND2 and ND5 the COI locus observed to be highly conserved followed by cyto b. [22].

In the investigation of animal species and population genetics the variation in the mitochondrial DNA are proved as significant tool. Recently, researcher's interest has increased in the structure, function and evolution of 13 encodes polypeptides of mtDNA with recognition of relationship between mutations in mtDNA and human disease [157, 179].

The main reasons for the selection of COI gene of mitochondrial genome is its effectiveness as a single marker irrespective of its small size it increases the similarity between the species in a tree that acts as a source of barcode locus for animals. This mitochondrial genome is helpful in different animal groups [180] of vertebrates [181] including fishes [182], birds and mammals [178]. The short sequence of COI gene can be has been used for identification up to specie level successfully in fishes [178], in Lepidoptera [183], in nematodes [180] and also in mammals and birds [40, 181]. The DNA barcoding region of many individuals, are used to construct the phylogenetic trees based on genetic distance [184]. COI gene, was used a rapid and accurate marker for species identification and phylogenetic reconstruction, because this portion of mtDNA have small rate of mutation. A sequence divergence at CO1 regularly enables the discriminations of closely allied species in all animal phyla [32, 40, 162] thus, the role of genetic analysis has become very significant for establishing phylogenies. The variation among different species is evaluated by

sequence comparisons of these genes. The comparison among mammals showed accurately of Cyt b for finding phylogeny than COI while in avian species COI region is more accurate to determine their phylogeny [185]. The main purpose for the taxonomic study indicates that a sequence divergence at CO1regularly enables the discriminations of closely allied species in all animal phyla except Cinidarian. Similarly Hajibabaei [32] reported that there were many mitochondrial markers used as a DNA barcodes such as COI, CO2 and 16 SAR ribosomal DNA are used as an authentic tool for species identification without using any taxonomic details.

4.9.7 Estimates of Average Evolutionary Divergence

Evolutionary divergences are the differences accumulated in the sequences of organisms over time. The analysis involved 235 nucleotide sequences including 43 newly generated sequences of Passeriformes of Pakistan and 192 sequences obtained from GenBank NCBI. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). Codon positions included were 1^{st} + 2^{nd} + 3^{rd}. All positions containing gaps and missing data were eliminated. There were a total of 992 positions in the final dataset. This evolutionary analyses was conducted in MEGA X.

The results show that average evolutionary divergence among species of Passeriformes was 0.18 the least average evolutionary divergence was 0.019 among *Lanius isabellinus* and *Lanius collurio* in the same way this average evolutionary divergence among other species of genus Lanius was very low as 0.022 among *Lanius tephronotus* and *Lanius cristatus* the highest was 0.263 among *Rhodospiza obsoleta* and *Ptyonoprogne rupestris* and among *Rhodospiza obsoleta* and *Cercotrichas galactotes*. All the differences were <2 therefore, meeting the criteria of intraspecific divergence Appendex E.

4.9.8 Phylogenetic Analysis

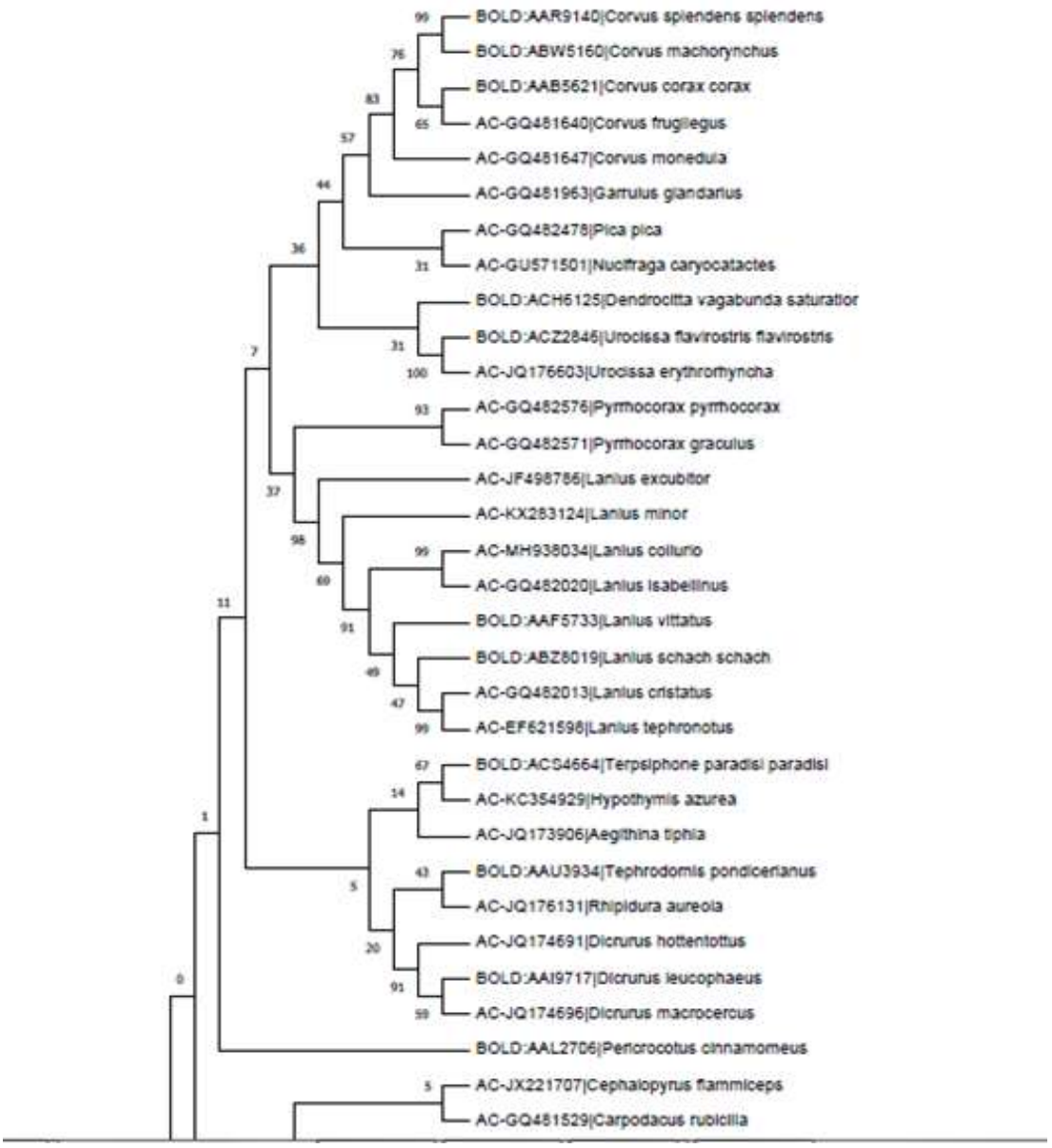
Birds broadly divide into main groups i-e Passeriformes and non-Passeriformes. The order Passeriformes is largest order of birds which further divided into 3 suborders. Suborder Acanthisitti with a single family Acanthisttidae (New Zeland Wrens), suborder Tyranni with 16 families and suborder Passeri with 121 families was found globally [164].

Phylogenetic analysis of organisms helps understand in-depth knowledge of evolution of species by genetic changes. Using phylogenetic analysis, present-day organism can be related with their ancestral origin and genetic divergence in the future may also be predicted.

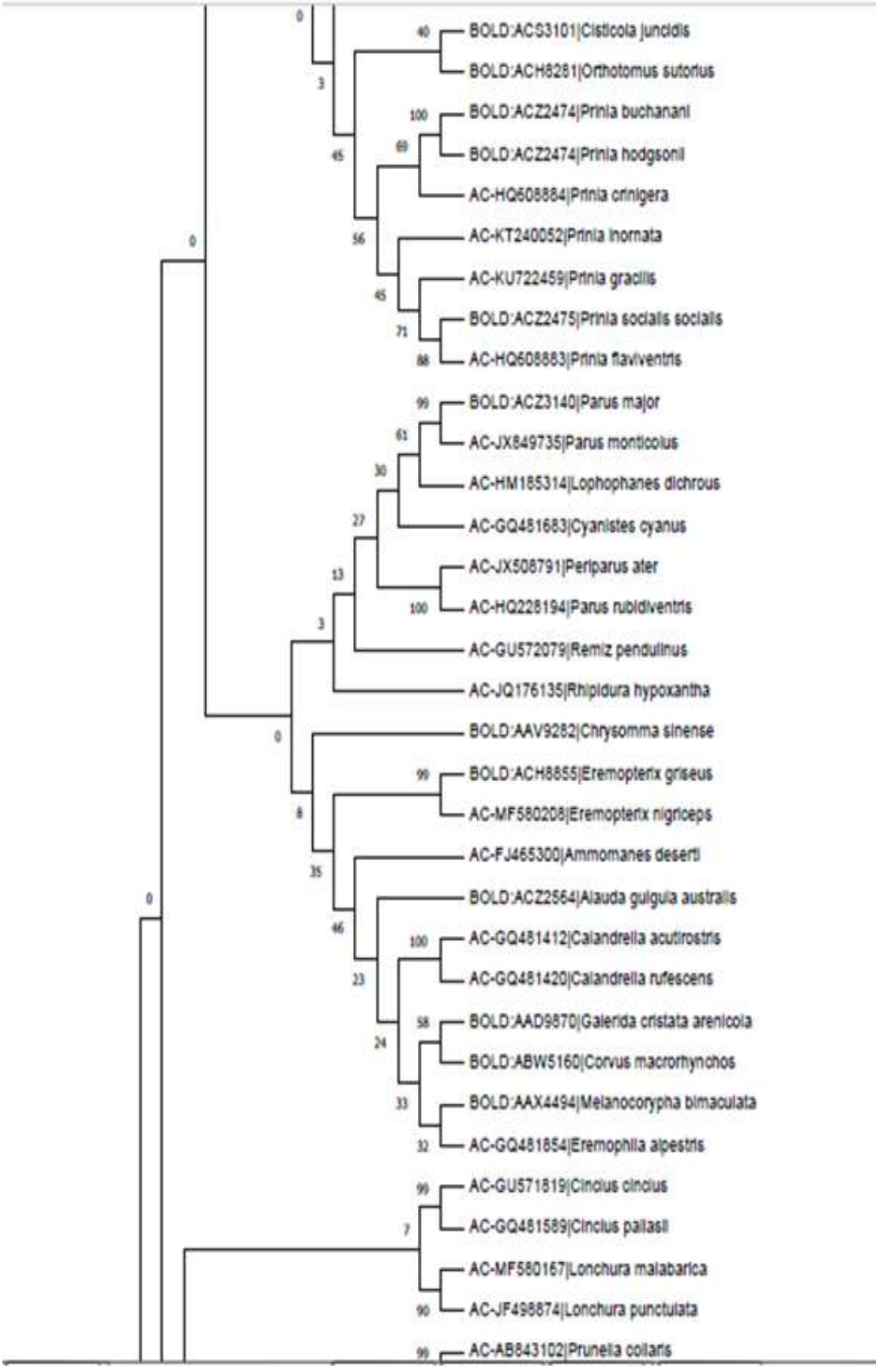
The principal of minium evolution works in the neighbor –joining technique and selects the tree based on branch length which is the minimum. This heuristic algorithm generates the sub-trees and their closest subtrees are ultimately joined togather in a stepwise manner to construct a final tree. In a true tree the total branch length is the shortest. The NJ method is normally applied for the large data of the taxa with variation in the degree of divergence and it help in correction of multiple substitutions.

Evolutionary analyses were conducted in MEGAX by Neibour joining method. The 236 nucleotide sequences of COI gene were involved in analysis including 43 newly generated sequences of Passeriformes and 192 sequences of other species of Passeriformes of Pakistan downloaded from NCBI GenBank and one species *Columbia livia* from another order of birds i-e Columbiformes as an out group. The results showed that the differences in COI sequences were usually higher among the species under family as compared to species under genus. Intraspecific hyplotypes were clustered under monophyletic clades with bootstrap support in thr NJ tree (Figure 4.3).

Following families were having single sample (Bombycillidae, Campephagidae, Certhiidae, Dicaeidae, Hypocoliidae, Nectariniidae, Pittidae, Panuridae, Paradoxornithidae, Vangidae, Regulidae, Remizidae, Rhipiduridae, Scotocercidae, Stenostiridae, Tichodromidae, Troglodytidae, Vangidae, Zosteropidae) while rest were



having multiple species. So far, the most robust phylogeny was attained using COI gene. The overall phylogeny of Passeriformes remained same as previous studies, forming similar split ups and clades [72, 76]. All 235 species of Passeriformes were first split into two clades. One comprising of the 5 species *Acrocephalus concinens*, *Aegithalos concinnus*, *Megalurus palustris*, *Locustella naevia* and rest of the specises in other clade which were further splited into sub clusters bases on the similarity and differences in theier COI gene sequences. The outgroup has made cluster with Pitta brachyura only which was a single species sequence from family pittidae based on bootstrap analysis.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BOLD:ACZ2474|Prinia buchanani
BOLD:ACZ2474|Prinia hodgsonii
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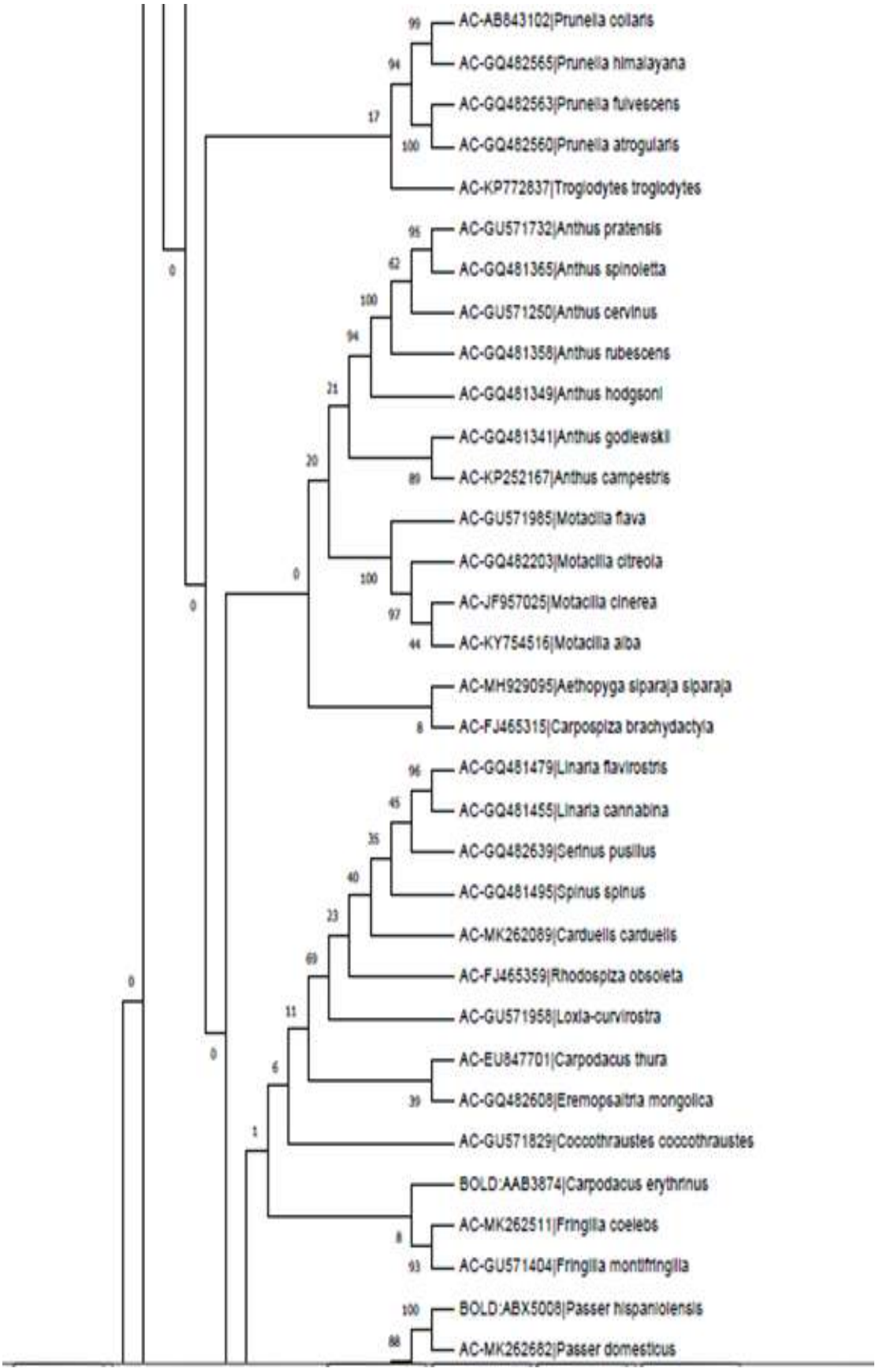
AC-KT240052|Prinia inornata
AC-KU722459|Prinia gracilis
BOLD:ACZ2475|Prinia socialis socialis
AC-HQ608883|Prinia flaviventris
BOLD:ACZ3140|Parus major
AC-JX849735|Parus monticolus
AC-HM185314|Lophophanes dichrous
AC-GQ481683|Cyanistes cyanus
AC-JX508791|Periparus ater
AC-HQ228194|Parus rubidiventris
AC-GU572079|Remiz pendulinus
AC-JQ176135|Rhipidura hypoxantha
BOLD:AAV9282|Chrysomma sinense
BOLD:ACH8855|Eremopterix griseus
AC-MF580208|Eremopterix nigriceps
AC-FJ465300|Ammomanes deserti
BOLD:ACZ2564|Alauda gulgula australis
AC-GQ481412|Calandrella acutirostris
AC-GQ481420|Calandrella rufescens
BOLD:AAD9870|Galerida cristata arenicola
BOLD:ABW5160|Corvus macrorhynchos
BOLD:AAX4494|Melanocorypha bimaculata
AC-GQ481854|Eremophila alpestris
AC-GU571619|Cinclus cinclus
AC-GQ481589|Cinclus pallasii
AC-MF580167|Lonchura malabarica
AC-JF498874|Lonchura punctulata
AC-AB843102|Prunella collaris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AC-GQ482563|Prunella fulvescens
AC-GQ482560|Prunella atrogularis
AC-KP772837|Troglodytes troglodytes
AC-GU571732|Anthus pratensis
AC-GQ481365|Anthus spinoletta
AC-GU571250|Anthus cervinus
AC-GQ481358|Anthus rubescens
AC-GQ481349|Anthus hodgsoni
AC-GQ481341|Anthus godlewskii
AC-KP252167|Anthus campestris
AC-GU571985|Motacilla flava
AC-GQ482203|Motacilla citreola
AC-JF957025|Motacilla cinerea
AC-KY754516|Motacilla alba
AC-MH929095|Aethopyga siparaja siparaja
AC-FJ465315|Carpospiza brachydactyla
AC-GQ481479|Linaria flavirostris
AC-GQ481455|Linaria cannabina
AC-GQ482639|Serinus pusillus
AC-GQ481495|Spinus spinus
AC-MK262089|Carduelis carduelis
AC-FJ465359|Rhodospiza obsoleta
AC-GU571958|Loxia-curvirostra
AC-EU847701|Carpodacus thura
AC-GQ482608|Eremopsaltria mongolica
AC-GU571829|Coccothraustes coccothraustes
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AC-GU571404|Fringilla montifringilla
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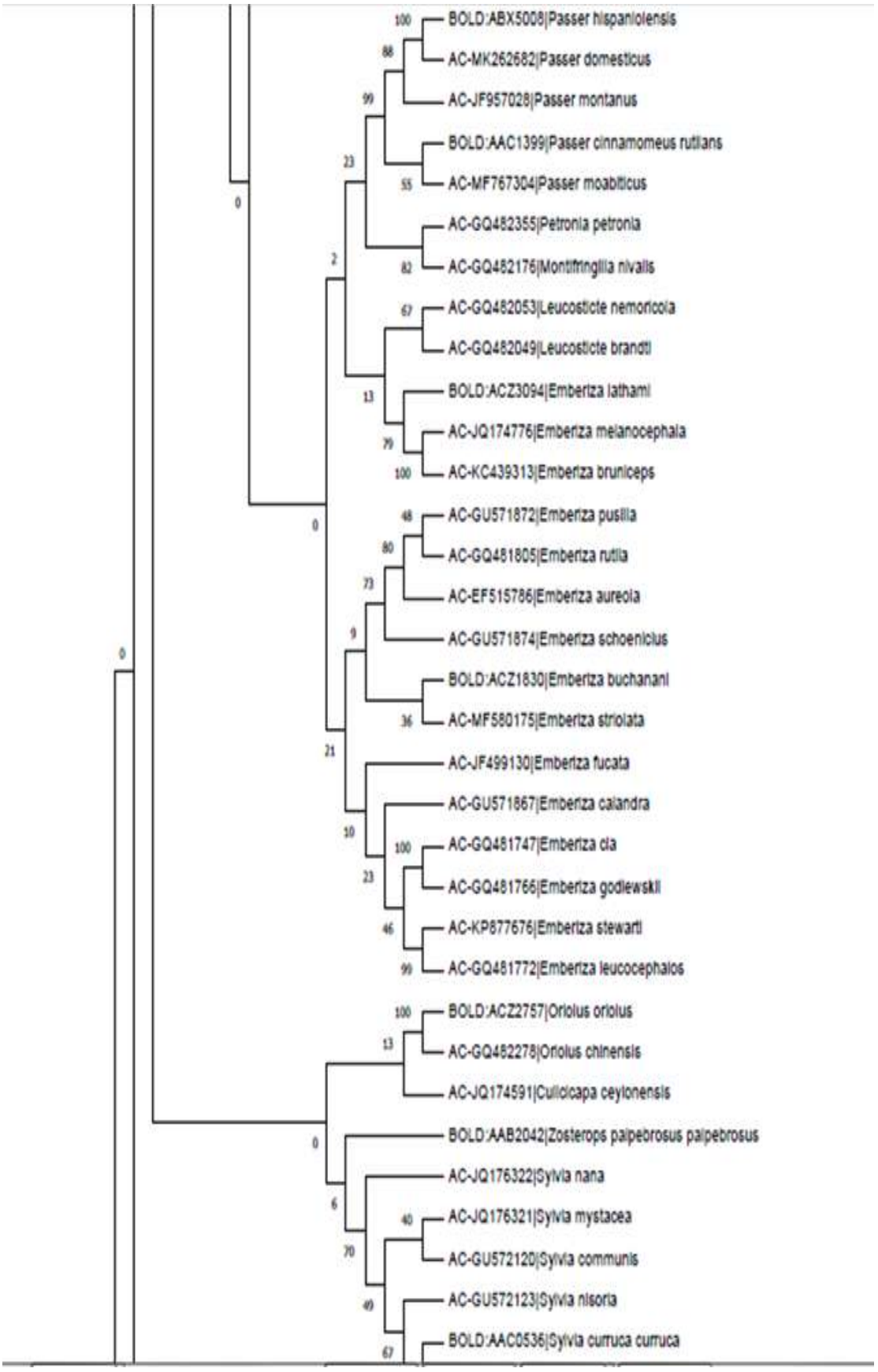
AC-MK262682|Passer domesticus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AC-GQ482053|Leucosticte nemoricola
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AC-MF580175|Emberiza striolata
AC-JF499130|Emberiza fucata
AC-GU571867|Emberiza calandra
AC-GQ481747|Emberiza cia
AC-GQ481766|Emberiza godlewskii
AC-KP877676|Emberiza stewarti
AC-GQ481772|Emberiza leucocephalos
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AC-GQ482278|Oriolus chinensis
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BOLD:AAB2042|Zosterops palpebrosus palpebrosus
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AC-GU572123|Sylvia nisoria
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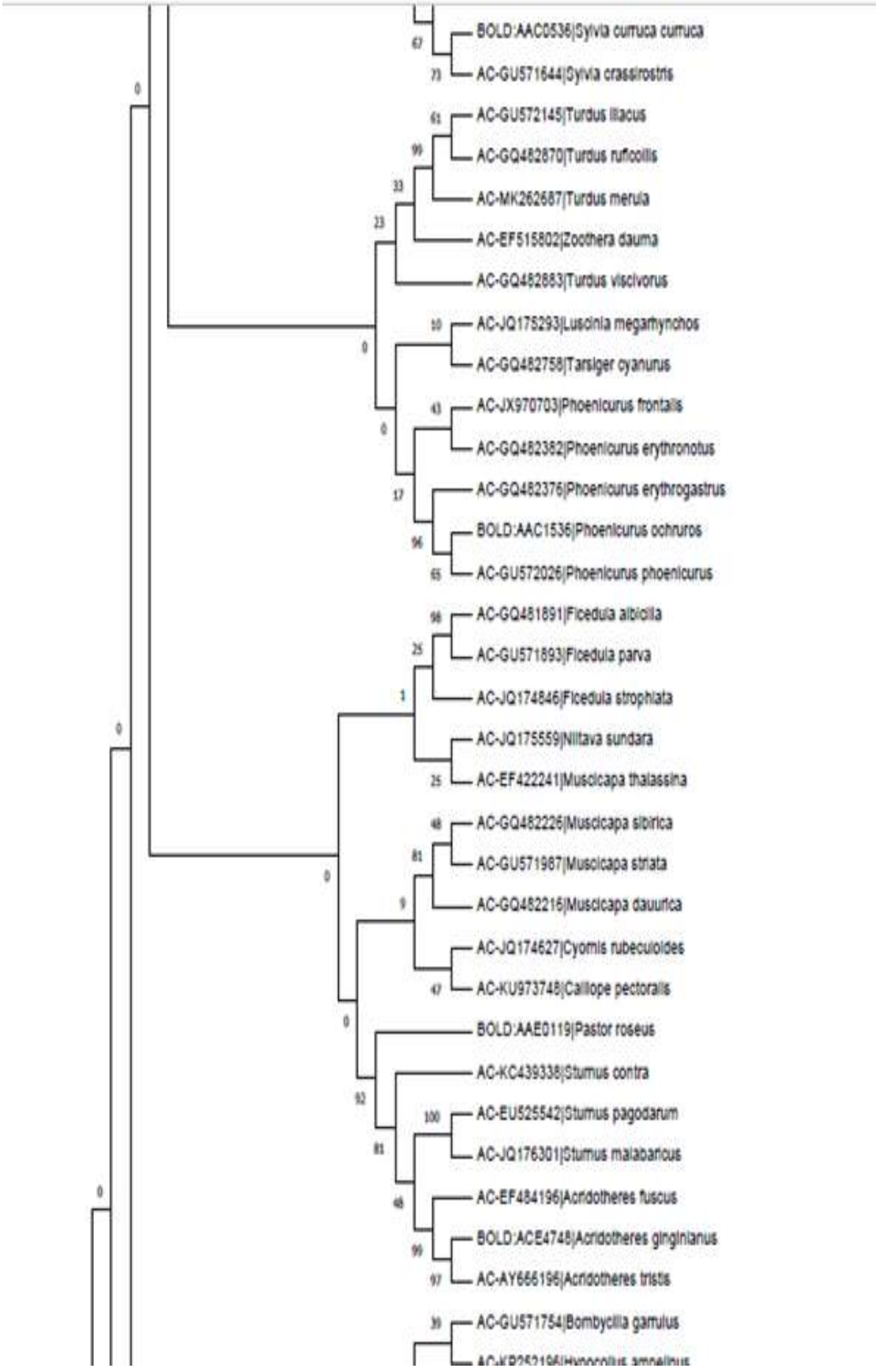
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AC-GU572145|Turdus iliacus
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AC-MK262687|Turdus merula
AC-EF515802|Zoothera dauma
AC-GQ482883|Turdus viscivorus
AC-JQ175293|Luscinia megarhynchos
AC-GQ482758|Tarsiger cyanurus
AC-JX970703|Phoenicurus frontalis
AC-GQ482382|Phoenicurus erythronotus
AC-GQ482376|Phoenicurus erythrogastrus
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AC-GQ481891|Ficedula albicilla
AC-GU571893|Ficedula parva
AC-JQ174846|Ficedula strophiata
AC-JQ175559|Niltava sundara
AC-EF422241|Muscicapa thalassina
AC-GQ482226|Muscicapa sibirica
AC-GU571987|Muscicapa striata
AC-GQ482216|Muscicapa dauurica
AC-JQ174627|Cyornis rubeculoides
AC-KU973748|Calliope pectoralis
BOLD:AAE0119|Pastor roseus
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AC-EU525542|Sturnus pagodarum
AC-JQ176301|Sturnus malabaricus
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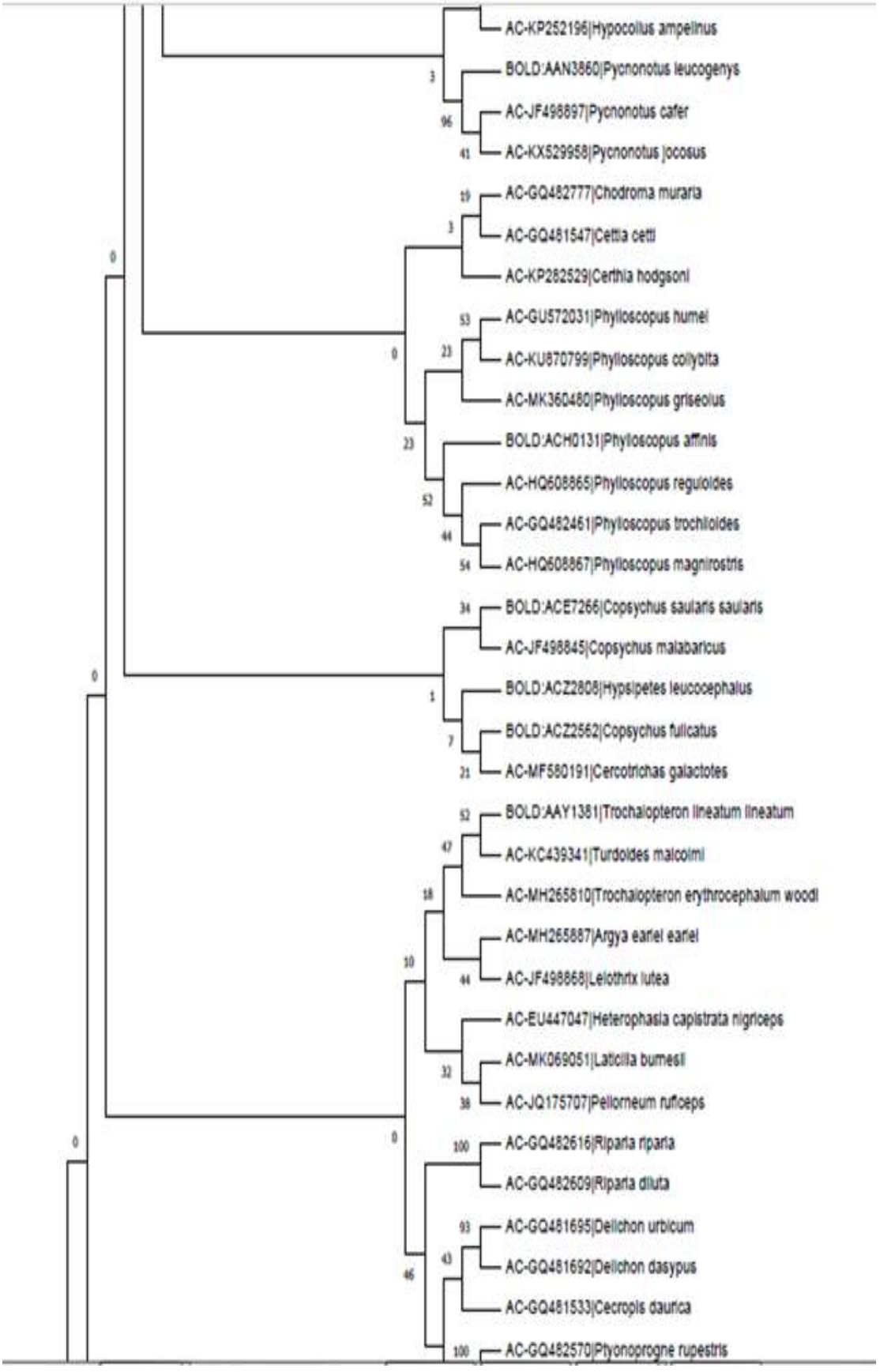
AC-AY666196|Acridotheres tristis
AC-GU571754|Bombycilla garrulus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AC-JF498897|Pycnonotus cafer
AC-KX529958|Pycnonotus jocosus
AC-GQ482777|Chodroma muraria
AC-GQ481547|Cettia cetti
AC-KP282529|Certhia hodgsoni
AC-GU572031|Phylloscopus humei
AC-KU870799|Phylloscopus collybita
AC-MK360480|Phylloscopus griseolus
BOLD:ACH0131|Phylloscopus affinis
AC-HQ608865|Phylloscopus reguloides
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AC-HQ608867|Phylloscopus magnirostris
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BOLD:ACZ2808|Hypsipetes leucocephalus
BOLD:ACZ2562|Copsychus fulicatus
AC-MF580191|Cercotrichas galactotes
BOLD:AAY1381|Trochalopteron lineatum lineatum
AC-KC439341|Turdoides malcolmi
AC-MH265810|Trochalopteron erythrocephalum woodi
AC-MH265887|Argya earlei earlei
AC-JF498868|Leiothrix lutea
AC-EU447047|Heterophasia capistrata nigriceps
AC-MK069051|Laticilla burnesii
AC-JQ175707|Pellorneum ruficeps
AC-GQ482616|Riparia riparia
AC-GQ482609|Riparia diluta
AC-GQ481695|Delichon urbicum
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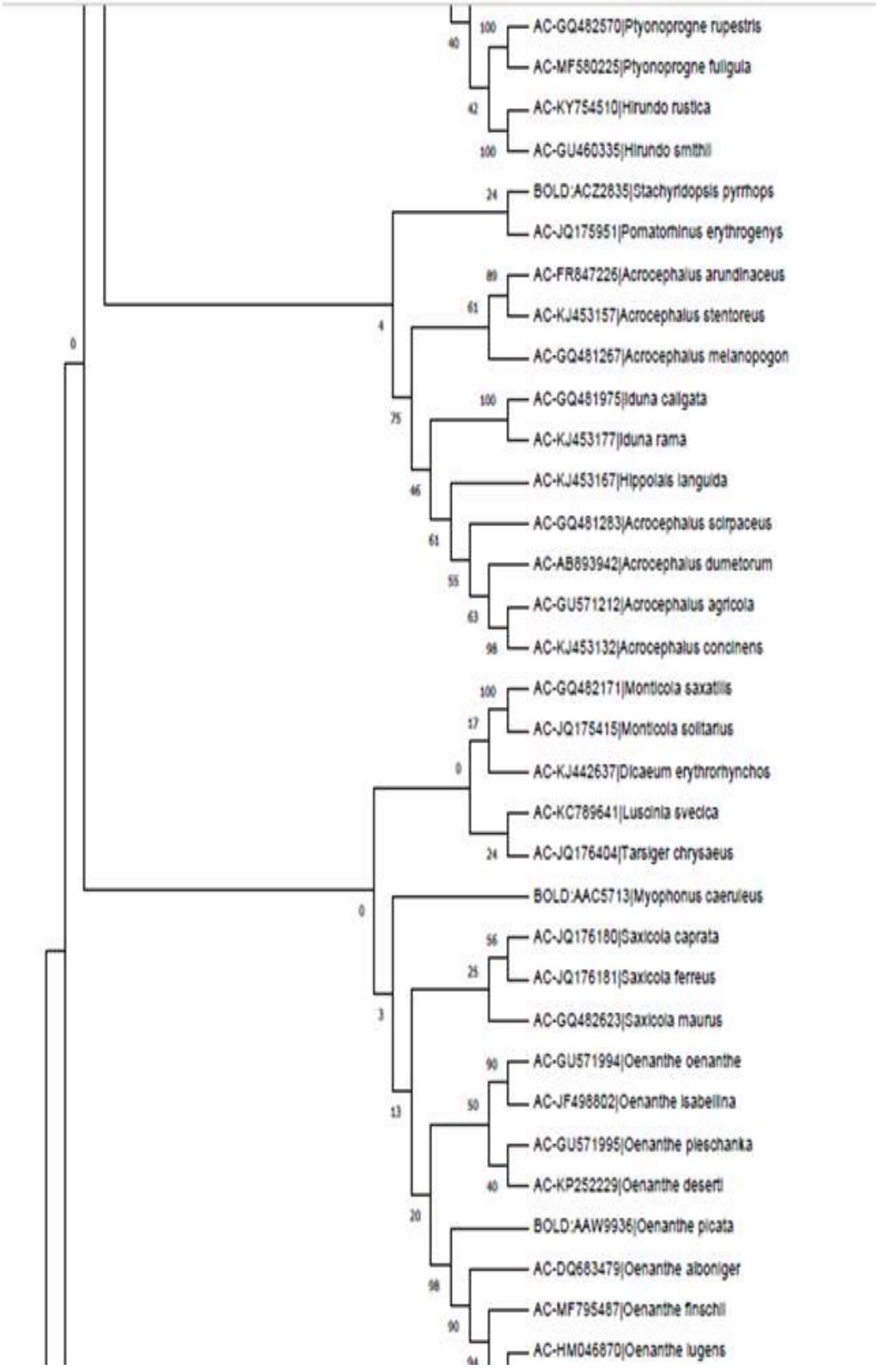
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AC-GU460335|Hirundo smithii
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AC-KJ453157|Acrocephalus stentoreus
AC-GQ481267|Acrocephalus melanopogon
AC-GQ481975|Iduna caligata
AC-KJ453177|Iduna rama
AC-KJ453167|Hippolais languida
AC-GQ481283|Acrocephalus scirpaceus
AC-AB893942|Acrocephalus dumetorum
AC-GU571212|Acrocephalus agricola
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AC-KC789641|Luscinia svecica
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AC-JQ176181|Saxicola ferreus
AC-GQ482623|Saxicola maurus
AC-GU571994|Oenanthe oenanthe
AC-JF498802|Oenanthe isabellina
AC-GU571995|Oenanthe pleschanka
AC-KP252229|Oenanthe deserti
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AC-HM046870|Oenanthe lugens

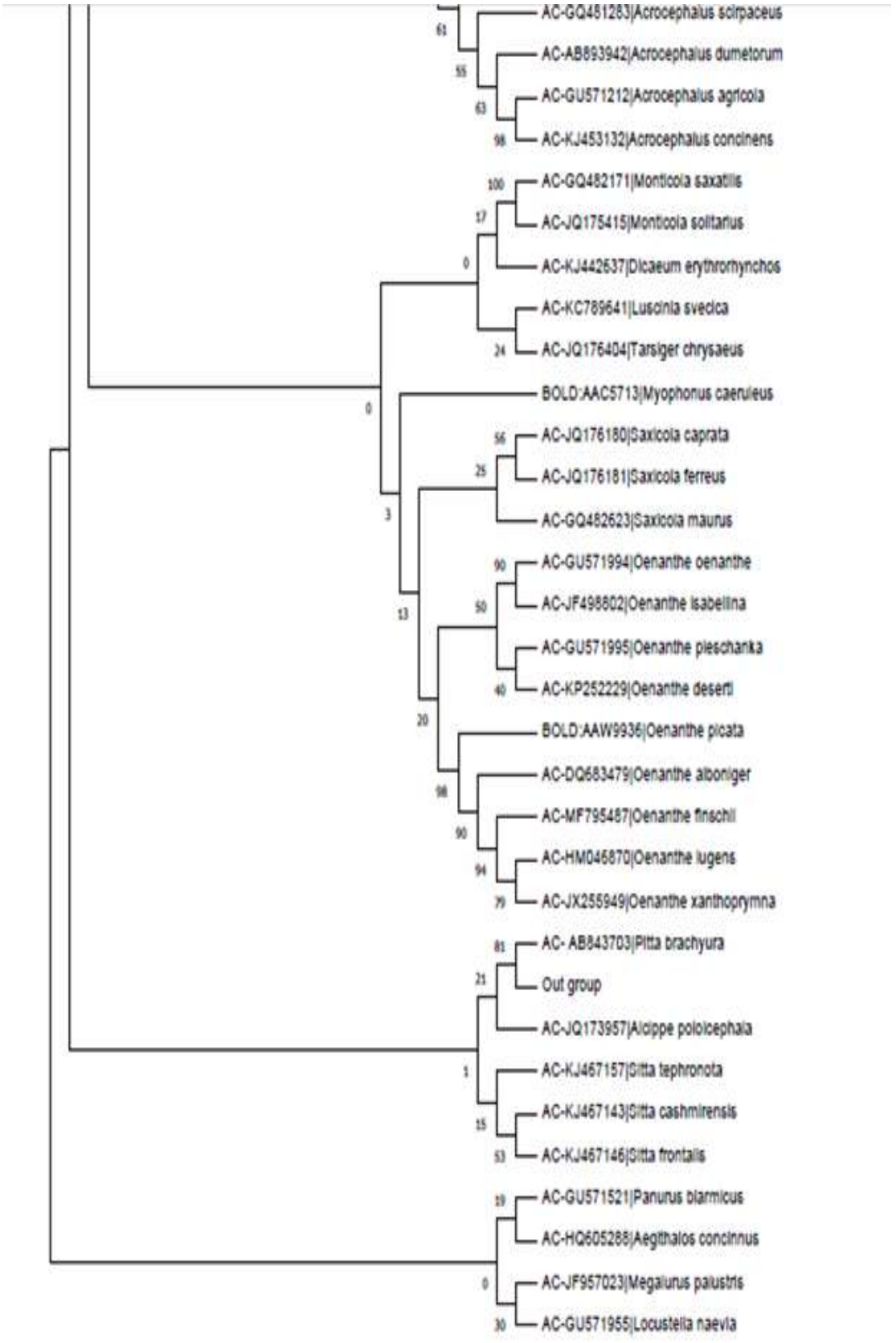

FIGURE 4.3: Evolutionary relationships of taxa by Neighbour Joining Method

The family wise phylogenetic analysis to explain evolutionary relationship among different species of Passeriformes under each family is explained below:

This family Acrocephalidae** of warblers is comprised of 60 species globally but from Pakistan 11 species were reported. COI gene sequence data of 10 species was available and downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. *Iduna caligata* (Booted Warbler) was formerly regarded as conspecific with *I. rama* (Sykes's Warbler), but now overlapping their breeding ranges, with difference in their mitochondrial DNA, morphology and habitat and is monotypic. *Hippolais languida* (Upcher's Warbler) is sister to *H. olivetorum* (Olive-tree Warbler), the two being fairly close to the pair formed by *H. polyglotta* (Melodious Warbler) and *H. icterina* (Icterine Warbler). E populations (E from E Iran) sometimes separated as subspecies magnirostris on basis mainly of slightly paler upper parts, but appear indistinguishable from birds elsewhere in range. Monotypic *Acrocephalus melanopogon* (Moustached Warbler) nominated birds from Austria differ genetically and morphologically from those of subspecies *mimicus* from Kazakhstan (L Alakol); further study needed. Subspecies *albiventris* sometimes included in mimicus, three subspecies are recognized [76, 92].

Acrocephalus agricola (Paddyfield Warbler) has been considered conspecific with one or both of *A. tangorum* and *A. concinens* (Blunt-winged Warbler); differs from each. Subspecies *capistratus* frequently merged with nominate, while initial discovery of substantial genetic difference (4.5%) between subspecies septimus and nominate [186]. is now considered erroneous, as more detailed DNA analysis found no evidence of molecular structure between these two; the species is perhaps best treated as monotypic [187, 188].Three subspecies recognized.

Acrocephalus concinens (Blunt-winged Warbler) closely related to *A. tangorum* and, to lesser extent, *A. agricola*; formerly treated as conspecific with latter. Subspecies *hokrae* (described from Hokra jheel, Kashmir) has been considered valid, but is also suggested to be a pale colour morph of subspecies *haringtoni* [188]. Three subspecies recognized. *Acrocephalus dumetorum* (Blyth's Reed Warbler) are probably closer to *A. palustris* than to *A. scirpaceus*; hybridization with former recorded in SE Finland and monotypic [76, 92].

Acrocephalus stentoreus (Clamorous Reed Warbler) considered conspecific with *A. australis*; populations in Indonesia from this taxa and Hybrids of this species and *A. arundinaceus* from S Kazakhstan [189]. Currently darker and larger birds from Levant, included as subspecies, and separated as *levantinus*. Four subspecies recognized [76, 92].

The phylogenetic analysis of these 11 species under study showed their monophalytic behavior. All of these species were clustered under one clade and were further grouped into small clades based on their similarity in the COI gene sequences. Both the species of genus Iduna (*Iduna caligata, Iduna rama*) had made one cluster while *Hippolais languida* being single species included from genus Hippolais was split separately.

This family Aegithalidae of Tits is reported with 4 species from Pakistan (avibase.bsc-eoc.org) [141]. COI gene sequence data of 1 species *Aegithalos concinnus* (Black-throated Tit) was available and downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. Results of our phylogentic analysis show that *Aegithalos concinnus* [76] has close association with *Acrocephalus concinens* and distinctly related to *Megalurus palustris* [76, 92].

This family Aegithinidae is comprised of 4 species globally, [169] but from Pakistan 2 species were reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence data of 1 species *Aegithina tiphia* (Common Iora) was available and downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes for this study. *Aegithina tiphia* has made a separate split from a clade of 3 species including *Terpsiphone paradisi paradise* and *Hypothymis azurea* therefore show close association with these species but has variation in sequence therefore not included in this clade [76, 92].

This family Alaudidae of larks and sparrows is comprised of 39 species globally, [164] but from Pakistan 18 species were reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of 5 species was processed in lab and COI gene sequence data of 5 species was available and downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. Current phylogenetic analysis show that all the

species are grouped in one big clade and further split into their groups on the bases of genus showing close association among species of one genus [76, 92].

Ammomanes deserti (Desert Lark) has shown four deep divergent lineages through a recent multilocus genetic study and has geographical variation [190]. Numerous subspecies are ranked on the bases of plumage coloration. Subspecies are widely distributed in Afghanistan and Pakistan too. A thorough taxonomic review is required for recognizing subspecies [164]. *Eremopterix nigriceps* (Black-crowned Sparrow-Lark) relationships was previously obscure, but recent genetic data show it closest to *E. griseus* [190]. *Eremopterix griseus* (Ashy-crowned Sparrow-Lark) apparently show close relation to *E. nigriceps* and *E. signatus* [190]. Birds of N India, Sri Lanka and S India are treated as monotypic. *Eremophila alpestris* (Horned Lark) many subspecies are described over vast range recent molecular studies suggest this taxa in Old World [191].

Calandrella brachydactyla (Greater Short-toed Lark) show close relation to *C. cinerea*, with which often merged, and to *C. blanfordi*, *C. eremica and C. acutirostris*; until recently considered conspecific with *C. dukhunensis*. *Melanocorypha bimaculata* (Bimaculated Lark) has dgeographic variation and monotypic. *Alaudala rufescens* (Lesser Short-toed Lark) was previously conspecific with *A. somalica*, and separately with *A. raytal*, but show considerable differences in their morphology, [192]. *Alauda arvensis* (Eurasian Skylark) has been considered conspecific with *A. gulgula* [164] and vice versa and are probably sisters [190]. *Galerida cristata* (Crested Lark) has extremely complex internal taxonomy having more than 60 subspecies but identified as two major clades. More research is required on the nomenclature of these taxa [9, 76, 164, 190].

The phylogenetic analysis of these 10 species under study showed their monophyletic behavior. All of these species were clustered under one clade and were further grouped into small clades based on their similarity in the COI gene sequences. Both the species of genus Eremopterix, *Eremopterix griseus* processed in Lab and *Eremopterix nigriceps* downloaded from NCBI grouped togather in single clade showing COI gene sequence similarity. On the other hand *Ammomanes deserti* being single species included from genus Ammomanes was split separately.

This family Bombycillidae is closly related to family Dulidae and is comprised of only 3 species under 1 genus Bombycilla globally [164] but from Pakistan only one species is reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of this species *Bombycilla garrulus* (Bohemian Waxwing) was downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. This species is closely related to *B. japonica* [193] with geographical variation and have only plumage differences [76, 92].

In phylogenetic analysis *Bombycilla garrulus* has made cluster with *Hypocolius ampelinus* the only sequence from family Hypocoliidae indicating close association among them.

This family Campephagidae of cuckoo shrikes is composed of two sub families with total 99 species globally [164] but from Pakistan only 8 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of one species *Pericrocotus cinnamomeus* (Small Minivet) collected from Pakistan was processed in lab for phylogenetic analysis of Passeriformes. Sister to *P. igneus* [194] sometimes considered conspecific, but little evidence of interbreeding where ranges meet in peninsular Thailand, and different habitat preferences may prevent direct contact [164]. *Pericrocotus cinnamomeus* being single species in analysis split separately [76, 92].

This family Certhiidae of Treecreepers is composed of one genus Certhia with total 9 species globally but from Pakistan only 2 species were reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of one species *Certhia hodgsoni* (Hodgson's Treecreeper) was downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. It was formerly treated as conspecific with *C. familiaris*, but showed difference genetically and vocally, showed significant genetic differences based on studies of cytochrome b [164]. During phylogenetic analysis *Certhia hodgsoni* was split alone as no other species from the same family was involved in this analysis but it has shown close association with *Cettia cetti* and *Chodroma muraria* [76, 92].

This family Cinclidae (Dipper) is composed of one genus Cinclus with total 5 species globally [164] but from Pakistan only 2 species are reported (avibase.bsc-eoc.org) [141]. COI gene sequence of these 2 species *Cinclus cinclus* (White-throated Dipper) *Cinclus pallasi* (Brown Dipper) was downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. *C. cinclus* is closest to *C. pallasii*. [164]. The evolutionary analysis in this study involved both species of this family which were grouped togather and showed close association with *Myophonus caeruleus* [76, 92].

This family Cisticolidae is comprised of 4 subfamilies with 161 species [164] but from Pakistan only 11 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 5 species *Orthotomus sutorius*, *Prinia buchanani*, *Prinia hodgsonii*, *Prinia socialis*, and *Cisticola juncidis* were collected from Pakistan and were sequenced in lab and COI gene sequence of 4 species *Prinia crinigera*, *Prinia gracilis*, *Prinia inornata* and *Prinia flaviventris* was downloaded from NCBI GenBank and total 9 species were used for phylogenetic analysis of Passeriformes. Nine subspecies are recognized [164]. Results of our analysis showed *Cisticola juncidis* as sister group of *Orthotomus sutorius* while all other species of genus Prinia were closely related to each other and grouped in one big clade. *Prinia socialis* assertion that Sri Lanka subspecies *brevicauda* differs vocally from others not strongly supported [195]. Four subspecies recognized. *Cisticola juncidis* sometimes considered conspecific with *C. haesitatus*, and close relationship with *C. cherina* also postulated. Considerable vocal variation across massive range and complex speculated to warrant treatment as at least three species [196]. Racial identity is uncertain of birds in S New Guinea [197] they show some characters of *laveryi*, but might belong to an as yet undescribed subspecies. Eighteen subspecies currently recognized [76, 92, 164].

The 19 species of family Corvidae are reported from Pakistan (https://avibase.bsc-eoc.org) [141]. *Corvus macrorhynchos* has shown close association with the COI sequence of *Galerida cristata arenicola* while rest of the species of family were clustered togather showing their monophyletic nature [70, 76, 92, 106, 107].

This family Dicaeidae of flower-peckers is comprised of 55 species globally [164] but from Pakistan only 2 species were reported [141]. COI gene sequence of 1 species *Dicaeum erythrorhynchos* (Pale-billed Flowerpecker was downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. Two subspecies recognized [164]. In our analysis this species has shown close association with species of genus Monticola of family Montacillidae [76, 92].

This family Dicruridae has one genus Dicrurus comprised of 26 species globally but from Pakistan only 4 species were reported (https://avibase.bsc-eoc.org) [141]. One species *Dicrurus leucophaeus* (Ashy Drongo) was sequences in lab whereas COI gene sequences of 2 species were downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. In our analysis species of this family have shown monophyletic behavior and all 3 species under analysis were grouped in single clade based on COI gene sequence similarity [76, 92].

This family Emberizidae of old world buntings has one genus Emberizia comprised of 44 species globally [164] but from Pakistan only 16 species were reported (https://avibase.bsc-eoc.org) [141]. Two species *Emberiza lathami* (Ashy Crested Bunting) and *Emberiza buchanani* (Gray-necked Bunting) were sequences in lab whereas COI gene sequence of 13 species sequences of family Emberizidae were downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. 12 species of this family were grouped togather in one big clade and showed its monophyletic nature but 3 species (*Emberiza lathami*, *Emberiza melanocephala, Emberiza bruniceps*) made a separate cluster and showed close association with the species of genus Leucosticte of family Fringillidae [69, 76, 198, 199].

This family Fringillidae consists of 3 subfamilies i-e Fringillinae, Euphoniinae, and Caruelinae with total 211 species globally [164] but from Pakistan only 30 species were reported (https://avibase.bsc-eoc.org) [141]. One species *Carpodacus erythrinus* (Common Rosefinch) was sequences in lab whereas COI gene sequences of 14 species were downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. All species of this family were grouped in one clade and shown close association [76, 92].

All the members of this family were grouped in one cluster but within cluster 3 species from gnus Carpodacus has shown more association with other members of other genera of the same family instead among themselves. This shows their non-monophylytic behavior *Carpodacus erythrinus* has shown close association with species of genus Fringilla (*Fringilla coelebs, Fringilla montifringilla*) of the same family but *Carpodacus thura* with *Eremopsaltria mongolica* and except *Carpodacus rubicilla* who was part of another cluster and shown association with C*ephalopyrus flammiceps*.

This family Hirundinidae consists of 89 species globally [164] but from Pakistan only 12 species were reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of 9 species *Riparia riparia* (Bank Swallow), *Ptyonoprogne rupestris* (Eurasian Crag-Martin),), *Riparia diluta* (Pale Sand Martin), *Ptyonoprogne fuligula* (Rock Martin), *Hirundo rustica* (Barn Swallow), *Cecropis daurica* (Red-rumped Swallow), *Hirundo smithii* (Wire-tailed Swallow), *Delichon urbicum* (Common House-Martin), *Delichon dasypus* (Asian House-Martin) were downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. All these 9 species of this family has close association with each other and were clustered into a single clade on the baisis of similarity in their COI gene sequence and also shown their monophyletic nature and sub clustered at genus level [76, 92].

This family Hypocoliidae consists of one genus Hypocolius with 1 species *Hypocolius ampelinus* (Hypocolius) globally [164] and same is reported from Pakistan (https://avibase.bsc-eoc.org) [141] and COI gene and phylogenetic analysis showed for Passeriformes that *Hypocolius ampelinus* has close association with *Bombycilla garrulus* [76, 92].

This family Laniidae of shrikes consists of 4 genera and 33 species globally [164] but from Pakistan only 9 species were reported from genus Lanius (https://avibase.bsc-eoc.org) [141]. Two species *Lanius vittatus* (Bay-backed Shrike) and *Lanius schach* (Long-tailed Shrike) were sequenced in lab whereas COI gene sequences of 6 were downloaded from NCBI GenBank for phylogenetic analysis of Passeriformes. During phylogenetic analysis we observed that all 8 species of genus Lanius were grouped together in one big clade and were further split into subclades based

on their respective genera on the bases of similarity in their COI gene sequences [76, 92].

This family Leiothrichidae of shrikes consists of 20 genera and 147 species globally globally [164] but from Pakistan only 14 species were reported (https://avibase.bsc-eoc.org) [141]. One species *Trochalopteron lineatum* (Streaked Laughingthrush) was sequences in lab whereas COI gene sequences of 5 species were available on NCBI GenBank for Passeriformes. Both the species under genus Trochalopteron were not sister species but showed association during evolutionary analysis and were clustered together but *Trochalopteron lineatum* have more association with *Turdoides malcolmi* from the same family rest of the sepecies were in same cluster but being single specimen from each genus split separated under same clade [76, 92].

This family Locustellidae of shrikes consists of 12 genera and 63 species globally [164] but from Pakistan only 4 species were reported (https://avibase.bsc-eoc.org) [141]. Two species *Megalurus palustris* (Striated Grassbird) and *Locustella naevia* (Common Grasshopper-Warbler) COI gene sequence were available on NCBI GenBank for Passeriformes analysis and both made one clade showing close association with each other being member of the same family [76, 92].

This family Monarchidae is comprised of 14 genera with 105 species globally [164] but from Pakistan only 2 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of 1 species *Terpsiphone paradisi* (Indian Paradise-Flycatcher) collected from Pakistan was sequenced in lab and COI gene sequence of 1 species *Hypothymis azurea* (Black-naped Monarch) was found on NCBI GenBank downloaded and used for analysis and both of these species showed close association and grouped in one clade [76, 92].

This family Motacillidae is comprised of 7 genera with 66 species globally [164] but from Pakistan only 18 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 2 species *Anthus richardi* (Richard's Pipit) and *Anthus trivialis* (Tree Pipit) collected from Pakistan were sequenced in lab but BOLD database did not assignes any BIN to them and at NCBI database sequences were not matched with

such species therefore were not included in this analysis and COI gene sequence of 11 species was found on NCBI GenBank retrieved and were used for phylogenetic analysis of Passeriformes. 11 species were under 2 genera Motacilla and Anthus and results of phylogenetic analysis showed clustering of 7 species of genus Anthus under one cluster and 4 species of genus Motacilla under another cluster showing their monophyletic nature [76, 92].

This family Muscicapidae is comprised of 4 subfamilies and 335 species globally [164] but from Pakistan only 57 species were reported (https://avibase.bsc-eoc.org) [141]. COI gene of 5 species *Copsychus fulicatus* (Indian Robin), *Copsychus saularis* (Oriental Magpie-Robin), *Myophonus caeruleus* (Blue Whistling-Thrush) *Phoenicurus ochruros* (Black Redstart) and *Oenanthe picata* (Variable Wheatear) collected from Pakistan were sequenced in lab and COI gene sequences of 32 species were downloaded from NCBI GenBank and used for phylogenetic analysis. Results showed close association among different species of one genus and they made different clades under genera [76, 92]. 5 species were grouped in one cluster from the same family *Hypsipetes leucocephalus* from other family. Members of genus muscicapa were clustered together with other members of genus Facedula of the same family. In the same way members of genus Phoenicurus, Monticola, Saxicola and Oenanthe were clustered togather in their respective clades based on their COI gene sequence similarity and showed their monophyletic nature [94].

This family Nectariniidae is comprised of 13 genera and 147 species globally [164] but from Pakistan only 2 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of these 1 species *Aethopyga siparaja* (Crimson Sunbird) was available NCBI GenBank downloaded and used in evolutionary analysis. It split separately but was showing close association with *Carpospiza brachydactyla* [76, 92].

This family Oriolidae is comprised of 4 subfamilies and 38 species globally [164] but from Pakistan only 5 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 1 species *Oriolus oriolus* (Eurasian Golden Oriole) collected from Pakistan was sequenced in lab and COI gene sequence of 1 species *Oriolus chinensis* (Black-naped Oriole) was found NCBI GenBank and downloaded and used to

find phylogenetic relationship with each other and other species. It was observed that both have close association with each other and made one clade [70][76, 92].

This family Paridae is comprised of 14 genera and 60 species globally [164] but from Pakistan only 10 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 1 species *Parus major* (Great Tit) collected from Pakistan was sequenced in lab and COI gene sequences of 6 species *Cephalopyrus flammiceps, Periparus ater Periparus rubidiventris, Lophophanes dichrous, Cyanistes cyanus Parus monticolus* were downloaded from NCBI GenBank and total 7 species were used for phylogenetic analysis of Passeriformes. All these species were grouped in one clade and further in sub clades genus wise except *Cephalopyrus flammiceps* has shown close association with *Carpodacus rubicilla* [76, 92].

This family Panuridae is comprised of 1 genus and 1 species *Acrocephalus concinens* (Bearded Reedling) globally [164] and same is reported from Pakistan (vibase.bsc-eoc.org) [141]. COI gene sequence of this species was downloaded from NCBI GenBank and used for phylogenetic analysis of Passeriformes. *Acrocephalus concinens* has shown close association with *Aegithalos concinnus* [76, 92].

Two species of this family Paradoxornithidae were reported from Pakistan which are vulnurable (https://avibase.bsc-eoc.org) [141]. COI gene of 1 species *Chrysomma sinense* (Yellow eyed Babbler) collected from Pakistan was sequenced in lab and used for phylogenetic analysis of Passeriformes. *Chrysomma sinense* was split separately as being one species present in analysis [76, 92].

This family Passeridae of old world sparrows is comprised of 8 genera and 43 species globally [164] but from Pakistan only 10 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 3 species *Passer hispaniolensis* (Spanish Sparrow), *Passer cinnamomeus* (Russet Sparrow), *Gymnornis xanthocollis* (Chestnut-shouldered Petronia) were collected from Pakistan and were sequenced in lab but BIN was not assigned to *Gymnornis xanthocollis* and match on the basis of similarity was not available at NCBI therefore was not included in analysis. COI gene sequence of 6 species was downloaded from NCBI GenBank and total 7 species were used for phylogenetic analysis of Passeriformes. They were all grouped in one big clade

and further grouped into small clades based on genus as Passer and other genera [76, 92, 95].

This family Pellorneidae is comprised of 14 genera and 64 species globally [164] but from Pakistan only 2 species are reported which are near threatened (avibase.bsc-eoc.org). COI gene sequences of 2 species *Laticilla burnesii* (Rufous-vented Prinia) and *Pellorneum ruficeps* (Puff-throated Babbler) were downloaded from NCBI GenBank and total 2 species were used for phylogenetic analysis of Passeriformes. *Laticilla burnesii* has shown close association with *Pellorneum ruficeps* during phylogenetic analysis being member of same family [76, 92].

This family Phylloscopidae is comprised of 1 genus Phylloscopus and 78 species globally [164] but from Pakistan only 17 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 2 species *Phylloscopus xanthoschistos* (Gray-hooded Warbler), *Phylloscopus affinis* (Tickell's Leaf Warbler) collected from Pakistan were sequenced in lab but no BIN as assigned to *Phylloscopus xanthoschistos* therefore was not included in analysis. The COI gene sequences of 6 species were downloaded from NCBI GenBank database and total 7 species were used for phylogenetic analysis of Passeriformes. All species made one big clade of these species during phylogenetic analysis and showed monophyletic behaviour [76, 92].

This family Pittidae is comprised of 48 species under genus Pittas globally [164] but from Pakistan only one species is reported (https://avibase.bsc-eoc.org) [141]. We downloaded COI gene sequence of this species *Pitta brachyura* (Indian Pitta) from NCBI GenBank for phylogenetic analysis of Passeriformes. Formerly this species was treated as conspecific with *P. nympha*, and with *P. elegans* and is monotypic [164]. *Pitta brachyura* in this study has split separately and made clade with out-group [76, 92].

This family Prunellidae is comprised of 1 genus Prunella and 12 species globally [164] but from Pakistan only 7 species are reported (https://avibase.bsc-eoc.org) [141]. We downloaded COI gene sequences of 4 species from NCBI GenBank based on their availability for phylogenetic analysis of Passeriformes. All species

were grouped into one clade based on COI gene sequence similarity and showed monophyletic nature [76, 92].

This family Pycnonotidae of bulbuls is comprised of 31 genera and 158 species globally [164] but from Pakistan only 5 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 2 species *Pycnonotus leucogenys* (Himalayan Bulbul), *Hypsipetes leucocephalus* (Black Bulbul), collected from Pakistan were sequenced in lab and COI gene sequence of 2 species *Pycnonotus cafer* (Red-vented Bulbul) and *Pycnonotus jocosus* (Red-whiskered Bulbul) was downloaded from NCBI database and total 4 species were used for phylogenetic analysis of Passeriformes. All 3 species under genus Pycnonotus were grouped together based on similarity in their COI gene sequences but *Hypsipetes leucocephalus* split separately and has shown association with members of genus Copsychus [76, 92].

This family Regulidae is comprised of 1 genus Regulus and 6 species globally [164] but from Pakistan only 1 species *Regulus regulus* (Goldcrest) is reported (https://avibase.bsc-eoc.org) [141]. COI gene of this species was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. It was split alone separately but has shown association with *Cisticola juncidis* [76, 92].

This family Remizidae is comprised of 3 genera and 12 species [164] but from Pakistan only 2 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 1 species *Remiz pendulinus* (Eurasian Penduline-Tit), was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. *Remiz pendulinus* was splited separately [76, 92].

This family Rhipiduridae is comprised of 3 genera and 65 species globally but from Pakistan only 2 species are reported. COI gene of 1 species *Rhipidura aureola* (White-browed Fantail) was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. It showed close association with *Tephrodornis pondicerianus* [76, 92].

This family Scotocercidae is comprised of 3 subfamilies 12 genera and 37 species

globally [164] but from Pakistan only 5 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 1 species *Cettia cetti* (Cetti's Warbler), was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. *Cettia cetti* has shown close association with *Chodroma muraria* being in one clade [76, 92].

This family Sittidae comprised of 3 subfamilies 3 genera and 32 species globally [164] but from Pakistan only 5 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 3 species *Sitta cashmirensis* (Kashmir Nuthatch), *Sitta tephronota* (Eastern Rock Nuthatch) and *Sitta frontalis* (Velvet-fronted Nuthatch) was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. Species of this family were grouped together into one clade showing monophyletic nature [76, 92].

This family Stenostiridae is comprised of 4 genera and 9 species globally [164] but from Pakistan only 2 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of 1 species *Culicicapa ceylonensis* was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. *Culicicapa ceylonensis* was splited separatly [76].

This family Sturnidae is comprised of 3 subfamilies of 32 genera and 123 species [164] but from Pakistan only 10 species were reported (https://avibase.bsc-eoc.org) [141]. COI gene of 3 species *Sturnus vulgaris*, *Pastor roseus*, *Acridotheres ginginianus* collected from Pakistan were sequenced in lab. *Sturnus vulgaris* was not identified by NCBI database and BOD database has not assigned any BIN therefore it was excluded from analysis. The COI gene sequence of 5 species was downloaded from NCBI database and total 7 species were used for phylogenetic analysis of Passeriformes. *Pastor roseus* was split separately but showed close association with species of genus Sturnus and all these grouped in one cluster [76].

This family Sylviidae is comprised of 17 genera and 67 species [164] but from Pakistan only 9 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene of 1 species was collected from Pakistan and was sequenced in lab. COI gene sequences of 5 species were downloaded from NCBI database and were used for

phylogenetic analysis of Passeriformes. All 6 species were grouped together in one big clade showing monophyletic nature [76].

One species *Tichodroma muraria* (Wallcreeper) of Tichodromidae family is reported from Pakistan (https://avibase.bsc-eoc.org) [141]. COI gene sequence of this species was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. It was splitted separately but showed association with *Cettia cetti* [76].

This family Timaliidae is comprised of 8 genera and 54 species globally [164] but from Pakistan only 2 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequences of these two species *Cyanoderma pyrrhops* (Black-chinned Babbler), *Pomatorhinus erythrogenys* (Rusty-cheeked Scimitar-Babbler) were downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. *Cyanoderma pyrrhops* clustered with *Pomatorhinus erythrogenys* being member of same family during this analysis and shows similarity in COI gene sequences. Stachyridopsis is synonym of Cyanoderma [76].

This family Troglodytidae is comprised of 19 genera and 93 species globally [164] but from Pakistan only 1 species *Troglodytes troglodytes* (Eurasian Wren) is reported (https://avibase.bsc-eoc.org) [141]. COI gene of species collected from Pakistan was sequenced in lab used for phylogenetic analysis of Passeriformes. It splitted separately but showed a close association with *Prunella atrogularis* [76].

This family Turdidae is comprised 2 subfamilies 20 genera and 176 species globally [164] but from Pakistan only 15 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of 5 species was downloaded from NCBI database were used for phylogenetic analysis of Passeriformes and all 5 were grouped in one clade but further split into smaller clades based on similarity in the COI gene sequences in their respective genus [76].

This family Turdidae is comprised of 2 subfamilies, 20 genera and 176 species globally [164] but from Pakistan only 15 species are reported (https://avibase.bsc-eoc.org) [141]. COI gene sequence of 5 species was downloaded from NCBI database and used for phylogenetic analysis of Passeriformes. All 5 were grouped in one

clade but further split into smaller clades based on similarity in the COI gene sequences in their respective genus [76].

This family Vangidae is comprised of 2 subfamilies, 21 genera and 40 species [164]. From Pakistan only 1 species Tephrodornis pondicerianus is reported [141]. COI gene of it was sequenced in lab and used for phylogenetic analysis of Passeriformes and it has shown close association with *Rhipidura aureola* [76].

This family Vangidae is comprised 2 subfamilies 21 genera and 40 species [164] but from Pakistan only 1 species *Tephrodornis pondicerianus* are reported (avibase.bsc-eoc.org) [141]. COI gene of it was sequenced in lab and used for phylogenetic analysis of Passeriformes and it has shown close association with *Rhipidura aureola* [76].

This family Zosteropidae is comprised 10 genera and 57 species globally [164] but from Pakistan only 1 species *Zosterops palpebrosus* was reported (avibase.bsc-eoc.org) [141] its COI gene sequence was processed in lab and used in phylogenetic analysis. During phylogentic analysis it showed close association with *Sylvia nana* [76, 111].

Chapter 5

Conclusion and Future Recommendations

5.1 Conclusion

Passeriformes is a morphologically diverse, species rich and largest (60%) order of the class Aves (birds). It is represented in all terrestrial habitats, distributed all over the world except Antarctica and generally well identified on morphological and molecular bases globally. Species characterization and phylogenetic analysis using DNA barcodes based on COI gene (cytochrome c oxidase subunit I) of mitochondrion was aimed for birds sampled from different area of Pakistan.

The first objective of the study was molecular characterization of species of Passeriformes. First of all collected samples of birds were morphologically identified referencing the T. J. Roberts [15] as 49 species from 39 genera and 18 familis of order Passeriformes. Then after successful extraction of mitochondrial DNA from keel tissues folmer region comprising 650 bps of CO1 gene were amplified using universal primers. The PCR products were then confirmed using 1% agarose gel electrophoresis.

BLAST analyzed of sample sequences were performed using NCBI and BOLD database. Comparison of classical and molecular taxonomy of samples of Passeriformes of Pakistan at genus, family and species level was inferred by referencing the literature by Roberts (1992) for classical taxonomy and BOLD (Barcode of Life Data System) databases for molecular taxonomy.

1. Samples were morphologically identified referencing the T. J. Roberts [15] as 49 species from 39 genera and 18 family of order Passeriformes Total 43 out of 49 sequences were identified as species of order Passeriformes based on the best matches by comparing their similarity percentage with other sequences in the database.

2. Out of 43 species sequences, 32 were ranging from 97-100 % identity with their respective species. Species sequences of 10 (*Hypsipetes leucocephalus*, *Urocissa flavirostris flavirostris, Phylloscopus affinis, Lanius vittatus Valenciennes, Stachyrdopsis pyrrhops, Emberiza lathami, Prinia hodgsonii, Copsychus fulicatus, Prinia buchanani, Myophonus caeruleus, Alauda gulgula australis*) were showing similarity < 97 % but with other species of same genus. Further verification at molecular level was performed using the Barcode of Life Data System (BOLD).

3. BINs were assigned to 43 sequences representing 42 BINs. Single BIN was assigned to 2 species (*Prinia buchanani Blyth, Prinia hodgsonii Blyth*, BOLD: ACZ2474). Out of these 42 BINs 32 records were taxonomically concordant. Rest of 11 (*Lanius vittatus, Copsychus fulicatus, Oriolus oriolus, Parus major, Stachyridopsis pyrrhops, Hypsipetes leucocephalus, Urocissa flavirostris flavirostris, Alauda gulgula australi, Prinia buchanani Blyth, Prinia hodgsonii Blyth, Cisticola juncidis*) records out of 42 showing unique BINs, as single new record of respective species Passeriformes of Pakistan and have not been submitted from another region of the world in the BOLD database. The comparison revealed changes at different taxonomic levels of 17 species by our study.

The second objective of the study was to compare the genetic diversity and to establish evolutionary relationship based on COI gene of the different species of Passeriformes from Pakistan and the world. Sequence analysis was performed to establish evolutionary relationship of study data and worldwide species of Passeriformes were retrieved from AVIBASE database using MEGAX. The worldwide COI gene sequence data of 192 species of order Passeriformes were retrieved from GenBank (NCBI) database and phylogenetic relationship was established of 236 barcodes including 43 sequences barcodes of Passeriformes along with out-group.

- Sequence analysis revealed variation in frequencies of each nucleotide (ATGC) in the barcode region of Passeriformes species of Pakistan at the three codon positions, 1^{st}, 2^{nd}, and 3^{rd}. K2-parameter distances indicates that that average evolutionary divergence among species of Passeriformes was 0.18 the least average evolutionary divergence was 0.019 among Lanius isabellinus and Lanius collurio in the same way this average evolutionary divergence among other species of genus Lanius was very low as 0.022 among *Lanius tephronotus* and *Lanius cristatus* the highest was 0.263 among *Rhodospiza obsoleta* and *Ptyonoprogne rupestris* and among *Rhodospiza obsoleta* and *Cercotrichas galactotes*.

- Evolutionary analyses showed that the differences in COI sequences were usually higher among the species under family as compared to species under genus. Intraspecific hyplotypes were clustered under monophyletic clades with bootstrap support in the NJ tree. The overall phylogeny of Passeriformes remained same as previous studies, forming similar split ups and clades. All 235 species of Passeriformes were first split into two clades. One comprising of the 5 species *Panurus biarmicus, Aegithalos concinnus, Megalurus palustris, Locustella naevia* and rest of the specises in other clade which were further splited into sub clusters bases on the similarity and differences in theier COI gene sequences. Species under one genus and species under one family were grouped together in each clade showing the similarity in their COI gene sequences and showing their monophyletic nature.

- DNA barcoding was proved to be an effective tool for species molecular identification and their phylogenetic analysis during this study which may help in identification and biogeographic studies of the birds in future.

5.2 Recommendations

DNA barcodes were proved as tool for genetic identification of different specimens of Passeriformes and for the contribution in generating a sequence library high quality data is required to answer different biological questions. The COI sequences of birds submitted in BOLD library have many applications they provide information about genetic, and biodiversity from all over the world. This information can help to device different conservation strategies for endangered and threatened species. The DNA barcodes of birds provide opportunity to answer questions related to avian evolution along with primary purpose of characterization of avian species. The following recommendations are proposed for future studies to get complete picture of avian biodiversity of Pakistan and the evolutionary relationship among themselves.

- Multiple numbers of the same specimens should be included in future studies to compute the interspecific genetic variation among species of passeriformes.

- Extensive collection at different time period of year and season within Pakistan should be conducted to study the maximum families and genera of order Passeriformes. It will help include unavailable species due to their migratory behavior and will help find complete picture of biodiversity and evolutionary relationship among species of Passeriformes from Pakistan.

- Only species level studies of Aves have been conducted in Pakistan so far. There is a need to cover other orders of class Aves form Pakistan to find the genetic diversity within the class and to establish evolutionary relationship. This can provide better understanding of genetic changes accumulated in Class of Aves of Pakistan and worldwide.

- Genetic markers other than COI, such as COII, COII and whole genome sequencing should be performed to have complete picture of genetic diversity of Passeriformes of Pakistan.

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Appendix A

TABLE 1: (a) Morphometric Data of study specimens used for morphological characterization

Species Names	**Weight** (grams)	**Body Color/Length** (cm)	**Wing Color/Length** (cm)	**Tail Color/Length** (cm)	**Bill Color/Length** (mm)	**Eye Color (Iris)**	**Legs & Feet Color**	**Nape** Color
Eurasian or Common Skylark	0.14-0.15	Pale sandy yellow 16.8-20	Blackish Brown M: 11.4-11.6 F: 9.8-10.6	Dark brown with creamy margins 6.5-7.6	Pinkish brown 15-16cm	Dark brown	Fleshy brown	Blackish Brown
Crested Lark	0.27-0.28	Pale Sandy with greyish black streaks on crown and back 17-18	Pale white M: 10.3-10.6 F: 9.5-9.8	Greyish Black 5.2.-6.3	Greyish white or horny brown 20-22	Yellowish brown	Fleshy brown	Grey

Oriental Skylark		Brown 16-16.5cm	8.5-10cm	4.9-6.8mm white	14-16mm Horney brown	Hazal brown	Fleshy brown	Grey
Fan- tailed warbler	6-8 g	Roufus brown 10cm	Blackish brown 4.5-5.5cm	Blackish brown 3.6-4.7cm	Dark brown	Hazal brown	Fleshy brown	black
Bimaculated Lark	0.907-0.964	white 16-19	Creamy with dark brown tips 11.6-12.6	Dark brown 5-6.1	Horney yellow with blackish tip 17-18	Brown	Yellowish Brown	
Ashy-crowned Sparrow-lark	0.60-0.69	Pale ashy grey 13-13.5	Greyish brown 7.2-8	Dark blackish brown 3.7-4.5	Black 1-1.3cm	Hazel to reddish brown	Fleshy brown	Greyish brown
Greater Short-toed Lark	19-25	Greyish brown 15-16.5	Blackish Brown M: 9.4-10.1 F: 9.8-10.6	Blackish Brown M: 5.4-6.5 F: 5.3-5.7	horny brown 37-42	Brown	Fleshy brown	Blackish Brown
Rufous-fronted Prinia	5-Sep	Mousy grey brown 12	Mousy grey brown .1-5.5	Dark Rufous Brown 5.3-6.0	Brown Dec-14	Orange brown	Pale Fleshy Brown	Mousy grey Brown
Ashy Prinia	6-7.5	Dark Grey 12-13	Rufous Brown with pale tips 4.7-5.8	Rufous Brown 5-5.4	Black 13-15	Hazel brown to yellow brown	Fleshy Brown	Grey
Grey-breasted Prinia	5-Jul	Grey Brown 11-13	Chestnut brown	Chestnut brown with blackish grey spots	Dark brown	Crimson	Fleshy Brown	Brownish

			4-May	307-5.0	13-14			
Rufous Tree pie	141-146	Dark sooty brown 36.5-45	Black 13.7-15.9	Dark grey 19.3-25.7	Slaty blue 32-37	Orange brown	Dark brown	Slaty grey
House Crow	266-280	Grey 43	Jet Black 25-275	Black 17	Black 51-56	Dark brown	Black	Grey
Common Raven	907-964	Black M:60-62	Purple, steel blue lights 40-44	White 24	Black 64-75	Dark brown	Shiny Black	Brownish
Carrion Crow	600-690	Black 50	Glossy Black 33-35	Dark brown 19	Black 58-60	Dark brown	Black	Black
Yellow-billed Blue Magpie	130-165	Pale lemon yellow 63-65	Slaty purplish 17.8-19	Blue 44-47	Paler orange yellow 37-42		Bright orange yellow	White
Common Woodshrike	141-146	Dark sooty brown 36.5-45	Black 13.7-15.9	Dark grey 19.3-25.7	Slaty blue 32-37	Orange brown	Dark brown	Slaty grey
Small Minivet	141-146	Dark blue - grey 15	Orange patches 6.4-7.0	Pale orange 6.4-76	Black 1.1-1.4cm	Dark brown	Black	Grey

Ashy Drongo	32-45	Glossy Black with lighter grey lower belly 25-26	Glossy Black 12.4-14.5	Dark slaty grey blue 12.7-17	Black 25-28	Red blood or crimson	Black	Dark slaty
Grey-necked Bunting	18-26	Pale creamy white with grey neck and roufus chest nut under parts 14.5	Black 8.5-9.1	Blackish with pale chestnut margins 7.5-7.9	Reddish brown 13.8-14.5	Dark brown	Yellowish brown	Grey brown
Crested Bunting	23.5-26	Steely blue black 16.5	Dull chestnut brown 7.9-8.8	Dull chestnut brown 6.7-7.1	Black with paler tints 11-Dec	Dark brown	Fleshy brown	Grey brown
Common Rosefinch	20-22	Pinkish Rosy 14.5	Dark grey brown ith paler grey margins 8.3=8.6	Dark grey brown ith paler grey margins 5.5-6	Browning horn-ing 9-Oct	Dark brown	Dark brown	Scarlet red

Bay-backed Shrike	0.27-0.28	Whitish	Black	Blackish in centre with whitish tips	Black	Dark brown	Black	Grey
		18-19	8.2-9.0	8.1-9.6	1.5-1.8 cm			
Long-tailed Shrike	0.90-0.96	Black	Blackish brown	Pale orange chestnut	Black	Dark brown	Black	Pale grey
		24-26	9.1-9.7	10.7-12.3	1.9-2.3			
Indian Robin	141-146	Dark sooty brown	Black	Dark grey	Slaty blue	Orange brown	Dark brown	Slaty grey
		36.5-45	13.7-15.9	19.3-25.7	32-37			
Black Redstart	266-280	Grey	Jet Black	Black	Black	Dark brown	Black	Grey
		43	25-275	17	51-56			
Oriental Magpie-robin	907-964	Black	Purple, steel blue lights	White	Black	Dark brown	Shiny Black	Brownish
		M:60-62	40-44	24	64-75			
Variable Wheatear	600-690	Black	Glossy Black	Dark brown	Black	Dark brown	Black	Black
		50	33-35	19	58-60			
Indian Paradise-flycatcher	18-22	White	black streaks	Blue	Bright cobalt blue	Dark brown	Bluish grey	Metallic blue black
		20-22	8.9-9.9	10-11.5	22-26			

Richard's Pipit	0.14-0.15	Pale Greyish 15	Dark Grey Brown 7.5-8.6	Dark brown 4.9-5.8	Horny brown 1.5-1.7cm	Brown	Pinkish yellow	Pale Greyish
Tree Pipit	0.25-0.28	Olive Brown 15-15.5	Creamy 8.3-9.4	Blackish Brown 5.4-6.6	Dark brown 1.4-1.6cm	Brown	Fleshy brown	Cream yellow
Eurasian Golden Oriole	65-70	Golden yellow 23-26	Black 14.1-14.3	yellow 8.5-9.9	Dull Crimson/ pinkish 29-33	Crimson	Bluish grey	Brownish
Spanish Sparrow	20-28	Dark liver chestnut 16	Dark brown 7.3-8.7	Dark brown 5.5-5.9	Horney brown Dec-14	Dark brown	Horney brown	Olive grey
Chestnut-Shouldered-Bush Sparrow	15-20	Grey brown 15	Bright chestnut shoulder patch 7.8-8.3	Dark greyish brown 4.9-5.4	Black Dec-13	Dark brown	Grey	Grey
Russet Sparrow	21	Bright cinnamon chestnut 13.5-15	Blackish brown 7-7.5	Blackish brown 4.6-5.5	Jet Black 10-Nov	Reddish brown	Fleshy brown	Bright cinnamon chestnut

Common Woodshrike	141-146	Dark brown 36.5-45	sooty	Black 13.7-15.9	Dark grey 19.3-25.7	Slaty blue 32-37	Orange brown	Dark brown	Slaty grey
Great Tit	266-280	Grey 43		Jet Black 25-275	Black 17	Black 51-56	Dark brown	Black	Grey
Himalayan bulbul	907-964	Black M:60-62		Purple, steel blue lights 40-44	White 24	Black 64-75	Dark brown	Shiny Black	Brownish
Black bulbul	600-690	Black 50		Glossy Black 33-35	Dark brown 19	Black 58-60	Dark brown	Black	Black
Tickell's Leaf-Warbler	141-146	Dark brown 36.5-45	sooty	Black 13.7-15.9	Dark grey 19.3-25.7	Slaty blue 32-37	Orange brown	Dark brown	Slaty grey
Lasser Whitethroat	266-280	Grey 43		Jet Black 25-275	Black 17	Black 51-56	Dark brown	Black	Grey
Grey hooded warbler	907-964	Black M:60-62		Purple, steel blue lights 40-44	White 24	Black 64-75	Dark brown	Shiny Black	Brownish

Common Tailor-bird	600-690	Black	Glossy Black	Dark brown	Black	Dark brown	Black	Black
		50	33-35	19	58-60			
Bank Myna	64-76	Slaty grey	Black	Black	Yellow/ orange yellow	Dark red	Paler yellow	Black
		22.8	11.9-12.5	6.8-7.3	18-21			
Rosy Starling	53-73	Pale brownish pink	Glossy Black	Glossy Black	Brown	Dark brown	Yellowish brown/red	Black
		22.8	12.4-13.5	6.5 – 7.5	21-23			
Common starling	65-72	Black with metallic purple green tints	Purple, steel blue lights	Black with metallic purple green tints	Greenish brown	Dark brown	Reddish brown	Purple
		19	10.8-11.8	5-Jun	25			
Bank Myna	64-76	Slaty grey	Black	Black	Yellow/ orange yellow	Dark red	Paler yellow	Black
		22.8	11.9-12.5	6.8-7.3	18-21			
Rosy Starling	53-73	Pale brownish pink	Glossy Black	Glossy Black	Brown	Dark brown	Yellowish brown/red	Black
		22.8	12.4-13.5	6.5 – 7.5	21-23			

Common starling	65-72	Black with metallic purple green tints	Purple, steel blue lights	Black with metallic purple green tints	Greenish brown	Dark brown	Reddish brown	Purple
		19	10.8-11.8	5-Jun	25			
Yellow -eyed Babbler	Dec-20	Chestnut brown	Chestnut brown	Brown	Black	Orange yellow	Fleshy yellow	Brown
		17-18	6.5-7					
				8.5-9	14.5-15			
Streaked Laughing thrush	38-46	Greyish Brown	Rufous Brown	Brown	Dusky brown	Reddish brown	Fleshy Brown	Greyish Brown
		20-21	7.4-7.7		18			
				9				
Blach-chinned babbler	10-11.5	Olive Brown	Olive Brown	Paler	Dull horny brown	Crimson	Pale brownish	Olive Brown
		12	5-5.3	5.5	15-16			
Blue Whistling-Thrush	141-146	Dark sooty brown	Black	Dark grey	Slaty blue	Orange brown	Dark brown	Slaty grey
		36.5-45						
			13.7-15.9	19.3-25.7				
					32-37			
Oriental White - eye	7.5-10.1	Bright golden greenish yellow	Dark brown	Dark brown ith olive green margins	Black	Yellow brown /hazal	Dark blue grey	Bright yellow
		10	5.5-5.7	3.3-3.7	9-Oct			

(a) *Alauda arvensis intermedia* Eurasian Skylark

(b) *Galerida cristata arenicola* Crested Lark

(c) *Melanocorypha bimaculata* Bimaculated Lark

(d) *Eremopterix griseus* Ashy-crowned Sparrow-lark

(e) *Calandrella brachydactyla* Greater Short-toed Lark

(f) *Prinia buchanani Blyth* Rufous-fronted Prinia

(g) *Prinia socialis socialis* Ashy Prinia

(h) *Prinia hodgsonii Blyth* Grey-breasted Prinia

(i) *Dendrocitta vagabunda saturatior* Rufous Tree pie

(j) *Corvus splendens splendens* House Crow

(a) *Corvus corax corax* Common Raven

(b) *Corvus corone* Carrion Crow

(c) *Urocissa flavirostris flavirostris* Yellow-billed Blue Magpie

(d) *Pericrocotus cinnamomeus* Small Minivet

(e) *Dicrurus leucophaeus* Ashy Drongo

(f) *Emberiza buchanani Blyth* Grey-necked Bunting

(g) *Emberiza lathami* Crested Bunting

(h) *Carpodacus erythrinus* Common Rosefinch

(i) *Lanius vittatus Valenciennes* Bay-backed Shrike

(j) *Lanius schach schach* Long-tailed Shrike

(a) *Saxicoloides fulicatus* Indian Robin

(b) *Phoenicurus ochruros* Black Redstart

(c) *Copsychus saularis saularis* Oriental Magpie-robin

(d) *Oenanthe picata* Variable Wheatear

(e) *Terpsiphone paradisi paradise* Indian Paradise-flycatcher

(f) *Anthus richardi* Richard's Pipit

(g) *Anthus trivialis* Tree Pipit

(h) *Oriolus oriolus* Eurasian Golden Oriole

(i) *Passer hispaniolensis* Spannish Sparrow

(j) *Petronia xanthocollis xanthocollis* Chestnut Shouldered-Bush Sparrow

(a) *Passer rutilans rutilans* Russet Sparrow

(b) *Tephrodornis pondicerianus* Common Woodshrike

(c) *Parus major* Great Tit

(d) *Pycnonotus leucogenys* Himalayan bulbul

(e) *Hypsipetes leucocephalus* Black bulbul

(f) *Phylloscopus affinis* Tickell's Leaf-Warbler

(g) *Sylvia curruca curruca* Lasser Whitethroat

(h) *Seicercus xanthoschistos* Grey hooded warbler

(i) *Orthotomus sutorius* Common Tailorbird

(j) *Acridotheres ginginianus* Bank Myna

(a) *Sturnus roseus* Rosy Starling

(b) *Sturnus vulgaris* Common starling

(c) *Chrysomma sinense* Yellow -eyed Babbler

(d) *Garrulax lineatus lineatus* Streaked Laughingthrush

(e) *Stachyris pyrrhops Blyth* Blach-chinned babbler

(f) *Myophonus caeruleus* Blue Whistling-Thrush

(g) *Zosterops palpebrosus palpebrosus* Oriental White eye

(h) *Alauda gulgula australis* Oriental Skylark

(i) *Cisticola juncidis* Streaked fantail warbler

FIGURE 1: Images of study specimens used for Morphological characterization

Appendix B

Lab Mixtures

1. **70% Ethanol**

   Ethanol 70ml

   Distilled water 30ml

   Final volume was made up to 100ml

2. **PBS Buffer**

   Use one tablet of PBS in 100ml of water

   Adjust volume to I L with addition of distilled water

3. **Proteinase-K (10mg/mL)**

   To prepare proteinase-K, 0.01g of proteinase-K was dissolved in 1ml distilled water by inverting eppendorf tube. Now solution was stored at -20^oC.

4. **10% (W/V) Sodium Dodecyl Sulphate (SDS)**

   Ten grams of sodium dodecyl sulphate was dissolved completely in 80 ml of distilled water by continuous stirring and final volume was made up to 100ml. The solution was stored at room temperature

5. **Chloroform/Isoamyl Alcohol Solution (24:1)**

   Chloroform 96ml

   Isoamyl alcohol 4ml

   Final volume was made up to 100ml. Mix them together to get of solution

6. **TE Buffer**

   Tris-HCI 10mM

EDTA 1mM

Tris-HCI (121 g) was added in about 800ml of deionized water and dissolved completely by continuous stirring. Then final volume was made up to 1000ml and the pH of the solution was adjusted at 8.2. Tris10mM was prepared from 1M Tris stock.

7. **5X Tris Borate EDTA (TBE) Buffer**

   Tris Base 27 g

   Boric Acid 13.75g

   EDTA 18.5g

   All the chemicals were dissolved and final volume made up to 1 liter. pH of solution was adjust at 8.

8. **Ethidium Bromide**

   Ethidium bromide 1g

   Distilled water 100ml

   Dissolved by stirring and stored at room temperature.

9. **6X Bromophenol Blue**

   Bromophenol blue 25mg

   Sucrose 4g

   Dissolved in 10ml of distilled water by stirring and stored at 4 ^oC

10. **1% Agarose Gel**

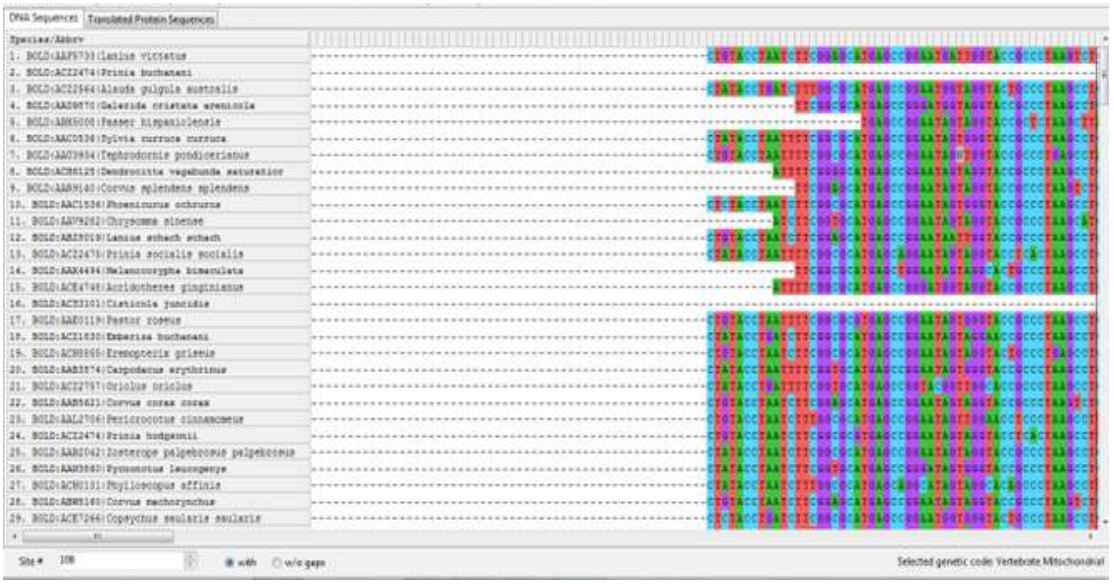
    Agarose gel 1g

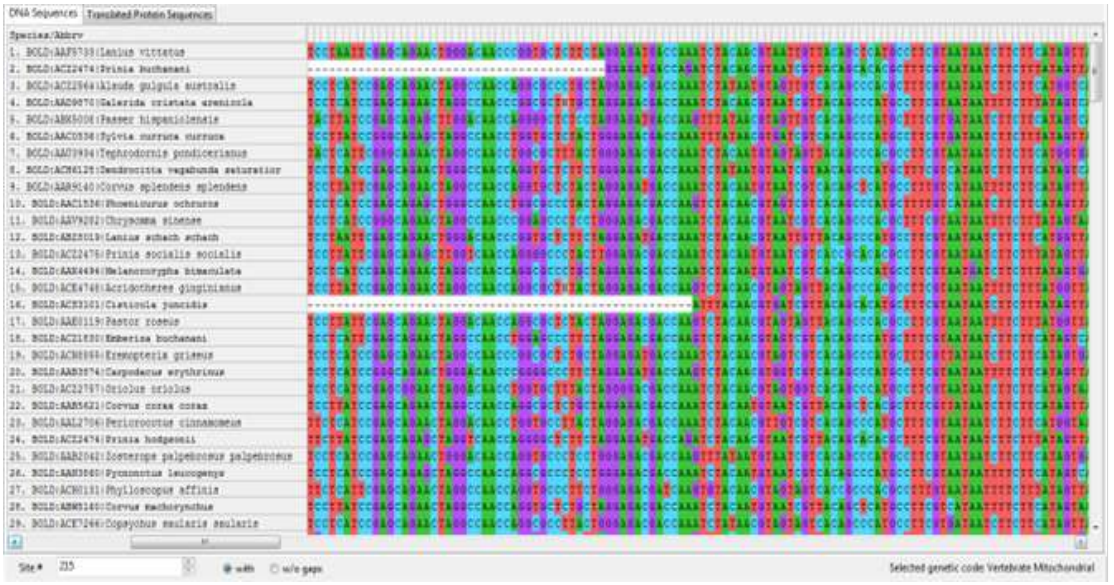
    TBE buffer (0.5X) 100ML

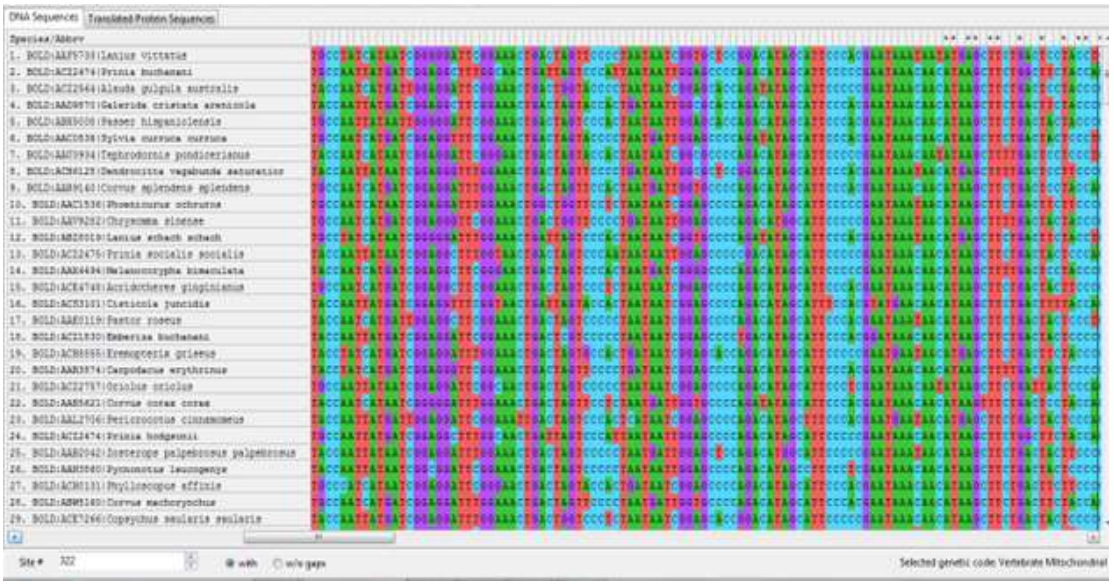
    One gram of pure agarose was dissolved in 100ml of 0.5X TBE. The mixture Was boiled and then poured into the casting tray and allowed to cool at room temperature.

Appendix C

Nucleotide Sequence Alignment of COI gene in 235 species of Passeriformes using ClastalW in MEGAX program.



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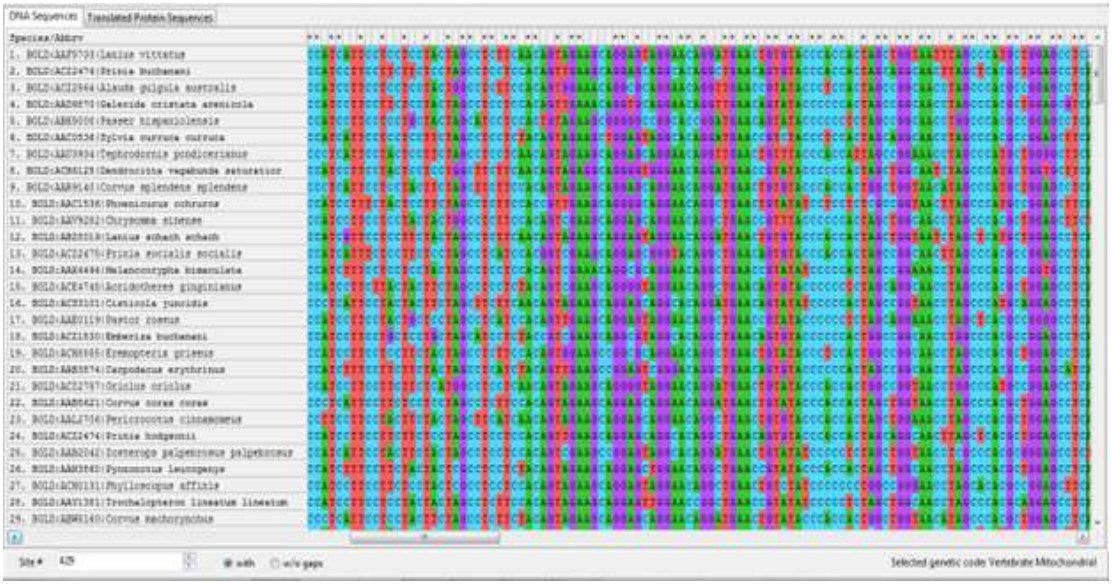
Translated Protein Sequences
Species/Abbrv
Site # 322
with
w/o gaps
Selected genetic code: Vertebrate Mitochondrial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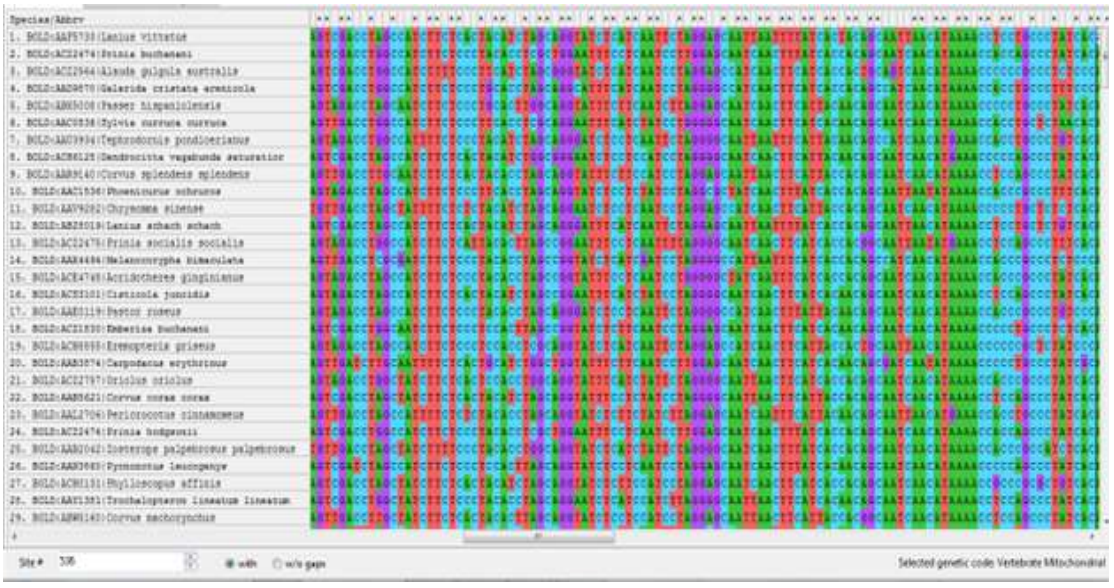
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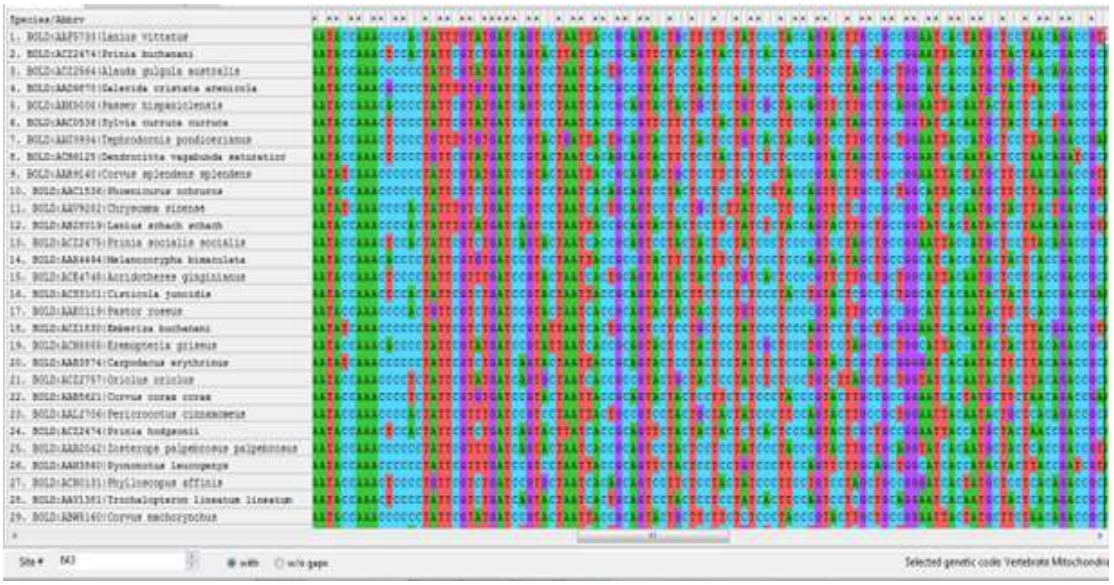

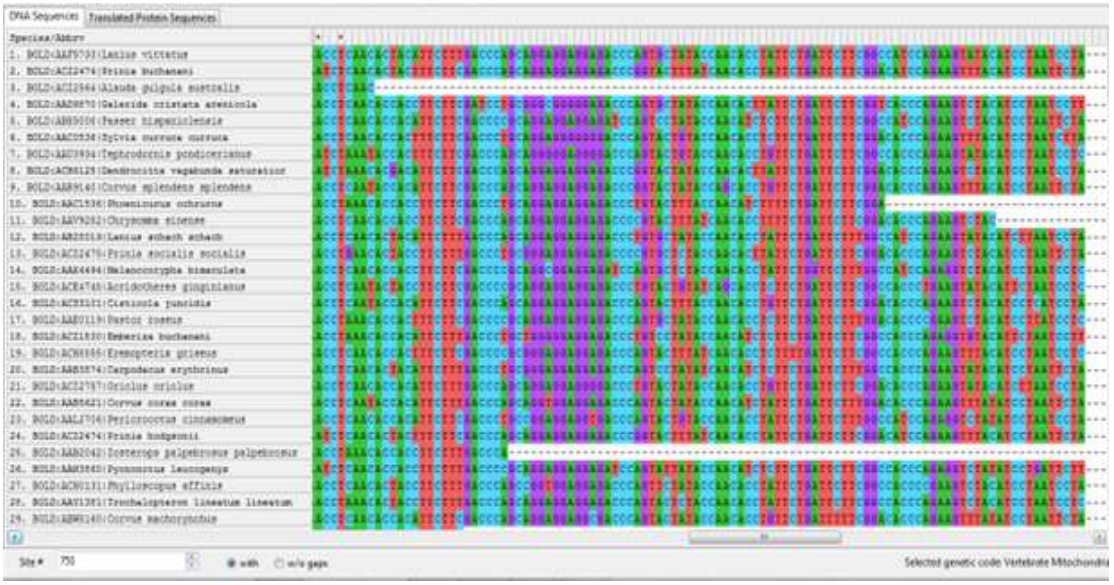

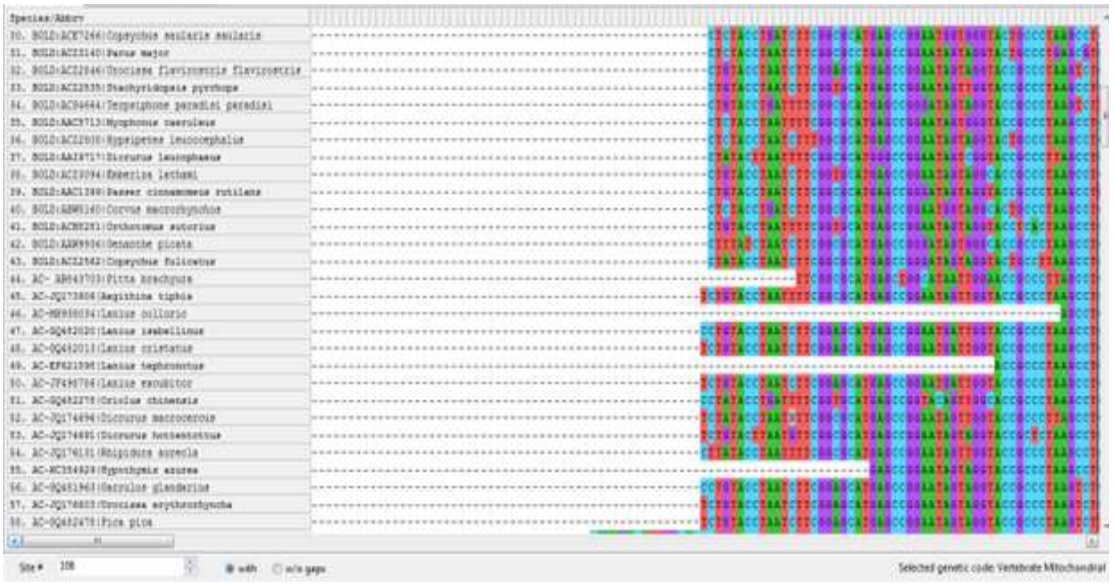

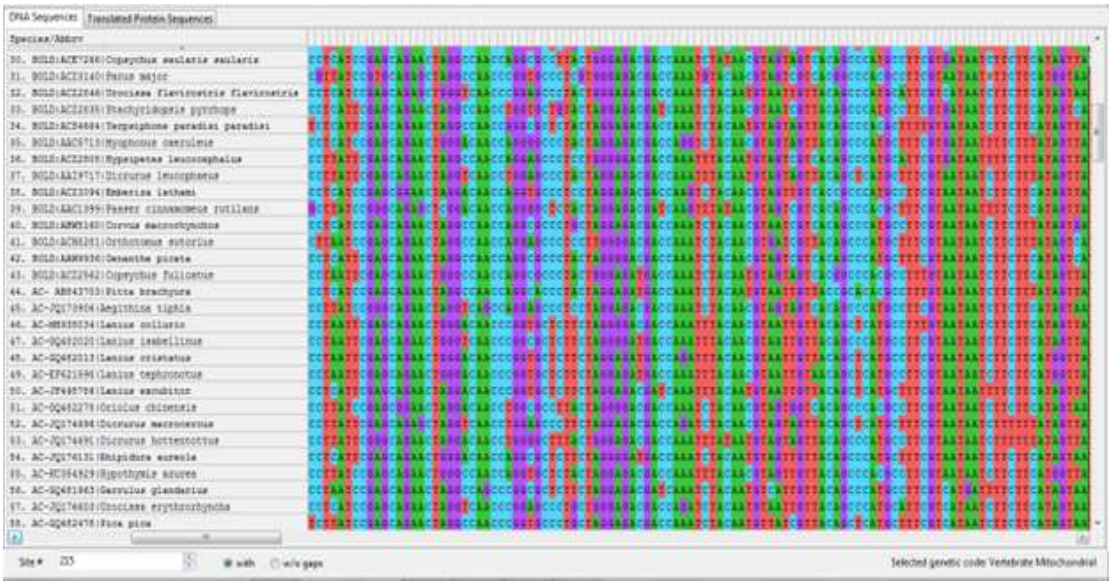

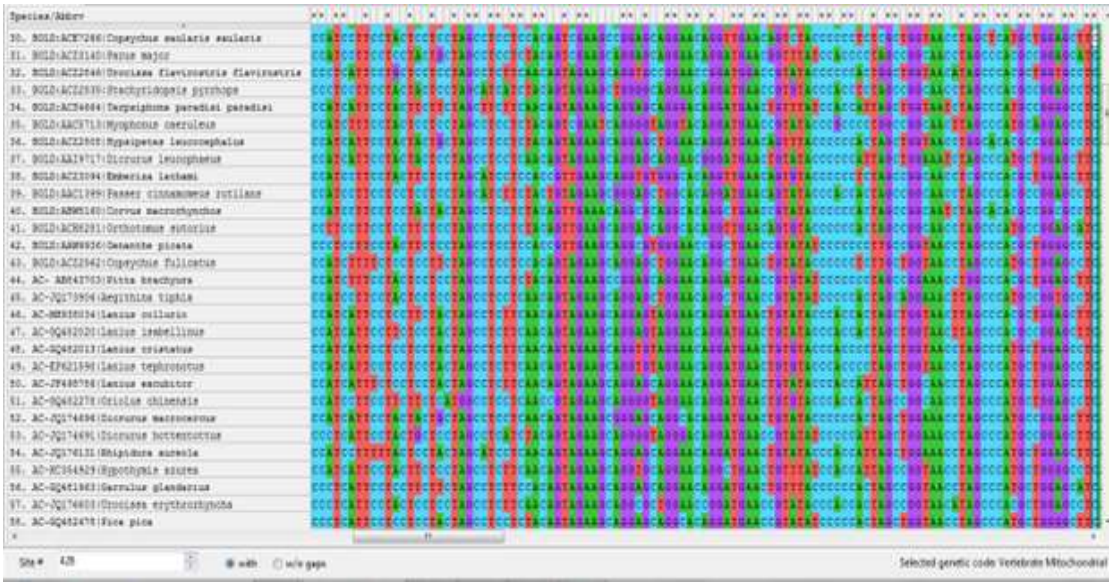

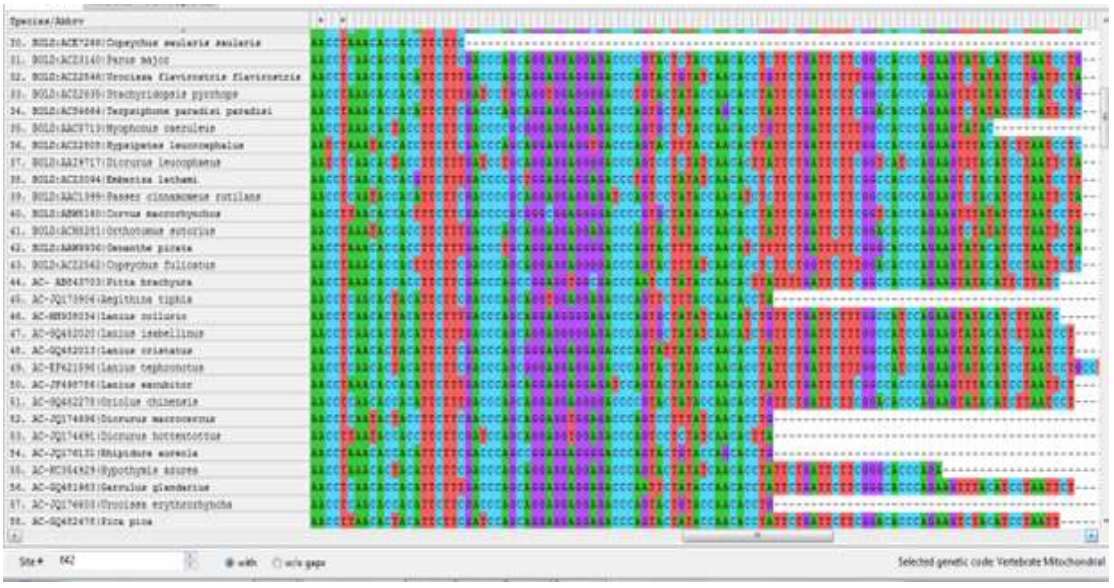

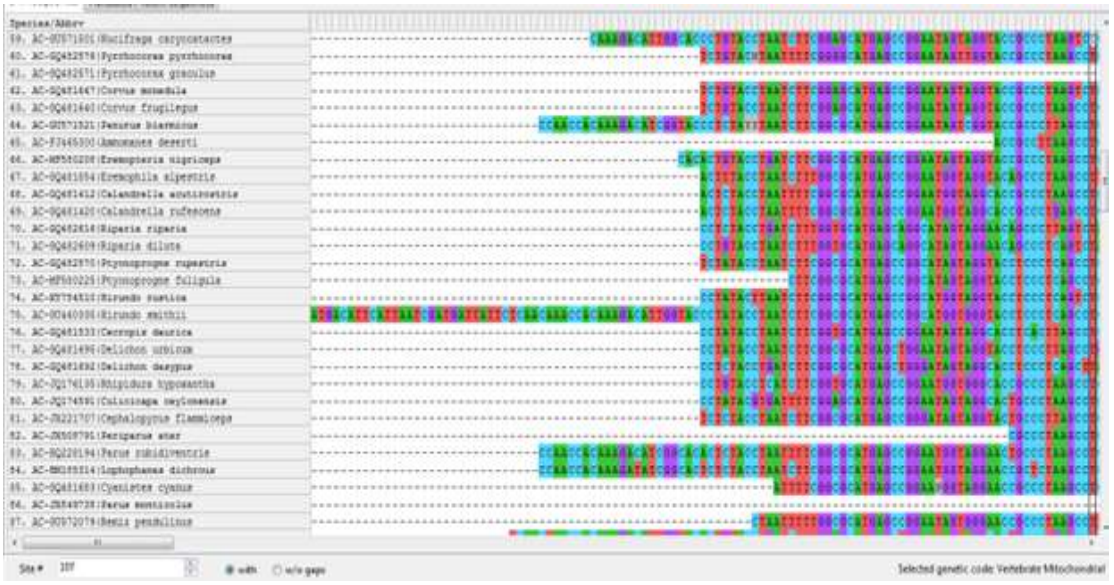

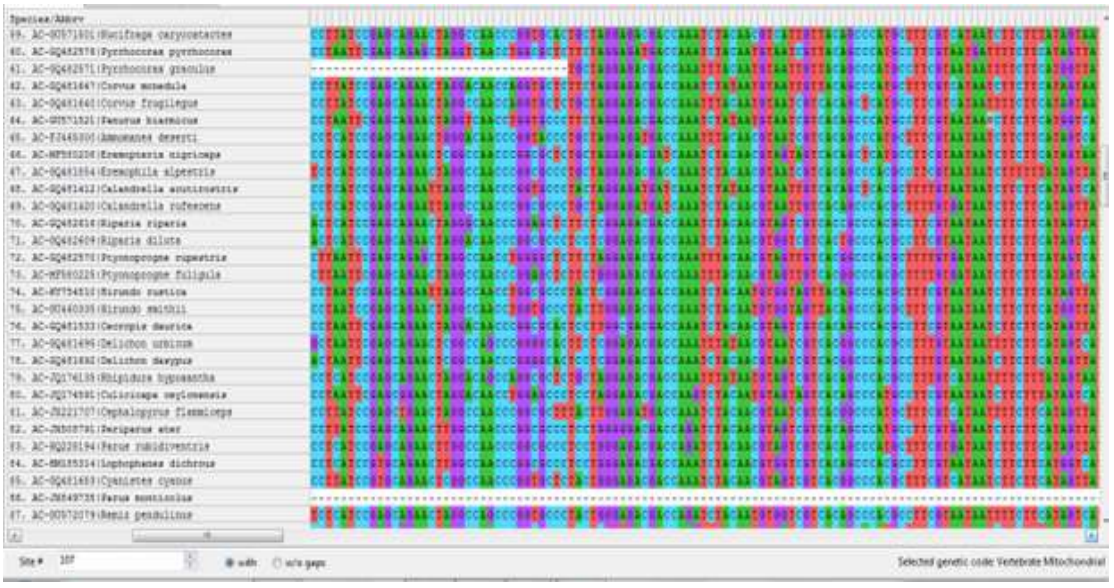

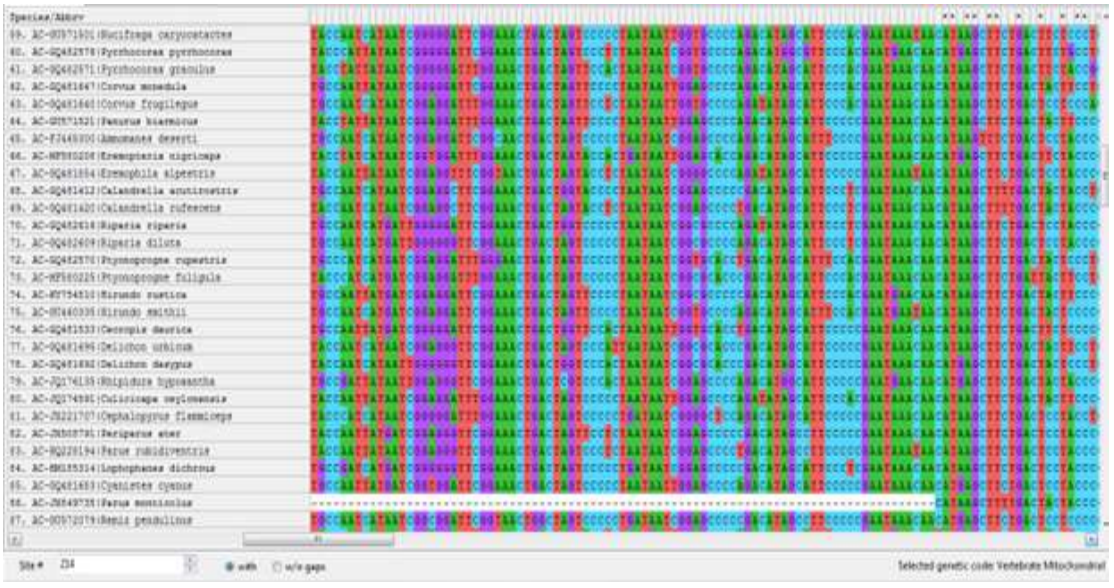

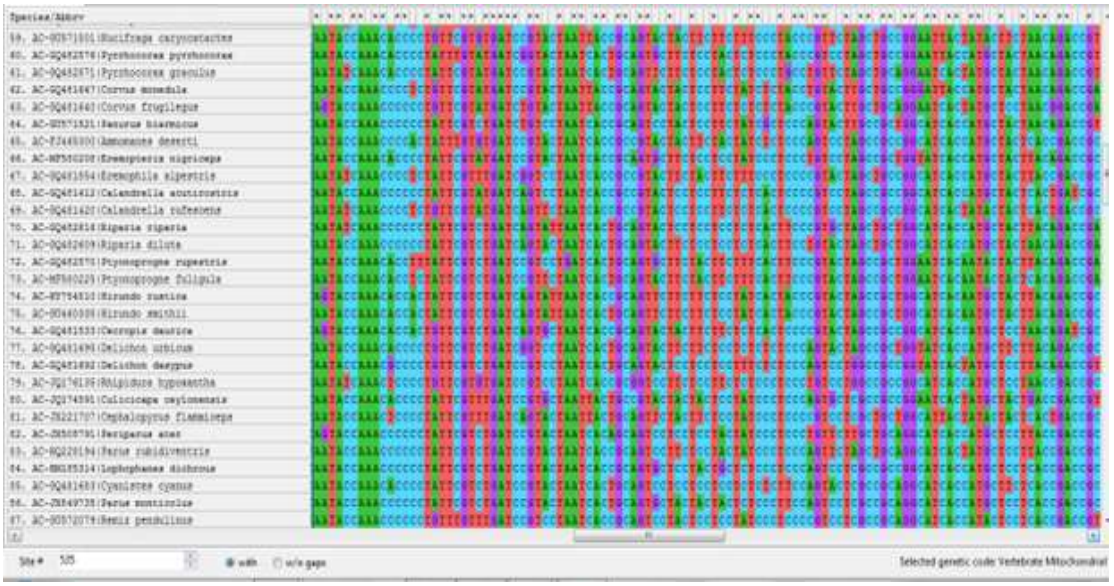

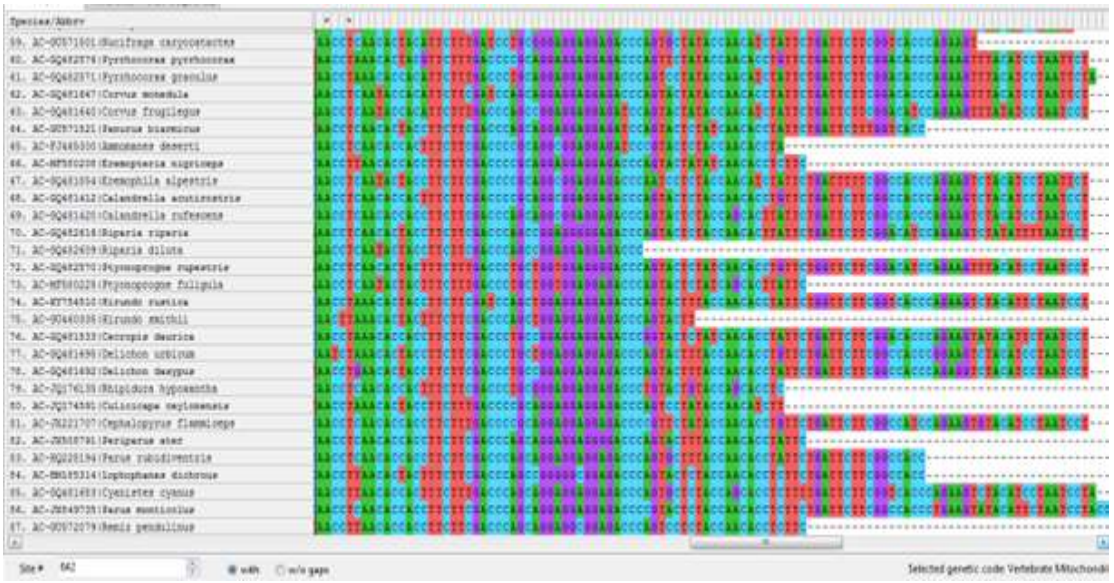

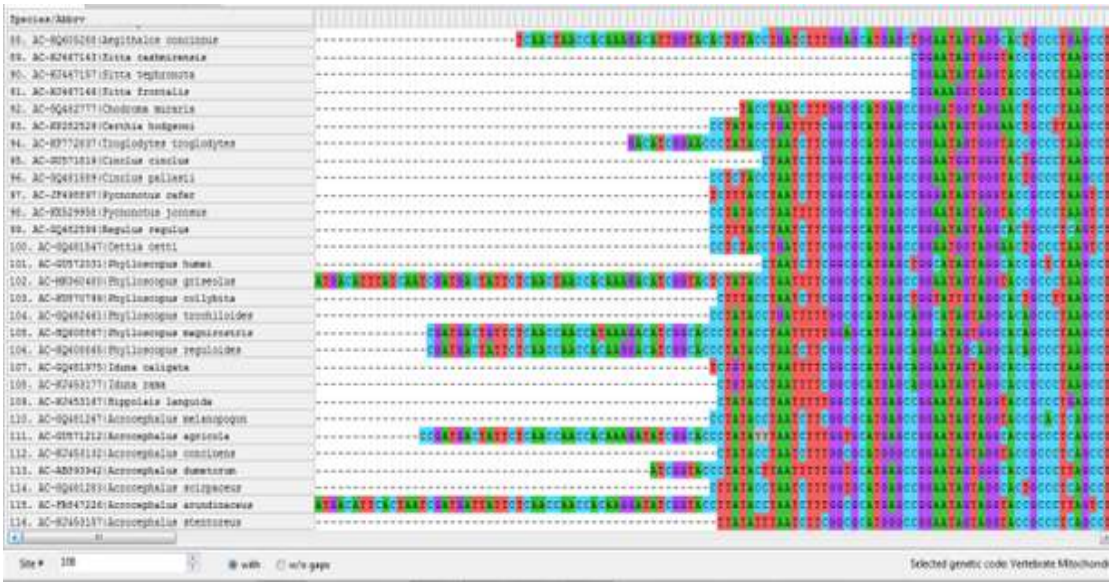

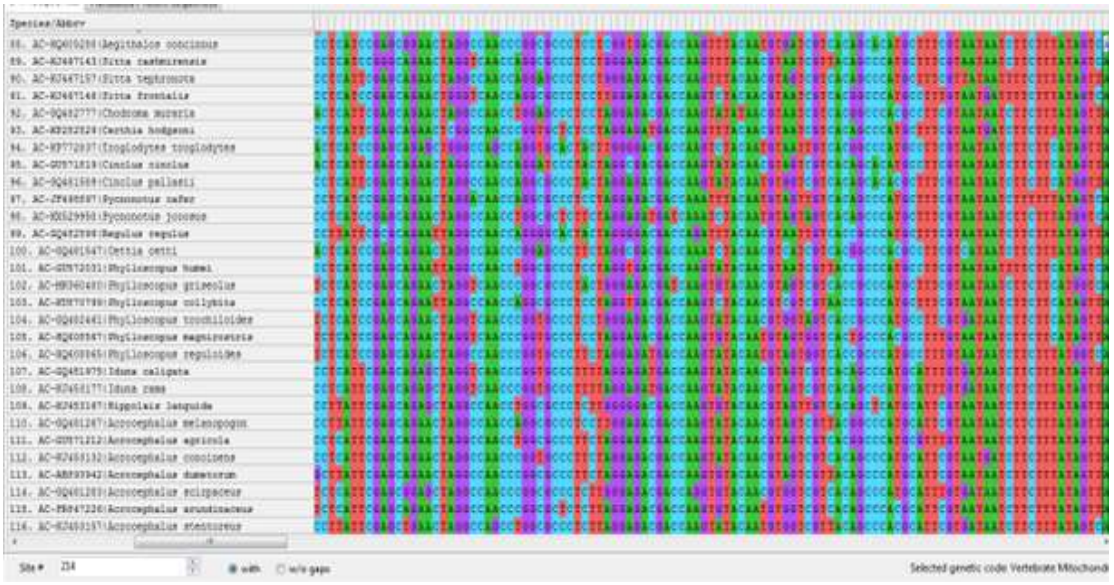

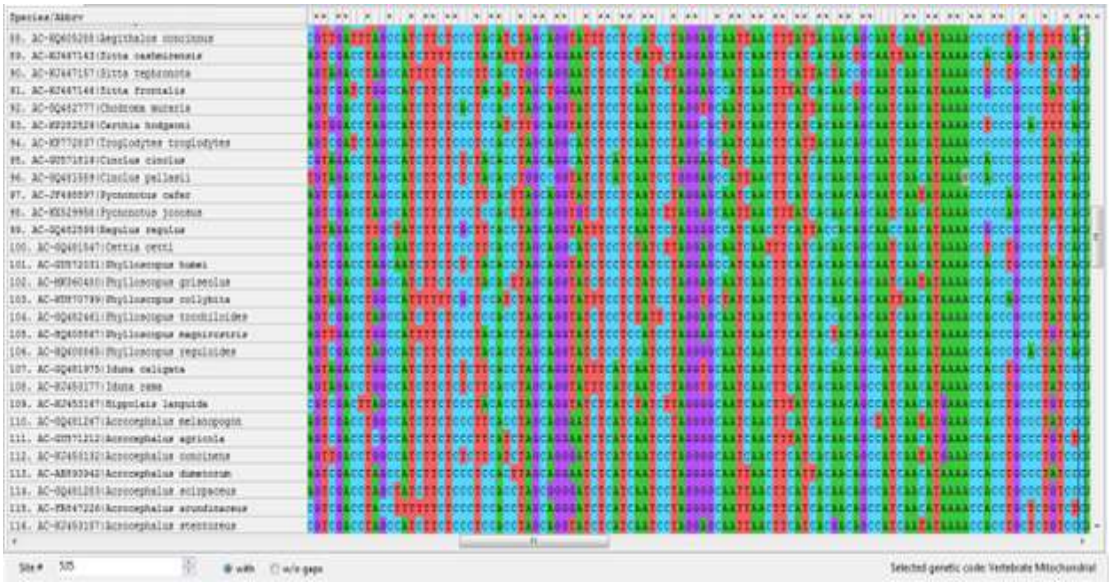

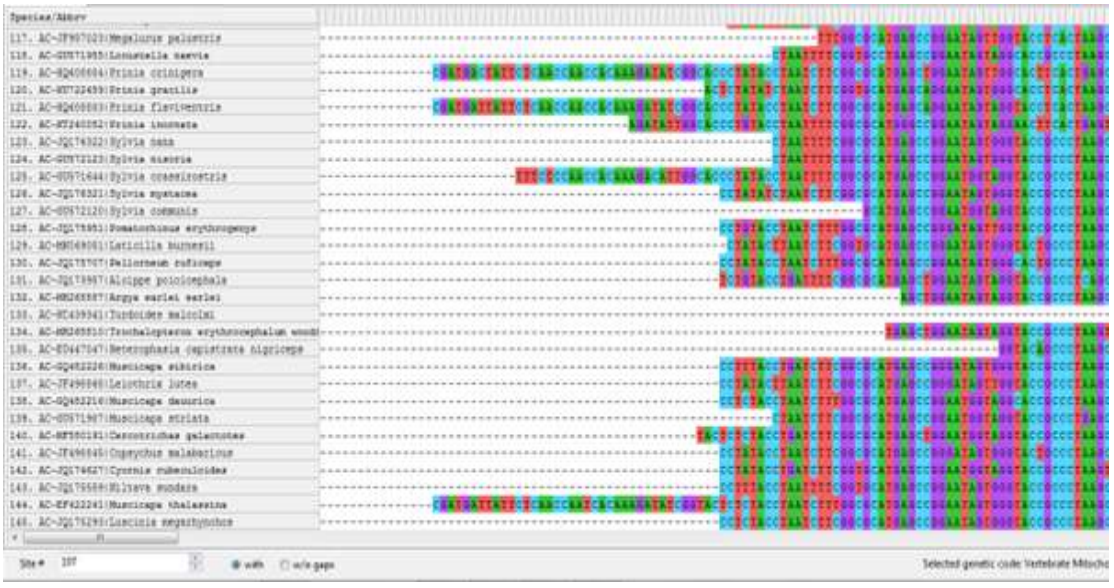

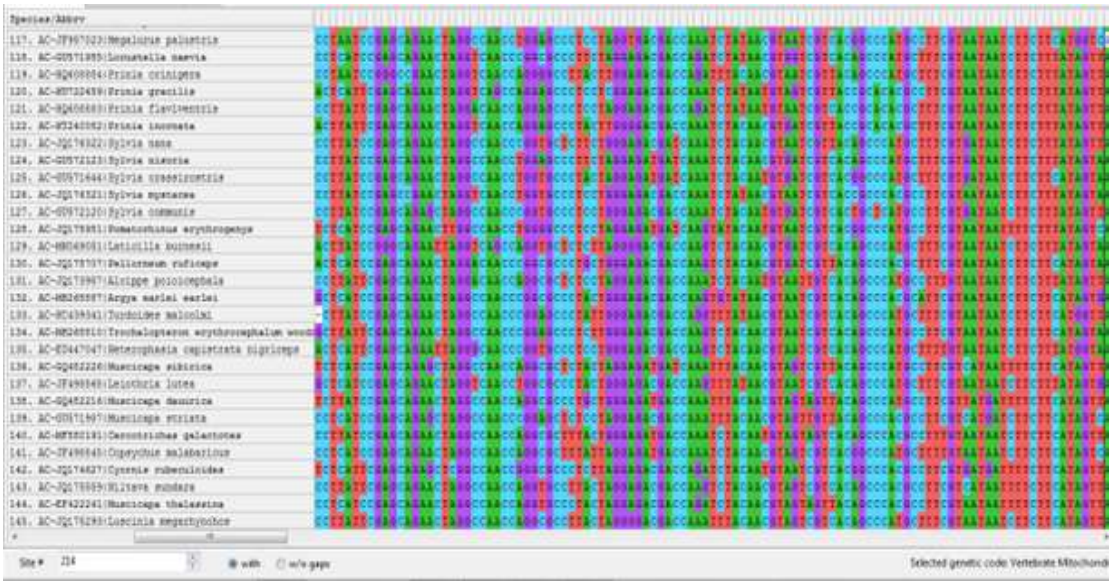

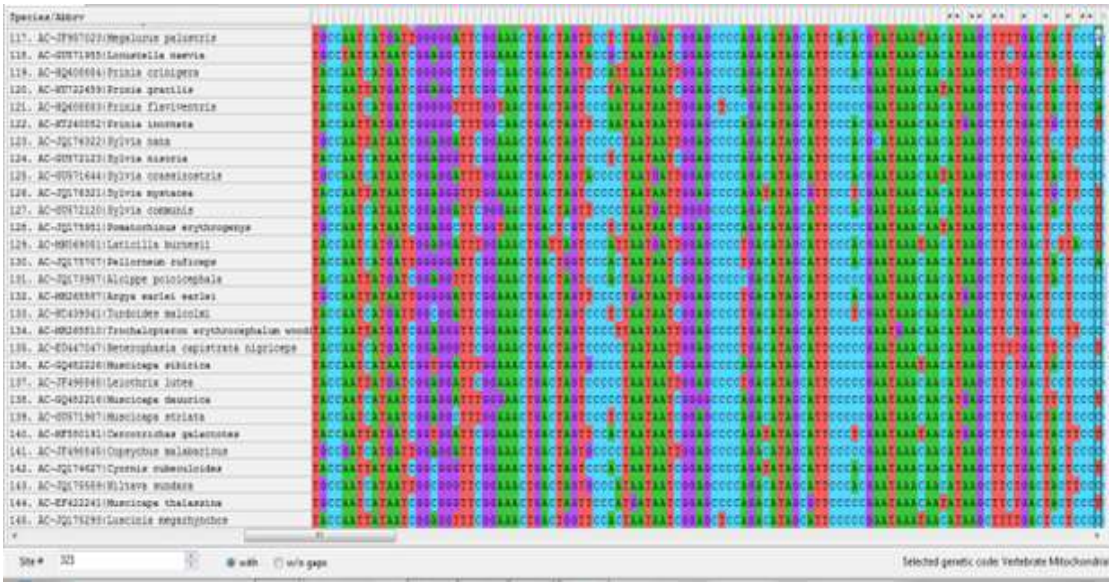

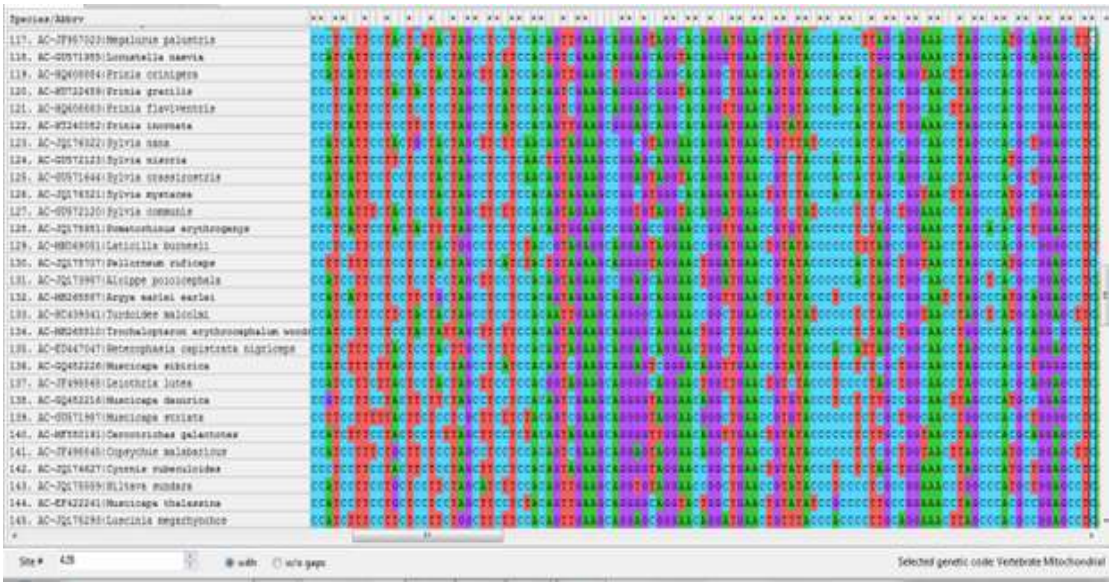

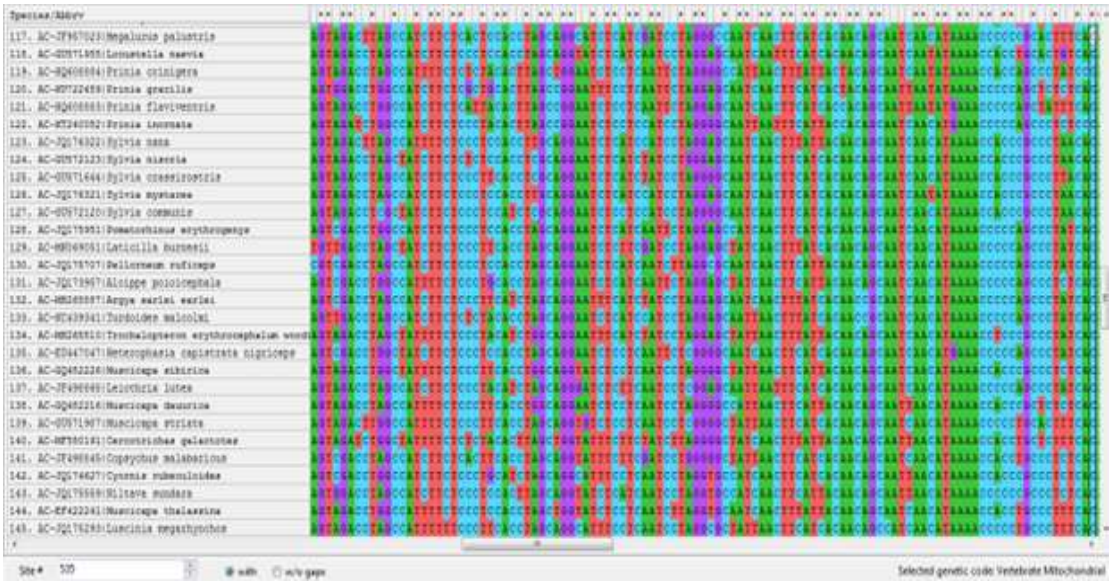

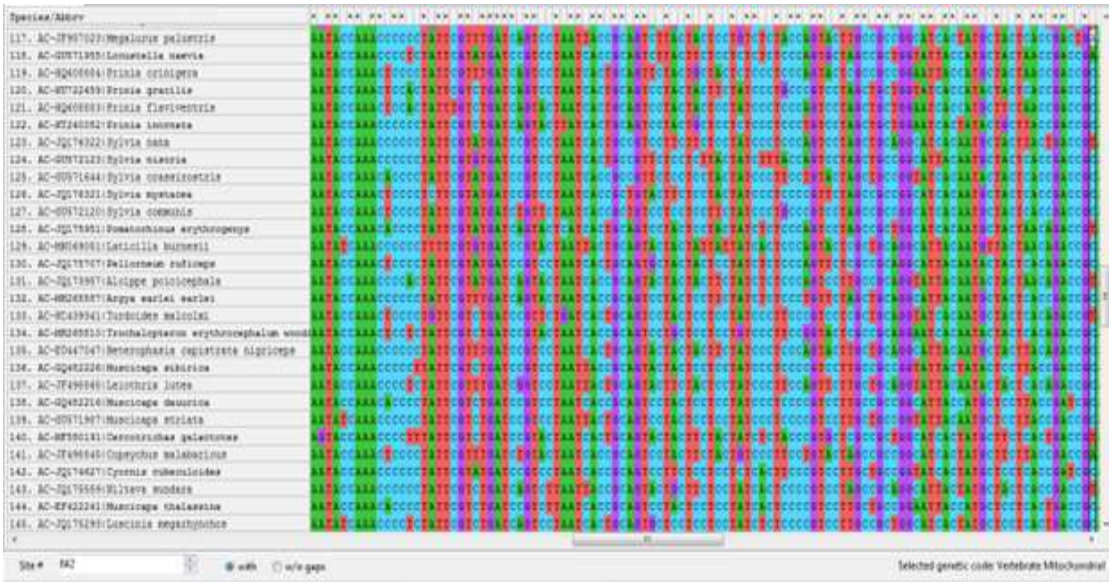

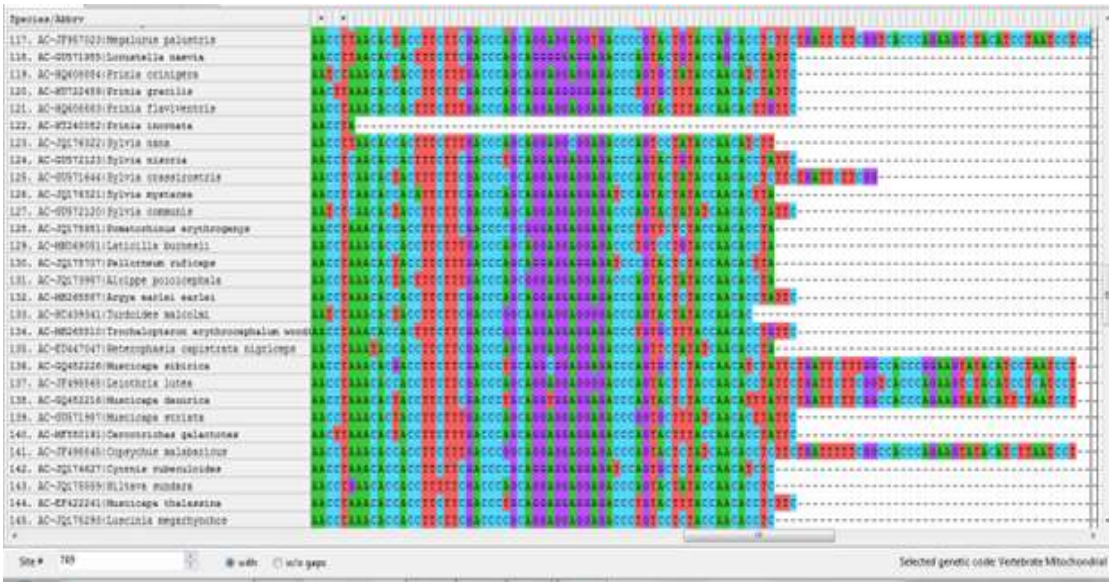

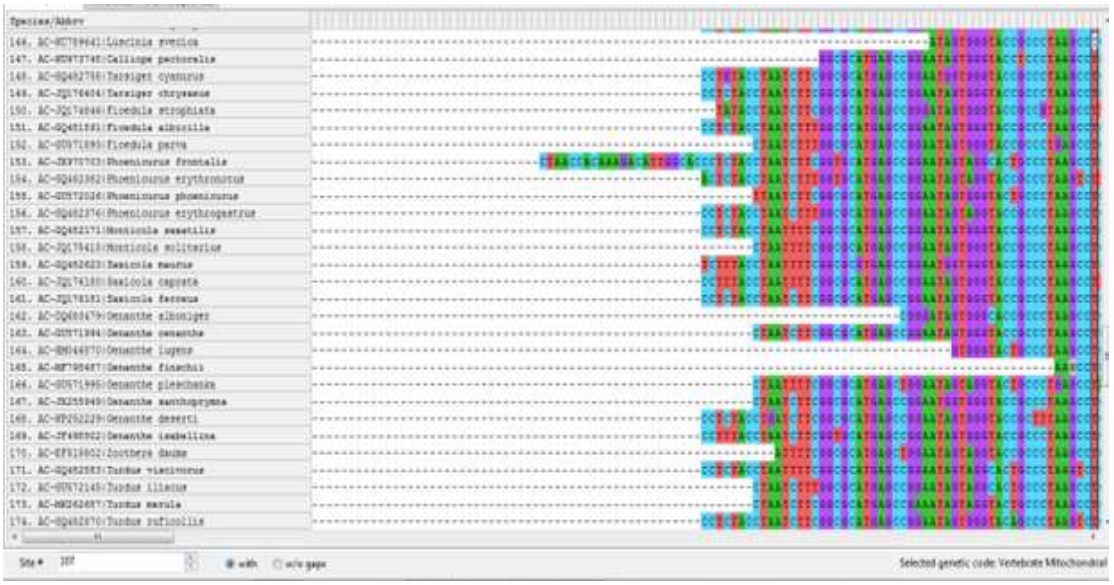

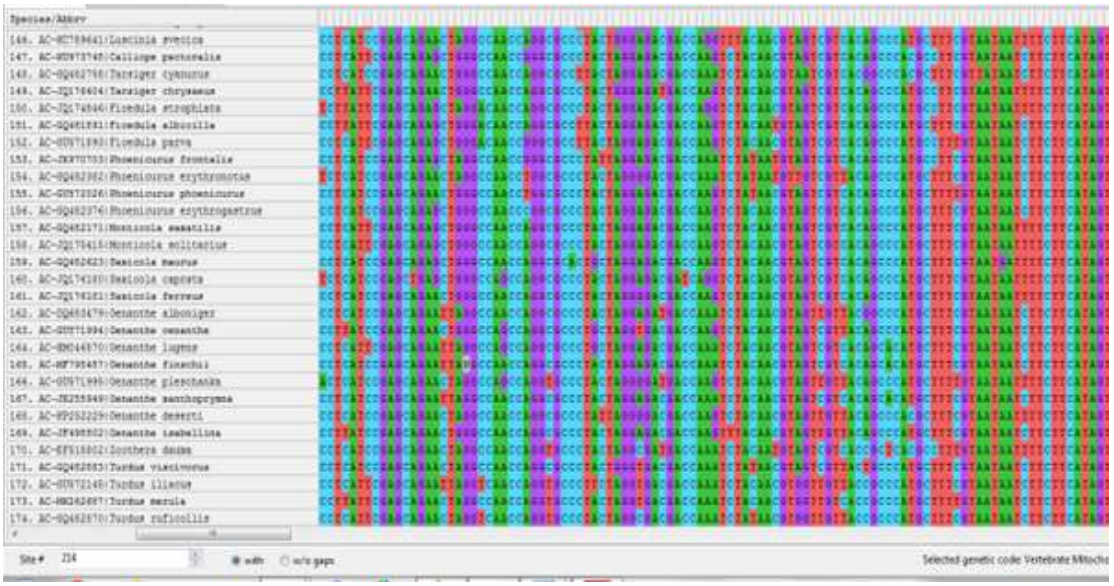

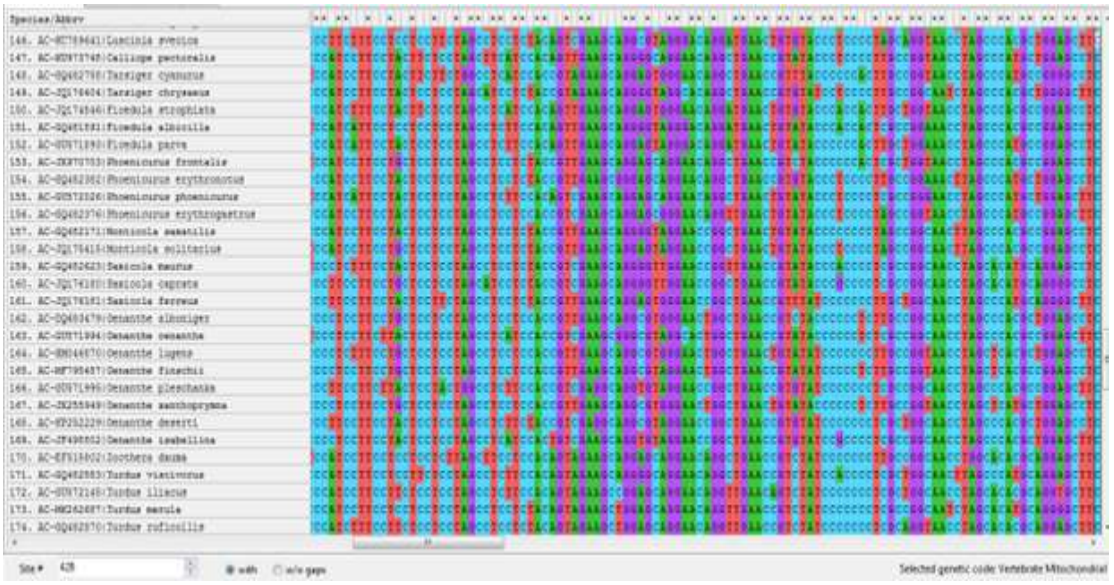

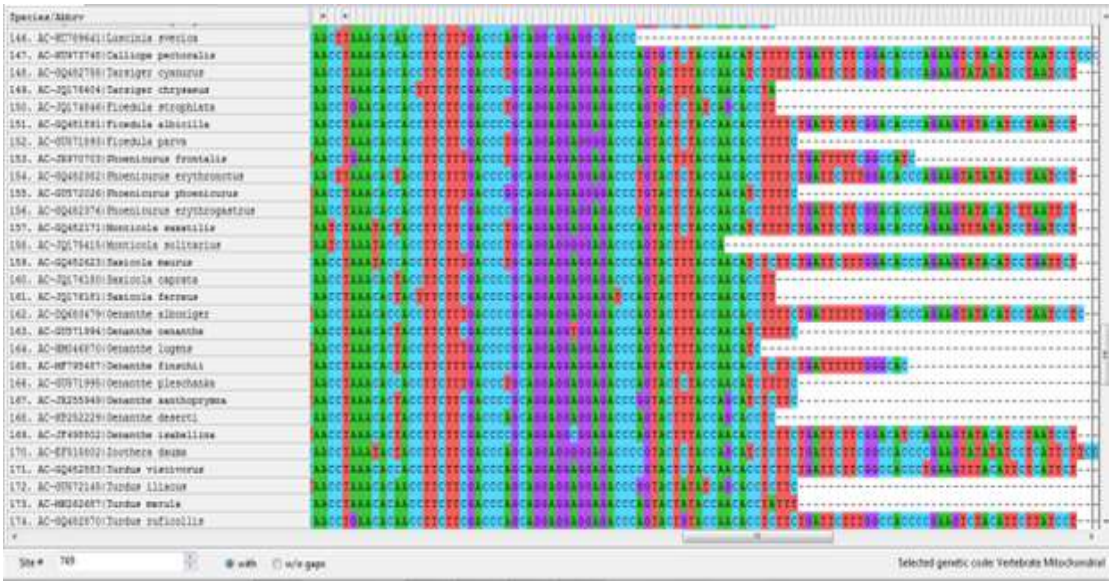

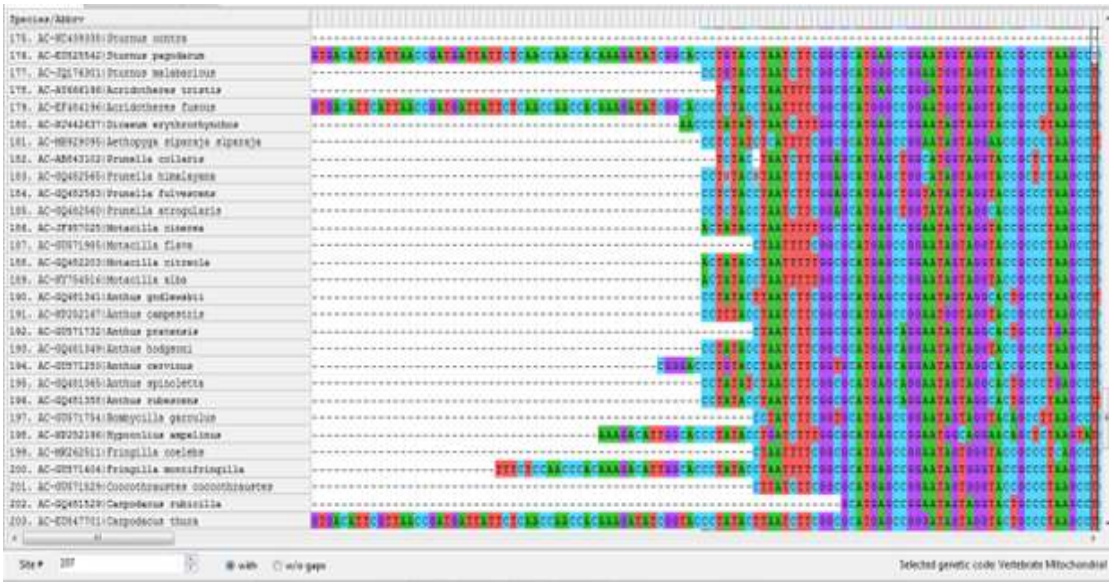

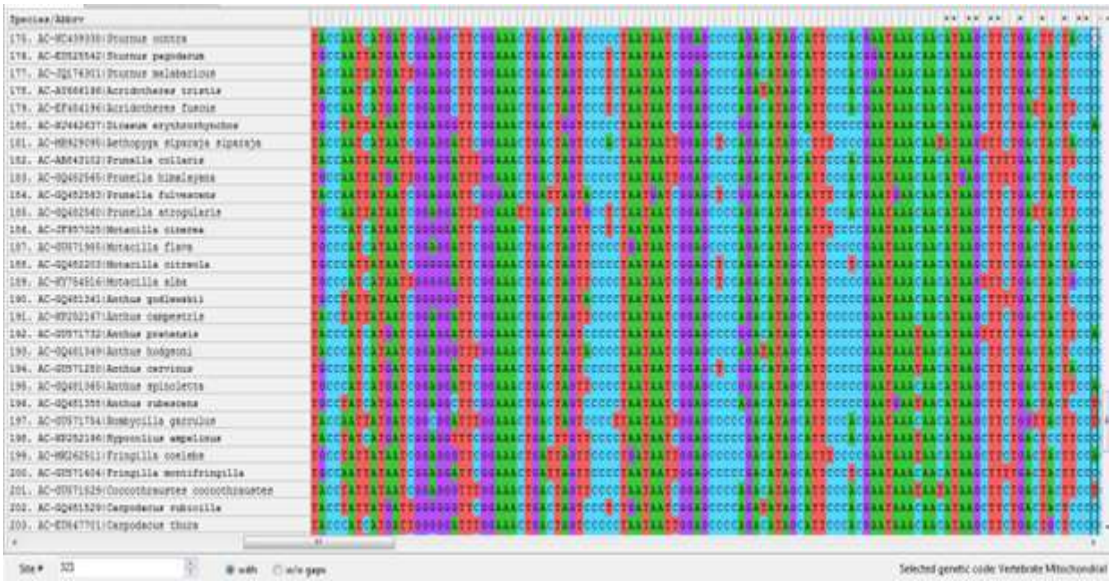

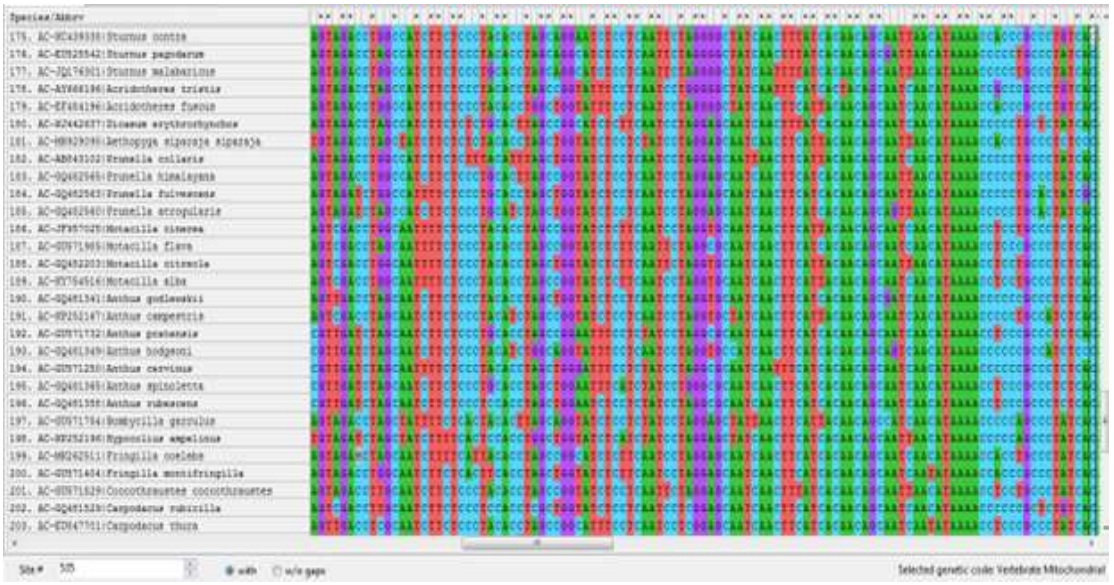

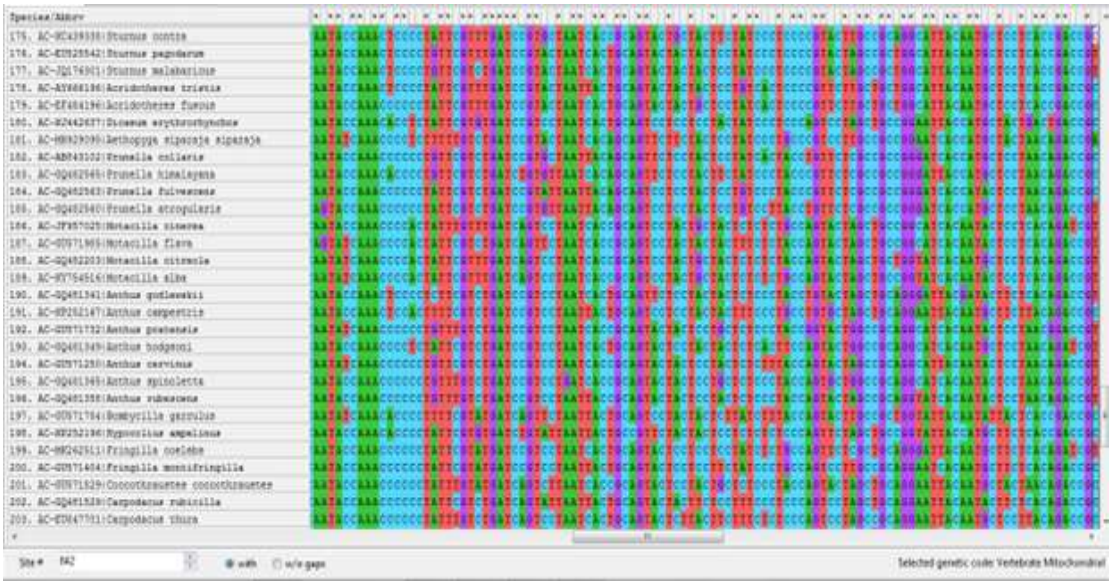

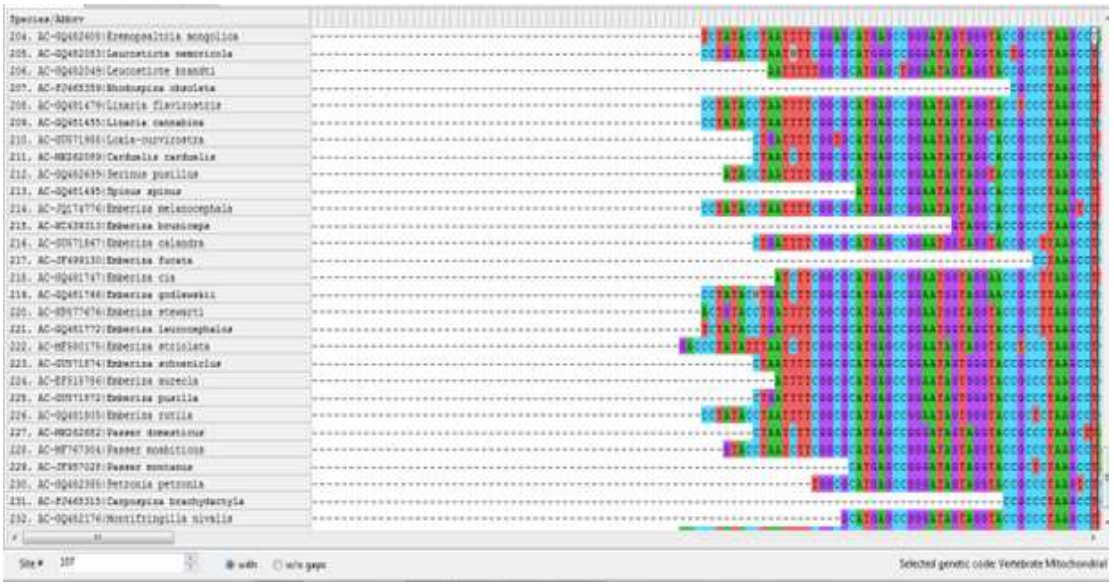

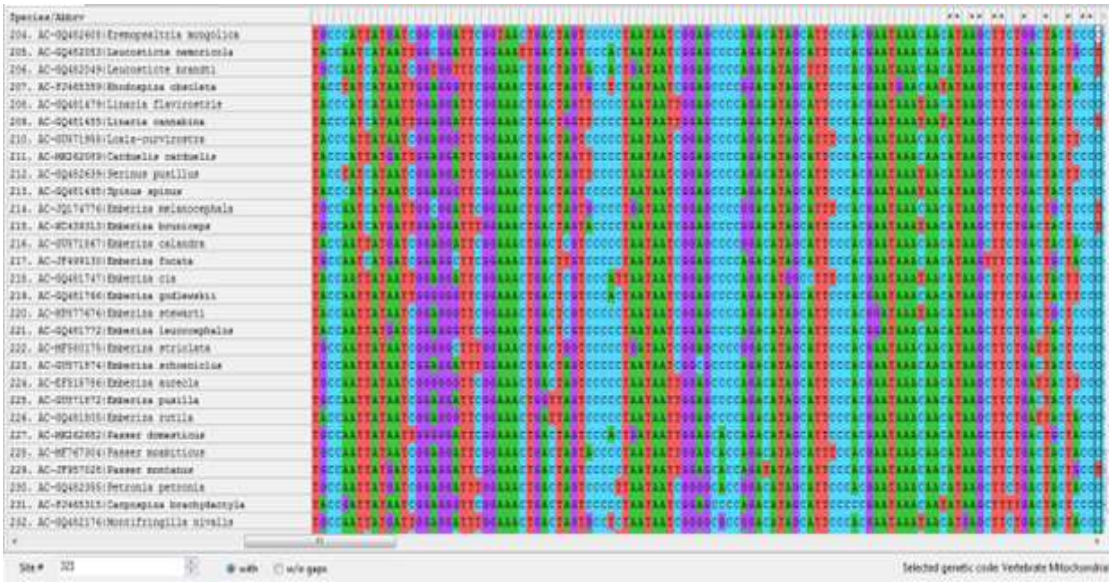

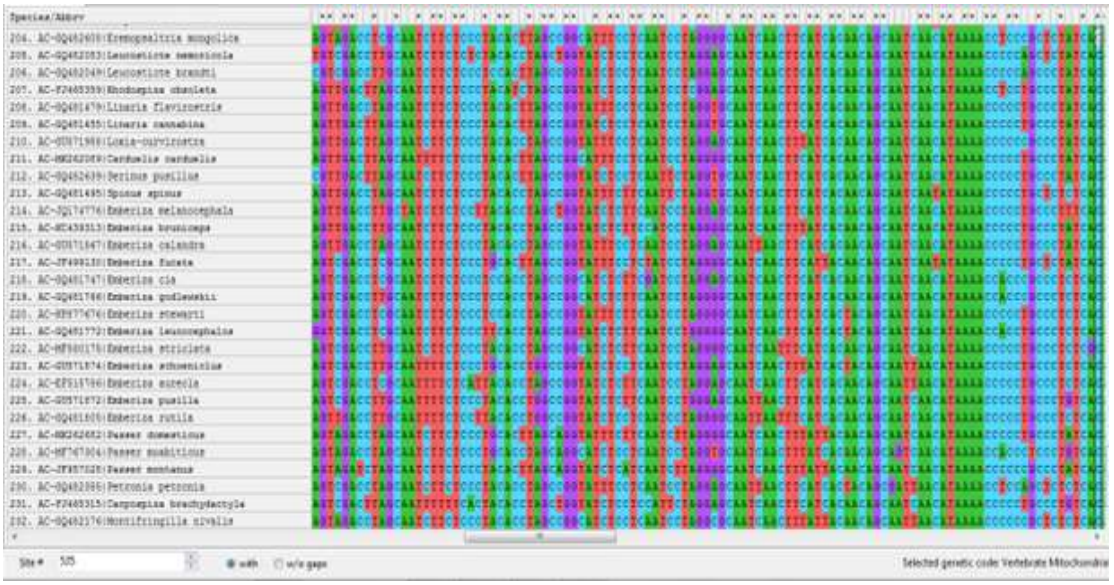

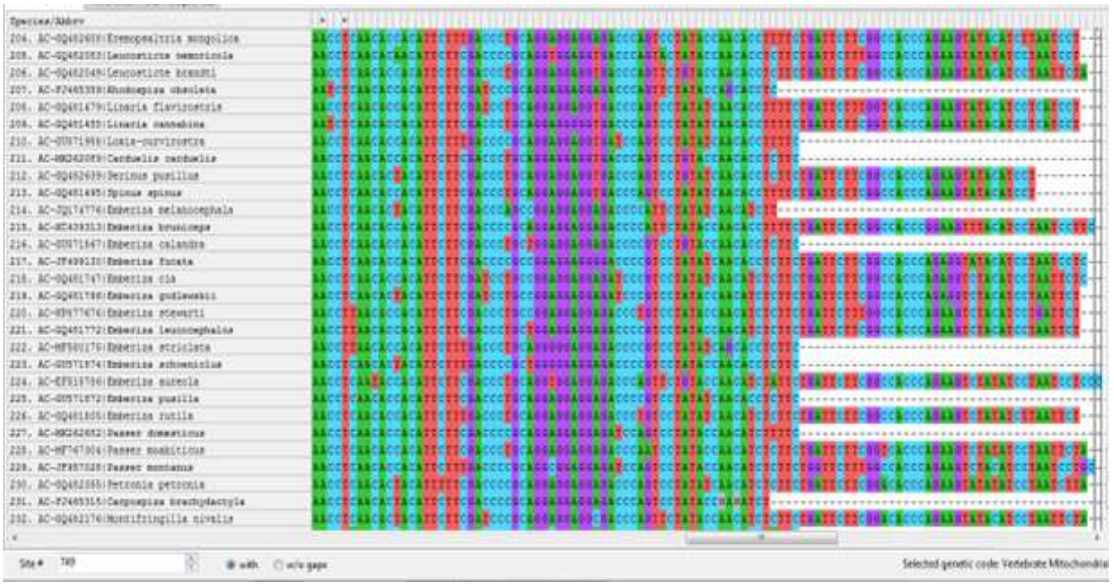

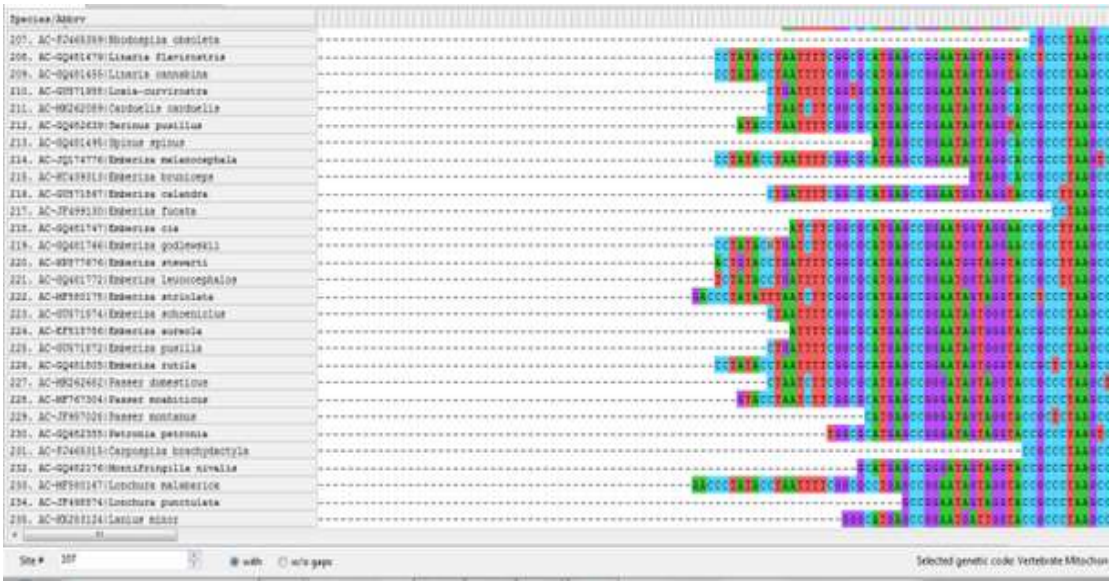

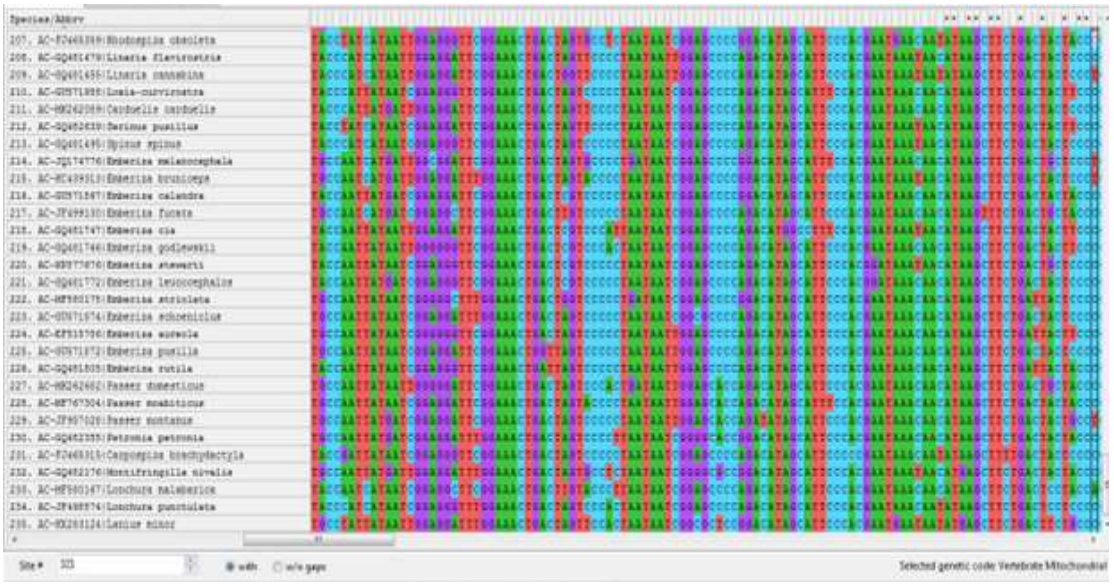

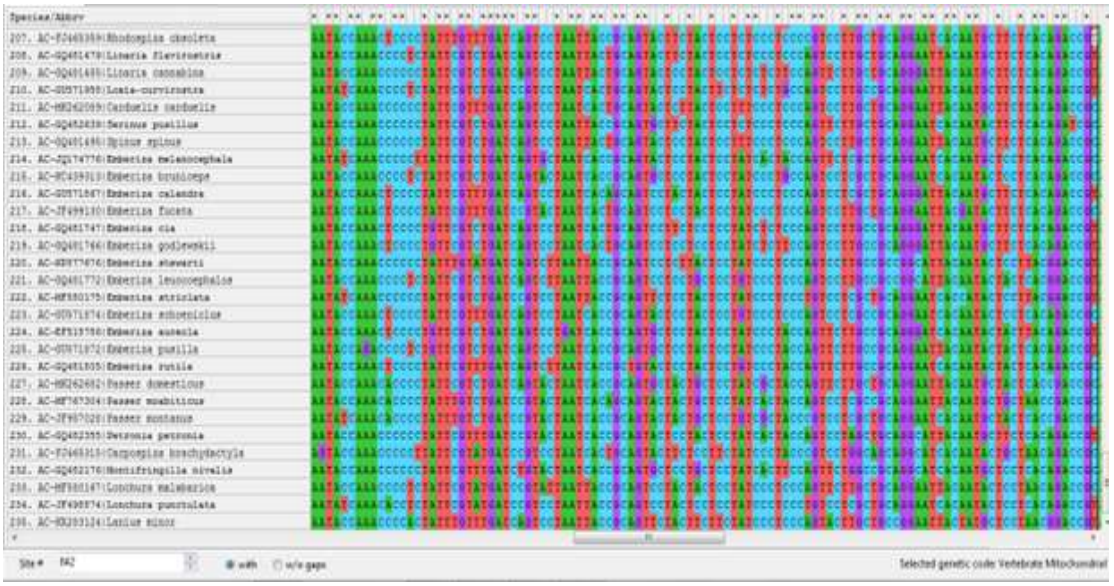

Appendix D

Nucleotide Composition of samples of Passeriforme of Pakistan

The nucleotide composition of COI gene of different species of Passeriformes shows how different or similar ATGC content is in the same gene of different species. MEGAX was used to compute the frequencies of each nucleotide (ATGC) in the barcode region of Passeriformes species of Pakistan.

The analysis involved 43 newly generated sequences of Passeriformes and 193 retrieved sequences from GenBank of NCBI database of Pakistan.

Cytosine showing highest average amount 32.5% followed by Adenine 25.9%, Tha mine 24.5% and least Guanine 17.1% of nucleotides and total average amount of nucleotides was 670.7 in all sequences.

It shows a clear variations in the frequencies of nucleotides Thyamine T(U) ranging from 21.1% (*Lophophanes dichrous*) to 28.4% (*Cercotrichas galactotes*) deviating from aveage values. Highest Cytosine value was 38.0% (*Remiz pendulinus*) and lowest was 27.0% (*Rhipidura aureola*).

Highest value of Adinine 29.1% (*Cisticola juncidis*) and lowest 22.4% (*Rhipidura hypoxantha*) was observed. Guanine value was highest 18.9% (*Hippolais languida*) and lowest 15.5% (*Prinia buchanani*) showing deviation range from average value of Guanine in the all 235 sequences.

Nucleotide composition of an organism reveals its genome. GC content varies among species and has several implications.

Data Filename: All Passiformes of Pakistan COI 19-7-23 _235 .meg
Data Title: All Passeriformes of Pakistan COI.
Nucleotide Frequencies
Sites Used: All selected
All frequencies are given in percent.
Domain: Data

	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
BOLD:AAF5733|Lanius vittatus	26.7	28.6	28.1	16.6	693	16.9	28.6	23.8	30.7	231	42.9	27.3	15.6	14.3	231	20.3	29.9	45.0	4.8	231
BOLD:ACZ2474|Prinia buchanani	25.7	31.2	27.7	15.5	600	19.0	26.5	24.5	30.0	200	42.5	28.5	16.5	12.5	200	15.5	38.5	42.0	4.0	200
BOLD:ACZ2564|Alauda gulgula australis	22.8	35.0	25.0	17.3	597	15.1	28.6	24.6	31.7	199	42.7	29.1	14.1	14.1	199	10.6	47.2	36.2	6.0	199
BOLD:AAD9870|Galerida cristata arenicola	23.2	34.1	25.1	17.5	680	16.8	27.9	23.9	31.4	226	41.9	28.2	15.4	14.5	227	11.0	46.3	36.1	6.6	227
BOLD:ABX5008|Passer hispaniolensis	24.1	31.0	27.4	17.6	672	17.4	27.7	22.8	32.1	224	42.0	28.1	15.6	14.3	224	12.9	37.1	43.8	6.3	224
BOLD:AAC0536|Sylvia curruca curruca	25.0	32.2	25.4	17.5	693	16.5	28.6	23.8	31.2	231	42.9	27.3	15.6	14.3	231	15.6	40.7	36.8	6.9	231
BOLD:AAU3934|Tephrodornis pondicerianus	25.3	30.1	26.2	18.5	692	17.3	28.1	22.9	31.6	231	42.2	27.8	15.7	14.3	230	16.5	34.2	39.8	9.5	231
BOLD:ACH6125|Dendrocitta vagabunda saturatior	24.6	30.7	26.8	18.0	684	16.7	28.1	23.7	31.6	228	42.5	27.6	15.4	14.5	228	14.5	36.4	41.2	7.9	228
BOLD:AAR9140|Corvus splendens splendens	26.0	29.4	27.8	16.9	681	16.3	28.2	23.8	31.7	227	41.9	28.2	15.4	14.5	227	19.8	31.7	44.1	4.4	227
BOLD:AAC1536|Phoenicurus ochruros	26.3	32.7	24.0	17.0	666	17.6	27.5	22.5	32.4	222	41.9	28.4	14.9	14.9	222	19.4	42.3	34.7	3.6	222
BOLD:AAV9282|Chrysomma sinense	24.3	33.0	25.4	17.3	672	17.0	27.2	23.7	32.1	224	41.1	28.6	15.6	14.7	224	14.7	43.3	37.1	4.9	224
BOLD:ABZ8019|Lanius schach schach	27.4	27.8	28.1	16.6	693	17.3	28.1	23.8	30.7	231	42.9	27.3	15.6	14.3	231	22.1	28.1	45.0	4.8	231
BOLD:ACZ2475|Prinia socialis socialis	25.0	31.5	26.7	16.9	693	19.0	26.4	23.8	30.7	231	42.4	27.7	15.6	14.3	231	13.4	40.3	40.7	5.6	231
BOLD:AAX4494|Melanocorypha bimaculata	22.3	35.7	24.7	17.3	681	16.3	28.6	23.8	31.3	227	41.9	28.2	15.4	14.5	227	8.8	50.2	34.8	6.2	227
BOLD:ACE4748|Acridotheres ginginianus	24.5	32.7	25.3	17.6	683	16.7	27.8	22.9	32.6	227	42.5	27.6	15.4	14.5	228	14.0	42.5	37.7	5.7	228
BOLD:ACS3101|Cisticola juncidis	25.7	30.6	29.1	14.6	588	18.9	27.0	25.0	29.1	196	43.4	29.1	15.3	12.2	196	14.8	35.7	46.9	2.6	196
BOLD:AAE0119|Pastor roseus	23.1	33.3	26.3	17.3	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	10.0	43.7	40.7	5.6	231
BOLD:ACZ1830|Emberiza buchanani	23.4	34.3	24.5	17.7	693	17.3	28.1	22.5	32.0	231	42.9	27.3	15.6	14.3	231	10.0	47.6	35.5	6.9	231
BOLD:ACH8855|Eremopterix griseus	24.2	33.5	25.1	17.2	693	16.9	28.6	22.9	31.6	231	42.4	27.7	15.6	14.3	231	13.4	44.2	36.8	5.6	231
BOLD:AAB3874|Carpodacus erythrinus	25.7	30.9	25.5	17.9	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	17.7	36.4	38.5	7.4	231
BOLD:ACZ2757|Oriolus oriolus	24.7	30.9	26.7	17.7	693	18.2	26.8	23.4	31.6	231	42.4	27.7	15.6	14.3	231	13.4	38.1	41.1	7.4	231
BOLD:AAB5621|Corvus corax corax	26.1	29.9	27.8	16.2	693	16.5	29.0	23.4	31.2	231	42.4	27.7	15.6	14.3	231	19.5	32.9	44.6	3.0	231
BOLD:AAL2706|Pericrocotus cinnamomeus	26.0	30.2	27.1	16.7	693	17.7	28.1	22.9	31.2	231	42.4	27.7	15.6	14.3	231	17.7	34.6	42.9	4.8	231
BOLD:ACZ2474|Prinia hodgsonii	25.5	30.7	27.0	16.7	693	18.2	27.7	23.4	30.7	231	42.4	27.7	15.6	14.3	231	16.0	36.8	42.0	5.2	231
BOLD:AAB2042|Zosterops palpebrosus palpebrosus	24.7	32.4	26.5	16.4	615	15.6	27.8	25.4	31.2	205	42.9	29.3	14.1	13.7	205	15.6	40.0	40.0	4.4	205
BOLD:AAN3860|Pycnonotus leucogenys	24.8	33.2	25.8	16.2	693	17.3	28.1	23.4	31.2	231	42.4	27.7	15.6	14.3	231	14.7	43.7	38.5	3.0	231
BOLD:ACH0131|Phylloscopus affinis	24.1	33.0	25.4	17.5	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	13.0	42.9	38.1	6.1	231
BOLD:ABW5160|Corvus corone	25.7	30.2	27.6	16.6	693	16.9	28.1	23.8	31.2	231	42.4	27.7	15.6	14.3	231	17.7	34.6	43.3	4.3	231
BOLD:ACE7266|Copsychus saularis saularis	25.3	33.3	25.0	16.4	609	17.2	26.6	25.1	31.0	203	42.9	29.6	13.8	13.8	203	15.8	43.8	36.0	4.4	203
BOLD:AAY1381|Trochalopteron lineatum lineatum	23.7	32.9	27.0	16.5	693	17.3	28.1	22.9	31.6	231	42.9	27.3	15.6	14.3	231	10.8	43.3	42.4	3.5	231
BOLD:ACZ3140|Parus major	22.3	34.7	25.1	17.9	692	16.9	27.3	22.9	32.9	231	42.4	27.7	15.6	14.3	231	7.4	49.1	37.0	6.5	230

BOLD:ACZ2846|Urocissa flavirostris flavirostris	25.1	30.7	26.8	17.3	693	16.9	28.1	23.8	31.2	231	42.4	27.7	15.6	14.3	231	16.0	36.4	41.1	6.5	231
BOLD:ACZ2835|Stachyridopsis pyrrhops	24.7	31.2	26.6	17.6	693	17.7	27.3	23.4	31.6	231	42.4	27.7	15.6	14.3	231	13.9	38.5	40.7	6.9	231
BOLD:ACS4664|Terpsiphone paradisi paradisi	26.1	29.9	26.4	17.6	693	16.9	28.6	22.9	31.6	231	42.4	27.7	15.6	14.3	231	19.0	33.3	40.7	6.9	231
BOLD:AAC5713|Myophonus caeruleus	25.0	32.2	25.0	17.9	681	18.1	27.8	22.0	32.2	227	41.9	27.8	15.9	14.5	227	15.0	41.0	37.0	7.0	227
BOLD:ACZ2808|Hypsipetes leucocephalus	25.5	30.4	26.6	17.5	693	18.2	27.3	22.9	31.6	231	42.4	27.7	15.6	14.3	231	16.0	36.4	41.1	6.5	231
BOLD:AAI9717|Dicrurus leucophaeus	27.6	28.4	27.7	16.3	693	18.2	27.3	22.9	31.6	231	42.4	27.7	15.6	14.3	231	22.1	30.3	44.6	3.0	231
BOLD:ACZ3094|Emberiza lathami	23.5	34.1	25.5	16.9	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	11.3	45.9	38.5	4.3	231
BOLD:AAC1399|Passer cinnamomeus rutilans	23.8	31.5	26.8	17.9	693	16.9	28.6	22.5	32.0	231	42.4	27.7	15.6	14.3	231	12.1	38.1	42.4	7.4	231
BOLD:ABW5160|Corvus macrorhynchos	23.1	35.1	25.1	16.7	693	17.3	28.1	23.8	30.7	231	42.4	27.7	15.6	14.3	231	9.5	49.4	35.9	5.2	231
BOLD:ACH8281|Orthotomus sutorius	25.0	31.6	27.3	16.2	693	18.2	27.7	23.4	30.7	231	42.4	27.7	15.6	14.3	231	14.3	39.4	42.9	3.5	231
BOLD:AAW9936|Oenanthe picata	24.1	34.9	24.0	17.0	693	16.5	29.0	22.9	31.6	231	42.9	27.3	15.6	14.3	231	13.0	48.5	33.3	5.2	231
BOLD:ACZ2562|Copsychus fulicatus	25.3	32.6	25.5	16.6	693	17.3	28.1	22.9	31.6	231	42.4	27.7	15.6	14.3	231	16.0	42.0	38.1	3.9	231
AC- AB843703|Pitta brachyura	24.5	33.0	27.0	15.5	678	17.3	27.4	24.8	30.5	226	41.6	28.3	15.5	14.6	226	14.6	43.4	40.7	1.3	226
AC-JQ173906|Aegithina tiphia	24.1	31.9	27.3	16.7	652	16.1	28.1	23.5	32.3	217	41.5	29.0	15.2	14.3	217	14.7	38.5	43.1	3.7	218
AC-MH938034|Lanius collurio	27.1	28.2	28.2	16.5	642	17.8	28.0	23.8	30.4	214	42.5	27.6	16.4	13.6	214	21.0	29.0	44.4	5.6	214
AC-GQ482020|Lanius isabellinus	26.7	28.7	27.3	17.3	693	17.7	27.7	23.8	30.7	231	42.9	27.3	15.6	14.3	231	19.5	31.2	42.4	6.9	231
AC-GQ482013|Lanius cristatus	27.0	28.9	27.3	16.9	693	17.7	27.7	23.8	30.7	231	42.9	27.3	15.6	14.3	231	20.3	31.6	42.4	5.6	231
AC-EF621598|Lanius tephronotus	27.1	28.9	27.4	16.6	693	17.7	28.1	23.4	30.7	231	42.4	27.7	16.5	13.4	231	21.2	30.7	42.4	5.6	231
AC-JF498786|Lanius excubitor	27.3	28.0	28.6	16.2	693	17.7	27.7	23.8	30.7	231	42.4	27.7	15.6	14.3	231	21.6	28.6	46.3	3.5	231
AC-GQ482278|Oriolus chinensis	24.0	32.0	26.8	17.2	693	17.7	27.3	23.4	31.6	231	42.4	27.7	15.6	14.3	231	11.7	41.1	41.6	5.6	231
AC-JQ174696|Dicrurus macrocercus	25.5	30.0	27.5	17.1	651	15.2	29.0	23.5	32.3	217	41.5	29.0	15.2	14.3	217	19.8	31.8	43.8	4.6	217
AC-JQ174691|Dicrurus hottentottus	27.8	27.0	27.8	17.5	652	17.5	26.7	23.5	32.3	217	41.9	28.6	15.2	14.3	217	23.9	25.7	44.5	6.0	218
AC-JQ176131|Rhipidura aureola	25.3	29.4	28.1	17.2	652	16.6	27.6	23.5	32.3	217	41.5	29.0	15.2	14.3	217	17.9	31.7	45.4	5.0	218
AC-KC354929|Hypothymis azurea	25.0	30.5	27.1	17.3	652	17.1	27.6	23.0	32.3	217	40.8	28.9	15.6	14.7	218	17.1	35.0	42.9	5.1	217
AC-GQ481963|Garrulus glandarius	26.0	31.0	26.3	16.7	693	16.5	29.0	23.8	30.7	231	42.4	27.7	15.6	14.3	231	19.0	36.4	39.4	5.2	231
AC-JQ176603|Urocissa erythrorhyncha	23.5	31.7	28.1	16.7	652	16.1	27.6	24.4	31.8	217	41.5	29.0	15.2	14.3	217	12.8	38.5	44.5	4.1	218
AC-GQ482478|Pica pica	26.8	29.7	27.1	16.5	691	16.5	28.7	23.5	31.3	230	42.2	27.8	15.7	14.3	230	21.6	32.5	42.0	3.9	231
AC-GU571501|Nucifraga caryocatactes	25.0	31.2	26.8	17.0	693	16.0	28.1	23.8	32.0	231	41.6	27.7	16.0	14.7	231	17.3	37.7	40.7	4.3	231
AC-GQ482576|Pyrrhocorax pyrrhocorax	25.7	29.6	26.7	17.9	692	17.0	28.3	23.5	31.3	230	42.9	27.3	15.6	14.3	231	17.3	33.3	41.1	8.2	231
AC-GQ482571|Pyrrhocorax graculus	26.8	29.3	28.3	15.7	605	17.4	28.4	24.4	29.9	201	43.6	27.7	16.3	12.4	202	19.3	31.7	44.1	5.0	202
AC-GQ481647|Corvus monedula	26.7	28.6	28.6	16.2	693	16.5	28.6	23.8	31.2	231	42.4	27.7	15.6	14.3	231	21.2	29.4	46.3	3.0	231
AC-GQ481640|Corvus frugilegus	26.1	29.7	27.6	16.6	693	16.5	29.0	23.4	31.2	231	42.4	27.7	15.6	14.3	231	19.5	32.5	43.7	4.3	231
AC-GU571521|Panurus biarmicus	24.5	33.0	26.2	16.2	690	16.1	28.3	24.3	31.3	230	40.4	28.3	16.5	14.8	230	17.0	42.6	37.8	2.6	230
AC-FJ465300|Ammomanes deserti	22.2	35.8	26.5	15.5	612	15.2	29.4	25.5	29.9	204	41.2	29.9	15.7	13.2	204	10.3	48.0	38.2	3.4	204
AC-MF580208|Eremopterix nigriceps	22.8	34.7	25.8	16.7	658	16.0	28.3	23.7	32.0	219	41.6	29.2	15.1	14.2	219	10.9	46.4	38.6	4.1	220
AC-GQ481854|Eremophila alpestris	24.0	35.1	25.0	16.0	693	16.5	29.0	24.2	30.3	231	42.4	27.7	15.6	14.3	231	13.0	48.5	35.1	3.5	231

AC-GQ481412|Calandrella acutirostris	22.8	36.2	24.8	16.2	693	16.9	28.6	23.8	30.7	231	42.4	27.7	15.6	14.3	231	9.1	52.4	35.1	3.5	231
AC-GQ481420|Calandrella rufescens	23.4	35.1	25.3	16.3	693	17.3	28.1	23.8	30.7	231	42.4	27.7	15.6	14.3	231	10.4	49.4	36.4	3.9	231
AC-GQ482616|Riparia riparia	25.1	32.6	25.4	16.9	693	18.2	27.3	22.9	31.6	231	42.4	27.7	15.6	14.3	231	14.7	42.9	37.7	4.8	231
AC-GQ482609|Riparia diluta	23.2	33.8	25.8	17.2	633	16.1	27.0	24.2	32.7	211	41.2	29.9	14.2	14.7	211	12.3	44.5	38.9	4.3	211
AC-GQ482570|Ptyonoprogne rupestris	26.4	30.9	25.0	17.7	693	17.7	28.1	22.9	31.2	231	42.9	27.3	15.6	14.3	231	18.6	37.2	36.4	7.8	231
AC-MF580225|Ptyonoprogne fuligula	25.8	31.3	25.7	17.3	643	17.3	27.1	23.4	32.2	214	41.6	29.0	15.0	14.5	214	18.6	37.7	38.6	5.1	215
AC-KY754510|Hirundo rustica	24.5	32.0	26.4	17.0	693	18.2	27.7	22.9	31.2	231	42.9	27.3	15.6	14.3	231	12.6	41.1	40.7	5.6	231
AC-GU460335|Hirundo smithii	25.0	30.9	27.0	17.2	693	17.3	26.0	26.0	30.7	231	41.6	28.6	15.2	14.7	231	16.0	38.1	39.8	6.1	231
AC-GQ481533|Cecropis daurica	24.0	32.9	25.7	17.5	693	17.3	28.6	22.9	31.2	231	42.9	27.3	15.6	14.3	231	11.7	42.9	38.5	6.9	231
AC-GQ481695|Delichon urbicum	24.5	32.9	25.3	17.3	693	17.7	28.1	23.4	30.7	231	42.9	27.3	15.6	14.3	231	13.0	43.3	36.8	6.9	231
AC-GQ481692|Delichon dasypus	23.5	34.3	24.5	17.6	693	17.7	28.1	23.4	30.7	231	42.9	27.3	15.6	14.3	231	10.0	47.6	34.6	7.8	231
AC-JQ176135|Rhipidura hypoxantha	23.0	34.7	22.4	19.9	652	15.7	28.6	23.5	32.3	217	41.9	28.6	15.2	14.3	217	11.5	46.8	28.4	13.3	218
AC-JQ174591|Culicicapa ceylonensis	24.2	31.4	26.4	17.9	652	15.2	28.6	23.0	33.2	217	41.9	28.6	15.2	14.3	217	15.6	37.2	40.8	6.4	218
AC-JX221707|Cephalopyrus flammiceps	26.1	32.5	24.4	17.0	693	16.9	28.6	23.8	30.7	231	42.9	27.3	15.6	14.3	231	18.6	41.6	33.8	6.1	231
AC-JX508791|Periparus ater	22.2	36.4	24.8	16.6	613	15.2	29.9	23.5	31.4	204	42.2	28.9	15.7	13.2	204	9.3	50.2	35.1	5.4	205
AC-HQ228194|Parus rubidiventris	22.2	35.4	25.5	16.9	693	16.0	28.6	23.8	31.6	231	41.1	27.7	16.5	14.7	231	9.5	49.8	36.4	4.3	231
AC-HM185314|Lophophanes dichrous	21.1	37.4	23.5	18.0	693	16.0	28.6	23.8	31.6	231	41.1	27.7	16.5	14.7	231	6.1	55.8	30.3	7.8	231
AC-GQ481683|Cyanistes cyanus	23.9	34.1	24.5	17.6	683	17.1	27.6	23.2	32.0	228	41.9	28.2	15.4	14.5	227	12.7	46.5	34.6	6.1	228
AC-JX849735|Parus monticolus	23.1	34.6	25.7	16.6	693	19.9	25.1	23.8	31.2	231	39.8	29.4	16.0	14.7	231	9.5	49.4	37.2	3.9	231
AC-GU572079|Remiz pendulinus	21.5	38.0	22.5	18.1	648	15.3	28.7	23.6	32.4	216	41.7	29.2	14.8	14.4	216	7.4	56.0	29.2	7.4	216
AC-HQ605288|Aegithalos concinnus	26.8	30.7	25.5	16.9	693	16.9	27.3	24.2	31.6	231	40.7	28.6	16.0	14.7	231	22.9	36.4	36.4	4.3	231
AC-KJ467143|Sitta cashmirensis	23.4	33.6	26.6	16.4	628	15.3	28.7	24.4	31.6	209	42.1	28.7	15.3	13.9	209	12.9	43.3	40.0	3.8	210
AC-KJ467157|Sitta tephronota	23.6	33.3	26.0	17.2	628	15.8	28.2	23.4	32.5	209	42.1	28.7	15.3	13.9	209	12.9	42.9	39.0	5.2	210
AC-KJ467146|Sitta frontalis	24.2	32.6	25.5	17.7	628	14.8	29.2	23.9	32.1	209	41.6	28.7	15.8	13.9	209	16.2	40.0	36.7	7.1	210
AC-GQ482777|chodroma muraria	25.5	31.6	25.8	17.1	690	17.0	28.3	23.0	31.7	230	42.6	27.4	15.7	14.3	230	17.0	39.1	38.7	5.2	230
AC-KP282529|Certhia hodgsoni	25.8	33.5	22.9	17.8	663	17.6	27.6	23.1	31.7	221	42.5	28.1	14.9	14.5	221	17.2	44.8	30.8	7.2	221
AC-KP772837|Troglodytes troglodytes	22.8	34.3	25.6	17.4	645	15.3	27.0	25.1	32.6	215	41.4	29.3	14.4	14.9	215	11.6	46.5	37.2	4.7	215
AC-GU571819|Cinclus cinclus	23.1	31.8	28.2	16.8	648	16.2	28.2	23.1	32.4	216	42.1	28.7	14.8	14.4	216	11.1	38.4	46.8	3.7	216
AC-GQ481589|Cinclus pallasii	23.7	31.9	27.0	17.4	689	17.0	27.9	22.7	32.3	229	42.9	27.3	15.6	14.3	231	10.9	40.6	42.8	5.7	229
AC-JF498897|Pycnonotus cafer	25.0	32.9	25.8	16.3	693	17.3	28.1	22.9	31.6	231	42.4	27.7	15.6	14.3	231	15.2	42.9	39.0	3.0	231
AC-KX529958|Pycnonotus jocosus	25.2	31.9	26.6	16.3	655	17.0	27.5	22.9	32.6	218	41.7	28.9	15.1	14.2	218	16.9	39.3	41.6	2.3	219
AC-GQ482599|Regulus regulus	26.8	30.6	25.5	17.0	693	17.7	27.7	23.4	31.2	231	42.4	27.7	15.6	14.3	231	20.3	36.4	37.7	5.6	231
AC-GQ481547|Cettia cetti	25.4	32.6	24.4	17.6	693	16.9	28.1	23.8	31.2	231	42.4	27.7	15.6	14.3	231	16.9	42.0	33.8	7.4	231
AC-GU572031|Phylloscopus humei	23.8	33.2	26.4	16.7	648	15.7	28.2	23.6	32.4	216	42.1	28.7	14.8	14.4	216	13.4	42.6	40.7	3.2	216
AC-MK360480|Phylloscopus griseolus	25.0	30.9	27.0	17.2	693	16.5	26.4	25.5	31.6	231	41.6	28.6	15.2	14.7	231	16.9	37.7	40.3	5.2	231
AC-KU870799|Phylloscopus collybita	25.0	32.4	26.0	16.6	655	18.3	26.5	22.8	32.4	219	42.2	28.4	15.1	14.2	218	14.7	42.2	39.9	3.2	218

AC-GQ482461|Phylloscopus trochiloides	24.8	32.9	25.1	17.2	693	16.5	29.0	22.9	31.6	231	43.3	26.8	15.6	14.3	231	14.7	42.9	36.8	5.6	231
AC-HQ608867|Phylloscopus magnirostris	22.9	34.3	25.7	17.0	693	16.0	28.6	23.8	31.6	231	41.1	28.1	16.0	14.7	231	11.7	46.3	37.2	4.8	231
AC-HQ608865|Phylloscopus reguloides	23.2	33.9	26.1	16.7	693	16.0	28.6	23.8	31.6	231	40.7	28.6	16.0	14.7	231	13.0	44.6	38.5	3.9	231
AC-GQ481975|Iduna caligata	25.5	31.9	24.7	17.9	693	17.3	28.1	22.5	32.0	231	42.4	27.7	15.6	14.3	231	16.9	39.8	35.9	7.4	231
AC-KJ453177|Iduna rama	25.2	32.2	24.5	18.1	662	17.6	27.6	22.6	32.1	221	42.1	28.5	14.9	14.5	221	15.9	40.5	35.9	7.7	220
AC-KJ453167|Hippolais languida	24.3	33.8	23.0	18.9	662	19.5	25.8	22.6	32.1	221	42.1	28.5	14.9	14.5	221	11.4	47.3	31.4	10.0	220
AC-GQ481267|Acrocephalus melanopogon	24.1	33.6	25.4	16.9	693	16.5	29.0	22.5	32.0	231	42.4	27.7	15.6	14.3	231	13.4	44.2	38.1	4.3	231
AC-GU571212|Acrocephalus agricola	23.1	33.4	25.7	17.7	688	16.6	27.5	24.5	31.4	229	40.9	28.7	16.1	14.3	230	11.8	44.1	36.7	7.4	229
AC-KJ453132|Acrocephalus concinens	23.7	33.2	24.6	18.4	662	17.2	28.1	22.6	32.1	221	42.1	28.5	14.9	14.5	221	11.8	43.2	36.4	8.6	220
AC-AB893942|Acrocephalus dumetorum	25.1	30.9	26.0	18.0	693	18.6	26.0	22.9	32.5	231	41.6	28.1	15.6	14.7	231	15.2	38.5	39.4	6.9	231
AC-GQ481283|Acrocephalus scirpaceus	23.4	33.0	24.2	19.3	693	17.7	27.7	22.5	32.0	231	42.4	27.7	15.6	14.3	231	10.0	43.7	34.6	11.7	231
AC-FR847226|Acrocephalus arundinaceus	25.1	31.2	26.3	17.5	693	18.2	26.0	24.7	31.2	231	40.7	29.4	15.2	14.7	231	16.5	38.1	39.0	6.5	231
AC-KJ453157|Acrocephalus stentoreus	26.0	31.3	24.3	18.4	662	19.0	26.2	22.6	32.1	221	42.1	28.5	14.9	14.5	221	16.8	39.1	35.5	8.6	220
AC-JF957023|Megalurus palustris	24.3	32.3	26.5	16.9	682	18.9	26.8	23.7	30.7	228	42.3	27.8	15.4	14.5	227	11.9	42.3	40.5	5.3	227
AC-GU571955|Locustella naevia	23.1	32.6	26.9	17.4	648	15.7	28.2	23.6	32.4	216	41.7	29.2	14.8	14.4	216	12.0	40.3	42.1	5.6	216
AC-HQ608884|Prinia crinigera	25.1	30.4	27.8	16.6	693	18.2	26.8	24.7	30.3	231	40.7	28.6	16.0	14.7	231	16.5	35.9	42.9	4.8	231
AC-KU722459|Prinia gracilis	23.9	32.3	26.6	17.2	657	16.9	27.4	24.2	31.5	219	41.6	29.2	15.1	14.2	219	13.2	40.2	40.6	5.9	219
AC-HQ608883|Prinia flaviventris	24.2	31.2	28.4	16.2	693	18.6	25.5	25.5	30.3	231	40.7	28.6	16.0	14.7	231	13.4	39.4	43.7	3.5	231
AC-KT240052|Prinia inornata	24.9	31.6	25.7	17.8	607	15.8	27.2	25.7	31.2	202	42.6	29.2	13.9	14.4	202	16.3	38.4	37.4	7.9	203
AC-JQ176322|Sylvia nana	24.7	32.1	26.8	16.4	645	14.9	28.4	24.7	32.1	215	41.9	28.8	14.9	14.4	215	17.2	39.1	40.9	2.8	215
AC-GU572123|Sylvia nisoria	22.8	33.0	27.5	16.7	648	15.7	27.8	24.5	31.9	216	41.7	29.2	14.8	14.4	216	11.1	42.1	43.1	3.7	216
AC-GU571644|Sylvia crassirostris	23.4	32.9	27.4	16.3	693	16.5	27.7	24.7	31.2	231	41.6	27.7	16.0	14.7	231	12.1	43.3	41.6	3.0	231
AC-JQ176321|Sylvia mystacea	23.0	33.9	26.7	16.4	652	15.7	28.1	24.4	31.8	217	41.9	28.6	15.2	14.3	217	11.5	45.0	40.4	3.2	218
AC-GU572120|Sylvia communis	23.7	33.5	25.6	17.1	636	14.6	28.8	24.5	32.1	212	41.5	29.2	15.1	14.2	212	15.1	42.5	37.3	5.2	212
AC-JQ175951|Pomatorhinus erythrogenys	23.0	34.0	25.8	17.2	652	15.2	29.0	23.5	32.3	217	41.5	29.0	15.2	14.3	217	12.4	44.0	38.5	5.0	218
AC-MK069051|Laticilla burnesii	26.0	29.8	26.6	17.7	651	19.8	24.4	23.5	32.3	217	41.9	28.6	15.2	14.3	217	16.1	36.4	41.0	6.5	217
AC-JQ175707|Pellorneum ruficeps	22.9	32.8	27.5	16.9	652	16.1	28.1	23.5	32.3	217	41.9	28.6	15.2	14.3	217	10.6	41.7	43.6	4.1	218
AC-JQ173957|Alcippe poioicephala	23.8	31.9	28.1	16.3	652	15.2	29.0	24.0	31.8	217	41.5	29.0	15.2	14.3	217	14.7	37.6	45.0	2.8	218
AC-MH265887|Argya earlei earlei	22.8	33.1	27.1	17.0	631	14.8	29.0	23.8	32.4	210	41.4	29.5	15.2	13.8	210	12.3	40.8	42.2	4.7	211
AC-KC439341|Turdoides malcolmi	24.1	33.0	26.4	16.5	594	15.7	28.8	24.2	31.3	198	40.9	29.8	16.2	13.1	198	15.7	40.4	38.9	5.1	198
AC-MH265810|Trochalopteron erythrocephalum woodi	24.2	32.1	26.1	17.7	633	16.6	27.5	23.7	32.2	211	41.2	29.4	15.2	14.2	211	14.7	39.3	39.3	6.6	211
AC-EU447047|Heterophasia capistrata nigriceps	23.4	32.7	27.3	16.6	615	15.6	28.8	23.9	31.7	205	41.0	29.8	15.6	13.7	205	13.7	39.5	42.4	4.4	205
AC-GQ482226|Muscicapa sibirica	25.3	33.2	24.4	17.2	693	17.3	28.1	22.9	31.6	231	42.9	27.3	15.6	14.3	231	15.6	44.2	34.6	5.6	231
AC-JF498868|Leiothrix lutea	24.8	32.2	26.3	16.7	693	17.3	28.1	22.9	31.6	231	42.4	27.7	15.6	14.3	231	14.7	40.7	40.3	4.3	231
AC-GQ482216|Muscicapa dauurica	25.0	33.5	24.4	17.2	693	17.3	28.1	22.9	31.6	231	42.9	27.3	15.6	14.3	231	14.7	45.0	34.6	5.6	231
AC-GU571987|Muscicapa striata	25.0	33.6	23.5	17.9	648	16.7	27.3	23.1	32.9	216	42.1	28.7	14.8	14.4	216	16.2	44.9	32.4	6.5	216

AC-MF580191|Cercotrichas galactotes	28.4	28.9	25.7	17.0	658	18.3	26.0	23.7	32.0	219	42.0	28.8	15.1	14.2	219	25.0	31.8	38.2	5.0	220
AC-JF498845|Copsychus malabaricus	25.4	32.0	25.3	17.3	693	18.2	27.3	22.9	31.6	231	42.9	27.3	15.6	14.3	231	15.2	41.6	37.2	6.1	231
AC-JQ174627|Cyornis rubeculoides	24.1	34.2	24.2	17.5	652	15.7	28.6	24.0	31.8	217	41.9	28.6	15.2	14.3	217	14.7	45.4	33.5	6.4	218
AC-JQ175559|Niltava sundara	23.8	33.3	25.8	17.2	652	16.6	27.2	23.5	32.7	217	41.9	28.6	15.2	14.3	217	12.8	44.0	38.5	4.6	218
AC-EF422241|Muscicapa thalassina	24.5	33.2	25.4	16.9	693	17.3	26.8	24.7	31.2	231	40.7	28.6	16.0	14.7	231	15.6	44.2	35.5	4.8	231
AC-JQ175293|Luscinia megarhynchos	25.2	33.6	24.7	16.6	652	16.1	28.1	23.5	32.3	217	41.5	29.0	15.2	14.3	217	17.9	43.6	35.3	3.2	218
AC-KC789641|Luscinia svecica	23.8	34.2	23.9	18.1	602	14.9	27.9	24.4	32.8	201	41.8	29.9	14.4	13.9	201	14.5	45.0	33.0	7.5	200
AC-KU973748|Calliope pectoralis	23.4	34.2	25.1	17.2	692	16.9	28.1	22.5	32.5	231	41.6	28.1	15.2	15.2	231	11.7	46.5	37.8	3.9	230
AC-GQ482758|Tarsiger cyanurus	24.2	34.5	24.5	16.7	693	17.3	28.1	23.4	31.2	231	42.9	27.3	15.6	14.3	231	12.6	48.1	34.6	4.8	231
AC-JQ176404|Tarsiger chrysaeus	23.9	34.2	24.2	17.6	652	15.2	29.0	23.0	32.7	217	41.9	28.6	15.2	14.3	217	14.7	45.0	34.4	6.0	218
AC-JQ174846|Ficedula strophiata	23.8	33.1	25.1	18.0	650	15.7	27.8	23.1	33.3	216	41.9	28.6	15.2	14.3	217	13.8	42.9	36.9	6.5	217
AC-GQ481891|Ficedula albicilla	23.7	33.6	25.5	17.2	693	17.7	27.7	22.5	32.0	231	42.9	27.3	15.6	14.3	231	10.4	45.9	38.5	5.2	231
AC-GU571893|Ficedula parva	22.7	34.1	25.5	17.7	648	15.7	28.2	23.1	32.9	216	42.1	28.7	14.8	14.4	216	10.2	45.4	38.4	6.0	216
AC-JX970703|Phoenicurus frontalis	23.7	34.8	24.8	16.7	693	16.9	27.7	23.8	31.6	231	40.7	28.1	16.5	14.7	231	13.4	48.5	34.2	3.9	231
AC-GQ482382|Phoenicurus erythronotus	26.0	32.6	25.0	16.5	693	17.7	27.7	22.9	31.6	231	42.4	27.7	15.6	14.3	231	17.7	42.4	36.4	3.5	231
AC-GU572026|Phoenicurus phoenicurus	24.2	34.1	23.9	17.7	648	15.7	28.2	23.1	32.9	216	41.7	29.2	14.8	14.4	216	15.3	44.9	33.8	6.0	216
AC-GQ482376|Phoenicurus erythrogastrus	24.0	34.9	24.8	16.3	693	16.9	28.6	22.5	32.0	231	42.4	27.7	15.6	14.3	231	12.6	48.5	36.4	2.6	231
AC-GQ482171|Monticola saxatilis	25.3	33.5	24.5	16.7	693	17.3	28.1	22.5	32.0	231	42.9	27.3	15.6	14.3	231	15.6	45.0	35.5	3.9	231
AC-JQ175415|Monticola solitarius	24.3	33.7	24.8	17.2	638	16.0	27.2	23.5	33.3	213	41.8	29.1	14.6	14.6	213	15.1	44.8	36.3	3.8	212
AC-GQ482623|Saxicola maurus	25.4	33.6	23.2	17.7	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	16.9	44.6	31.6	6.9	231
AC-JQ176180|Saxicola caprata	24.2	34.4	23.8	17.6	652	15.7	28.6	23.0	32.7	217	41.9	28.6	15.2	14.3	217	15.1	45.9	33.0	6.0	218
AC-JQ176181|Saxicola ferreus	24.1	34.7	23.9	17.3	652	16.6	27.6	23.0	32.7	217	41.9	28.6	15.2	14.3	217	13.8	47.7	33.5	5.0	218
AC-DQ683479|Oenanthe alboniger	23.7	35.8	23.4	17.1	667	16.2	28.8	23.4	31.5	222	42.8	27.5	15.8	14.0	222	12.1	51.1	30.9	5.8	223
AC-GU571994|Oenanthe oenanthe	24.1	35.0	24.1	16.8	648	16.7	27.3	23.1	32.9	216	42.1	28.7	14.8	14.4	216	13.4	49.1	34.3	3.2	216
AC-HM046870|Oenanthe lugens	24.4	35.1	23.7	16.9	616	15.0	29.1	23.8	32.0	206	41.5	29.3	15.6	13.7	205	16.6	46.8	31.7	4.9	205
AC-MF795487|Oenanthe finschii	23.5	36.5	24.2	15.8	620	17.0	29.1	23.3	30.6	206	42.2	28.2	16.0	13.6	206	11.5	51.9	33.2	3.4	208
AC-GU571995|Oenanthe pleschanka	25.0	34.0	23.9	17.1	648	16.7	27.3	23.1	32.9	216	42.1	28.7	14.8	14.4	216	16.2	45.8	33.8	4.2	216
AC-JX255949|Oenanthe xanthoprymna	23.9	35.3	23.1	17.6	648	15.7	28.2	23.6	32.4	216	42.1	28.7	14.8	14.4	216	13.9	49.1	31.0	6.0	216
AC-KP252229|Oenanthe deserti	23.5	35.3	24.2	17.0	652	16.6	27.6	23.0	32.7	217	41.9	28.6	15.2	14.3	217	11.9	49.5	34.4	4.1	218
AC-JF498802|Oenanthe isabellina	24.1	34.9	24.1	16.9	693	16.9	28.6	22.5	32.0	231	42.9	27.3	15.6	14.3	231	12.6	48.9	34.2	4.3	231
AC-EF515802|Zoothera dauma	24.3	34.3	25.1	16.3	686	16.6	28.4	23.1	31.9	229	41.9	28.4	15.3	14.4	229	14.5	46.1	36.8	2.6	228
AC-GQ482883|Turdus viscivorus	25.0	33.9	24.4	16.7	693	17.3	28.1	22.9	31.6	231	42.4	27.7	15.6	14.3	231	15.2	45.9	34.6	4.3	231
AC-GU572145|Turdus iliacus	24.1	33.6	25.2	17.1	648	16.2	27.8	23.6	32.4	216	41.7	29.2	14.8	14.4	216	14.4	44.0	37.0	4.6	216
AC-MK262687|Turdus merula	23.9	33.5	25.8	16.8	648	15.7	28.2	23.6	32.4	216	41.7	29.2	15.3	13.9	216	14.4	43.1	38.4	4.2	216
AC-GQ482870|Turdus ruficollis	24.7	33.3	25.1	16.9	693	17.3	28.1	22.9	31.6	231	42.4	27.7	15.6	14.3	231	14.3	44.2	36.8	4.8	231
AC-KC439338|Sturnus contra	22.8	33.8	26.3	17.1	624	16.3	29.3	22.1	32.2	208	41.8	28.4	16.3	13.5	208	10.1	43.8	40.4	5.8	208

AC-EU525542|Sturnus pagodarum	22.7	33.3	26.3	17.7	693	16.0	26.8	25.1	32.0	231	41.6	28.6	15.2	14.7	231	10.4	44.6	38.5	6.5	231
AC-JQ176301|Sturnus malabaricus	22.5	34.0	25.5	17.9	652	15.2	29.0	23.0	32.7	217	41.9	28.6	15.2	14.3	217	10.6	44.5	38.1	6.9	218
AC-AY666196|Acridotheres tristis	24.3	33.0	25.2	17.5	691	17.0	28.3	22.6	32.2	230	42.6	27.4	15.7	14.3	230	13.4	43.3	37.2	6.1	231
AC-EF484196|Acridotheres fuscus	24.1	32.0	26.4	17.5	693	16.9	26.0	25.1	32.0	231	41.6	28.6	15.2	14.7	231	13.9	41.6	39.0	5.6	231
AC-KJ442637|Dicaeum erythrorhynchos	24.6	31.5	26.1	17.8	658	17.4	26.9	23.3	32.4	219	42.0	28.8	15.1	14.2	219	14.5	38.6	40.0	6.8	220
AC-MH929095|Aethopyga siparaja siparaja	24.2	32.7	26.1	17.0	652	15.2	29.0	23.0	32.7	217	41.9	28.6	15.2	14.3	217	15.6	40.4	39.9	4.1	218
AC-AB843102|Prunella collaris	25.7	31.5	26.3	16.6	693	17.8	27.4	23.0	31.7	230	42.7	27.6	15.5	14.2	232	16.5	39.4	40.3	3.9	231
AC-GQ482565|Prunella himalayana	24.7	31.9	26.3	17.1	689	17.5	27.5	23.1	31.9	229	42.6	27.4	15.7	14.3	230	13.9	40.9	40.0	5.2	230
AC-GQ482563|Prunella fulvescens	26.0	31.0	25.1	17.9	693	17.3	28.1	22.5	32.0	231	42.9	27.3	15.6	14.3	231	17.7	37.7	37.2	7.4	231
AC-GQ482560|Prunella atrogularis	25.5	31.3	25.4	17.7	693	17.7	27.7	22.1	32.5	231	42.9	27.3	15.6	14.3	231	16.0	39.0	38.5	6.5	231
AC-JF957025|Motacilla cinerea	25.3	32.0	26.0	16.7	693	16.9	28.6	22.5	32.0	231	42.4	27.7	15.6	14.3	231	16.5	39.8	39.8	3.9	231
AC-GU571985|Motacilla flava	23.8	33.2	26.7	16.4	648	15.7	28.2	23.1	32.9	216	41.7	29.2	14.8	14.4	216	13.9	42.1	42.1	1.9	216
AC-GQ482203|Motacilla citreola	26.4	30.7	26.4	16.5	693	17.3	28.1	22.5	32.0	231	42.4	27.7	15.6	14.3	231	19.5	36.4	41.1	3.0	231
AC-KY754516|Motacilla alba	25.3	31.9	26.1	16.7	693	16.9	28.6	22.5	32.0	231	42.4	27.7	15.6	14.3	231	16.5	39.4	40.3	3.9	231
AC-GQ481341|Anthus godlewskii	24.7	32.8	26.0	16.6	693	17.3	28.1	23.4	31.2	231	42.4	27.7	15.6	14.3	231	14.3	42.4	39.0	4.3	231
AC-KP252167|Anthus campestris	24.8	32.5	26.1	16.6	652	15.7	28.1	24.4	31.8	217	41.5	29.0	15.2	14.3	217	17.4	40.4	38.5	3.7	218
AC-GU571732|Anthus pratensis	22.4	34.3	26.1	17.3	648	15.3	28.7	23.6	32.4	216	42.1	28.7	14.8	14.4	216	9.7	45.4	39.8	5.1	216
AC-GQ481349|Anthus hodgsoni	23.5	33.9	26.0	16.6	693	16.9	28.1	22.5	32.5	231	42.4	27.7	15.6	14.3	231	11.3	45.9	39.8	3.0	231
AC-GU571250|Anthus cervinus	23.0	33.2	26.4	17.4	660	15.9	28.2	23.6	32.3	220	41.8	28.6	15.0	14.5	220	11.4	42.7	40.5	5.5	220
AC-GQ481365|Anthus spinoletta	22.8	34.2	25.3	17.7	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	9.1	46.3	37.7	6.9	231
AC-GQ481358|Anthus rubescens	22.9	34.6	25.8	16.6	693	16.5	29.0	22.5	32.0	231	42.9	27.3	15.6	14.3	231	9.5	47.6	39.4	3.5	231
AC-GU571754|Bombycilla garrulus	27.2	29.8	27.0	16.0	648	18.5	25.5	23.6	32.4	216	41.2	29.6	14.8	14.4	216	21.8	34.3	42.6	1.4	216
AC-KP252196|Hypocolius ampelinus	26.8	29.9	26.7	16.6	679	16.7	26.4	25.1	31.7	227	41.6	28.3	15.5	14.6	226	22.1	35.0	39.4	3.5	226
AC-MK262511|Fringilla coelebs	26.0	30.6	25.8	17.6	647	16.2	27.8	23.1	32.9	216	42.1	28.7	14.8	14.4	216	19.5	35.3	39.5	5.6	215
AC-GU571404|Fringilla montifringilla	24.7	31.9	26.7	16.7	693	16.9	27.7	23.4	32.0	231	41.6	28.6	15.6	14.3	231	15.6	39.4	41.1	3.9	231
AC-GU571829|Coccothraustes coccothraustes	24.8	30.9	27.6	16.7	648	16.2	27.8	23.6	32.4	216	42.1	28.7	14.8	14.4	216	16.2	36.1	44.4	3.2	216
AC-GQ481529|Carpodacus rubicilla	24.4	31.7	26.8	17.0	675	16.9	28.0	23.1	32.0	225	42.2	28.0	15.6	14.2	225	14.2	39.1	41.8	4.9	225
AC-EU847701|Carpodacus thura	24.0	32.0	26.8	17.2	693	16.9	26.0	25.1	32.0	231	41.6	28.6	15.2	14.7	231	13.4	41.6	40.3	4.8	231
AC-GQ482608|Eremopsaltria mongolica	23.7	32.9	26.8	16.6	693	17.7	27.7	22.5	32.0	231	42.9	27.3	15.6	14.3	231	10.4	43.7	42.4	3.5	231
AC-GQ482053|Leucosticte nemoricola	24.7	31.9	26.4	16.9	692	16.9	28.6	22.5	32.0	231	42.9	27.3	15.6	14.3	231	14.3	40.0	41.3	4.3	230
AC-GQ482049|Leucosticte brandti	23.6	33.1	26.9	16.4	685	17.1	27.6	22.8	32.5	228	42.5	27.6	15.4	14.5	228	11.4	44.1	42.4	2.2	229
AC-FJ465359|Rhodospiza obsoleta	24.8	32.3	26.1	16.9	610	15.8	29.1	23.2	32.0	203	41.9	29.1	15.8	13.3	203	16.7	38.7	39.2	5.4	204
AC-GQ481479|Linaria flavirostris	25.7	32.3	26.0	16.0	693	17.3	28.6	22.5	31.6	231	42.9	27.3	15.6	14.3	231	16.9	41.1	39.8	2.2	231
AC-GQ481455|Linaria cannabina	26.0	31.9	25.7	16.5	693	17.3	28.1	22.5	32.0	231	42.9	27.3	15.6	14.3	231	17.7	40.3	39.0	3.0	231
AC-GU571958|Loxia-curvirostra	24.8	32.3	26.4	16.5	648	15.7	28.2	23.6	32.4	216	42.1	28.7	14.8	14.4	216	16.7	39.8	40.7	2.8	216
AC-MK262089|Carduelis carduelis	24.1	33.0	26.1	16.8	648	16.7	27.3	23.1	32.9	216	42.1	28.7	14.8	14.4	216	13.4	43.1	40.3	3.2	216
AC-GQ482639|Serinus pusillus	25.3	32.3	26.2	16.2	684	17.5	27.6	22.8	32.0	228	42.1	27.6	15.8	14.5	228	16.2	41.7	39.9	2.2	228

AC-GQ481495|Spinus spinus	24.9	32.6	26.4	16.1	666	16.7	28.4	23.0	32.0	222	41.9	27.9	15.8	14.4	222	16.2	41.4	40.5	1.8	222
AC-JQ174776|Emberiza melanocephala	24.4	33.0	25.2	17.5	652	16.1	28.1	23.5	32.3	217	41.9	28.6	15.2	14.3	217	15.1	42.2	36.7	6.0	218
AC-KC439313|Emberiza bruniceps	23.8	34.3	25.3	16.6	661	15.8	29.9	23.1	31.2	221	42.7	27.7	15.9	13.6	220	12.7	45.5	36.8	5.0	220
AC-GU571867|Emberiza calandra	23.1	34.0	25.9	17.0	648	15.7	28.2	23.1	32.9	216	42.1	28.7	14.8	14.4	216	11.6	44.9	39.8	3.7	216
AC-JF499130|Emberiza fucata	24.0	34.4	25.0	16.6	649	17.1	29.2	22.7	31.0	216	43.1	27.3	16.2	13.4	216	12.0	46.5	35.9	5.5	217
AC-GQ481747|Emberiza cia	24.1	34.1	24.9	17.0	684	17.1	27.6	22.8	32.5	228	42.5	27.6	15.4	14.5	228	12.7	46.9	36.4	3.9	228
AC-GQ481766|Emberiza godlewskii	24.1	33.8	25.0	17.1	692	17.0	28.3	22.6	32.2	230	42.9	27.3	15.6	14.3	231	12.6	45.9	36.8	4.8	231
AC-KP877676|Emberiza stewarti	24.8	33.2	25.1	16.9	693	18.2	27.3	22.5	32.0	231	42.9	27.3	15.6	14.3	231	13.4	45.0	37.2	4.3	231
AC-GQ481772|Emberiza leucocephalos	23.8	33.9	24.7	17.6	693	17.3	28.1	22.5	32.0	231	42.9	27.3	15.6	14.3	231	11.3	46.3	35.9	6.5	231
AC-MF580175|Emberiza striolata	24.0	34.3	24.5	17.2	658	16.9	27.9	23.7	31.5	219	42.0	28.8	15.1	14.2	219	13.2	46.4	34.5	5.9	220
AC-GU571874|Emberiza schoeniclus	23.5	34.4	25.2	17.0	648	15.3	28.7	23.1	32.9	216	42.1	28.7	14.8	14.4	216	13.0	45.8	37.5	3.7	216
AC-EF515786|Emberiza aureola	24.3	32.7	25.4	17.6	686	17.0	27.9	22.7	32.3	229	42.4	27.9	15.3	14.4	229	13.6	42.1	38.2	6.1	228
AC-GU571872|Emberiza pusilla	23.8	33.2	25.5	17.6	648	15.7	28.2	23.1	32.9	216	42.1	28.7	14.8	14.4	216	13.4	42.6	38.4	5.6	216
AC-GQ481805|Emberiza rutila	25.4	32.2	25.3	17.2	693	18.6	26.8	22.5	32.0	231	42.9	27.3	15.6	14.3	231	14.7	42.4	37.7	5.2	231
AC-MK262682|Passer domesticus	23.6	31.2	26.9	18.4	648	16.7	27.3	23.1	32.9	216	41.7	29.2	14.8	14.4	216	12.5	37.0	42.6	7.9	216
AC-MF767304|Passer moabiticus	22.7	31.7	28.2	17.4	691	17.0	28.7	22.6	31.7	230	42.2	27.8	15.7	14.3	230	9.1	38.5	46.3	6.1	231
AC-JF957028|Passer montanus	23.4	31.6	27.4	17.6	675	16.9	28.4	22.7	32.0	225	41.8	28.4	15.6	14.2	225	11.6	37.8	44.0	6.7	225
AC-GQ482355|Petronia petronia	24.6	30.5	28.0	16.9	679	16.8	27.9	22.6	32.7	226	42.0	27.9	15.5	14.6	226	15.0	35.7	45.8	3.5	227
AC-FJ465315|Carpospiza brachydactyla	22.9	32.9	27.8	16.4	608	15.8	28.7	23.3	32.2	202	41.4	30.0	15.3	13.3	203	11.3	39.9	44.8	3.9	203
AC-GQ482176|Montifringilla nivalis	24.6	31.0	26.4	18.1	675	16.0	28.4	23.1	32.4	225	42.2	28.0	15.6	14.2	225	15.6	36.4	40.4	7.6	225
AC-MF580167|Lonchura malabarica	23.7	31.5	28.9	16.0	658	16.9	27.4	23.3	32.4	219	42.0	28.8	15.1	14.2	219	12.3	38.2	48.2	1.4	220
AC-JF498874|Lonchura punctulata	23.3	32.1	28.6	16.0	669	16.6	28.3	22.9	32.3	223	42.6	27.8	15.7	13.9	223	10.8	40.4	47.1	1.8	223
AC-KX283124|Lanius minor	27.0	28.2	26.9	17.9	655	17.4	27.5	23.9	31.2	218	41.1	28.8	15.1	15.1	219	22.5	28.4	41.7	7.3	218
Avg.	24.5	32.5	25.9	17.1	670.7	16.8	28.0	23.4	31.8	223.5	42.1	28.2	15.4	14.3	223.5	14.6	41.4	38.9	5.1	223.7

Appendix E

Estimates of Evolutionary Divergence between Sequences of Passeriformes of Pakistan.

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Title: ALL PASSERIFORMES 24-7-23
Description
  Analysis ====================
    Analysis                                = ====================
      Scope                                 = Pairs of taxa
    Estimate Variance                       = ====================
      Variance Estimation Method            = None
    Substitution Model                      = ====================
      Substitutions Type                    = Nucleotide
      Model/Method                          = Kimura 2-parameter model
      Substitutions to Include              = d: Transitions + Transversions
    Rates and Patterns                      = ====================
      Rates among Sites                     = Gamma Distributed (G)
        Gamma Parameter                     = 1.00
      Pattern among Lineages                = Same (Homogeneous)
    Data Subset to Use                      = ====================
      Gaps/Missing Data Treatment           = Pairwise deletion
      Select Codon Positions                = 1st,2nd,3rd,Non-Coding
  No. of Sites = 992
  d = Estimate

[  1] #BOLD:AAF5733|Lanius_vittatus
[  2] #BOLD:ACZ2474|Prinia_buchanani
[  3] #BOLD:ACZ2564|Alauda_gulgula_australis
[  4] #BOLD:AAD9870|Galerida_cristata_arenicola
[  5] #BOLD:ABX5008|Passer_hispaniolensis
[  6] #BOLD:AAC0536|Sylvia_curruca_curruca
[  7] #BOLD:AAU3934|Tephrodornis_pondicerianus
[  8] #BOLD:ACH6125|Dendrocitta_vagabunda_saturatior
[  9] #BOLD:AAR9140|Corvus_splendens_splendens
[ 10] #BOLD:AAC1536|Phoenicurus_ochruros
[ 11] #BOLD:AAV9282|Chrysomma_sinense
[ 12] #BOLD:ABZ8019|Lanius_schach_schach
[ 13] #BOLD:ACZ2475|Prinia_socialis_socialis
[ 14] #BOLD:AAX4494|Melanocorypha_bimaculata
[ 15] #BOLD:ACE4748|Acridotheres_ginginianus
[ 16] #BOLD:ACS3101|Cisticola_juncidis
[ 17] #BOLD:AAE0119|Pastor_roseus
[ 18] #BOLD:ACZ1830|Emberiza_buchanani
[ 19] #BOLD:ACH8855|Eremopterix_griseus
[ 20] #BOLD:AAB3874|Carpodacus_erythrinus
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[ 21] #BOLD:ACZ2757|Oriolus_oriolus
[ 22] #BOLD:AAB5621|Corvus_corax_corax
[ 23] #BOLD:AAL2706|Pericrocotus_cinnamomeus
[ 24] #BOLD:ACZ2474|Prinia_hodgsonii
[ 25] #BOLD:AAB2042|Zosterops_palpebrosus_palpebrosus
[ 26] #BOLD:AAN3860|Pycnonotus_leucogenys
[ 27] #BOLD:ACH0131|Phylloscopus_affinis
[ 28] #BOLD:ABW5160|Corvus_machorynchus
[ 29] #BOLD:ACE7266|Copsychus_saularis_saularis
[ 30] #BOLD:AAY1381|Trochalopteron_lineatum_lineatum
[ 31] #BOLD:ACZ3140|Parus_major
[ 32] #BOLD:ACZ2846|Urocissa_flavirostris_flavirostris
[ 33] #BOLD:ACZ2835|Stachyridopsis_pyrrhops
[ 34] #BOLD:ACS4664|Terpsiphone_paradisi_paradisi
[ 35] #BOLD:AAC5713|Myophonus_caeruleus
[ 36] #BOLD:ACZ2808|Hypsipetes_leucocephalus
[ 37] #BOLD:AAI9717|Dicrurus_leucophaeus
[ 38] #BOLD:ACZ3094|Emberiza_lathami
[ 39] #BOLD:AAC1399|Passer_cinnamomeus_rutilans
[ 40] #BOLD:ABW5160|Corvus_macrorhynchos
[ 41] #BOLD:ACH8281|Orthotomus_sutorius
[ 42] #BOLD:AAW9936|Oenanthe_picata
[ 43] #BOLD:ACZ2562|Copsychus_fulicatus
[ 44] #AC-_AB843703|Pitta_brachyura
[ 45] #AC-JQ173906|Aegithina_tiphia
[ 46] #AC-MH938034|Lanius_collurio
[ 47] #AC-GQ482020|Lanius_isabellinus
[ 48] #AC-GQ482013|Lanius_cristatus
[ 49] #AC-EF621598|Lanius_tephronotus
[ 50] #AC-JF498786|Lanius_excubitor
[ 51] #AC-GQ482278|Oriolus_chinensis
[ 52] #AC-JQ174696|Dicrurus_macrocercus
[ 53] #AC-JQ174691|Dicrurus_hottentottus
[ 54] #AC-JQ176131|Rhipidura_aureola
[ 55] #AC-KC354929|Hypothymis_azurea
[ 56] #AC-GQ481963|Garrulus_glandarius
[ 57] #AC-JQ176603|Urocissa_erythrorhyncha
[ 58] #AC-GQ482478|Pica_pica
[ 59] #AC-GU571501|Nucifraga_caryocatactes
[ 60] #AC-GQ482576|Pyrrhocorax_pyrrhocorax
[ 61] #AC-GQ482571|Pyrrhocorax_graculus
[ 62] #AC-GQ481647|Corvus_monedula
[ 63] #AC-GQ481640|Corvus_frugilegus
[ 64] #AC-GU571521|Panurus_biarmicus
[ 65] #AC-FJ465300|Ammomanes_deserti
[ 66] #AC-MF580208|Eremopterix_nigriceps
[ 67] #AC-GQ481854|Eremophila_alpestris
[ 68] #AC-GQ481412|Calandrella_acutirostris
[ 69] #AC-GQ481420|Calandrella_rufescens
[ 70] #AC-GQ482616|Riparia_riparia
[ 71] #AC-GQ482609|Riparia_diluta
[ 72] #AC-GQ482570|Ptyonoprogne_rupestris
[ 73] #AC-MF580225|Ptyonoprogne_fuligula
[ 74] #AC-KY754510|Hirundo_rustica
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[ 75] #AC-GU460335|Hirundo_smithii
[ 76] #AC-GQ481533|Cecropis_daurica
[ 77] #AC-GQ481695|Delichon_urbicum
[ 78] #AC-GQ481692|Delichon_dasypus
[ 79] #AC-JQ176135|Rhipidura_hypoxantha
[ 80] #AC-JQ174591|Culicicapa_ceylonensis
[ 81] #AC-JX221707|Cephalopyrus_flammiceps
[ 82] #AC-JX508791|Periparus_ater
[ 83] #AC-HQ228194|Parus_rubidiventris
[ 84] #AC-HM185314|Lophophanes_dichrous
[ 85] #AC-GQ481683|Cyanistes_cyanus
[ 86] #AC-JX849735|Parus_monticolus
[ 87] #AC-GU572079|Remiz_pendulinus
[ 88] #AC-HQ605288|Aegithalos_concinnus
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[ 90] #AC-KJ467157|Sitta_tephronota
[ 91] #AC-KJ467146|Sitta_frontalis
[ 92] #AC-GQ482777|Chodroma_muraria
[ 93] #AC-KP282529|Certhia_hodgsoni
[ 94] #AC-KP772837|Troglodytes_troglodytes
[ 95] #AC-GU571819|Cinclus_cinclus
[ 96] #AC-GQ481589|Cinclus_pallasii
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[ 98] #AC-KX529958|Pycnonotus_jocosus
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[103] #AC-KU870799|Phylloscopus_collybita
[104] #AC-GQ482461|Phylloscopus_trochiloides
[105] #AC-HQ608867|Phylloscopus_magnirostris
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[107] #AC-GQ481975|Iduna_caligata
[108] #AC-KJ453177|Iduna_rama
[109] #AC-KJ453167|Hippolais_languida
[110] #AC-GQ481267|Acrocephalus_melanopogon
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[115] #AC-FR847226|Acrocephalus_arundinaceus
[116] #AC-KJ453157|Acrocephalus_stentoreus
[117] #AC-JF957023|Megalurus_palustris
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[120] #AC-KU722459|Prinia_gracilis
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[127] #AC-GU572120|Sylvia_communis
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[133] #AC-KC439341|Turdoides_malcolmi
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[135] #AC-EU447047|Heterophasia_capistrata_nigriceps
[136] #AC-GQ482226|Muscicapa_sibirica
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[155] #AC-GQ482376|Phoenicurus_erythrogastrus
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[193] #AC-GQ481349|Anthus_hodgsoni
[194] #AC-GU571250|Anthus_cervinus
[195] #AC-GQ481365|Anthus_spinoletta
[196] #AC-GQ481358|Anthus_rubescens
[197] #AC-GU571754|Bombycilla_garrulus
[198] #AC-KP252196|Hypocolius_ampelinus
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[203] #AC-EU847701|Carpodacus_thura
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[205] #AC-GQ482053|Leucosticte_nemoricola
[206] #AC-GQ482049|Leucosticte_brandti
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[231] #AC-FJ465315|Carpospiza_brachydactyla
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[53] 0.22 0.23 0.25 0.22 0.20 0.18 0.19 0.19 0.17 0.24 0.21 0.21 0.22 0.23 0.20 0.19 0.18 0.23 0.24 0.22 0.21 0.17 0.21 0.23 0.20 0.22 0.22 0.17 0.21 0.21 0.22 0.19 0.21 0.18 0.20 0.19 0.13 0.22 0.19 0.23 0.20 0.23
0.22 0.21 0.17 0.21 0.21 0.20 0.20 0.20 0.22 0.14
[54] 0.15 0.21 0.19 0.20 0.18 0.19 0.14 0.17 0.15 0.17 0.19 0.15 0.22 0.21 0.16 0.17 0.17 0.18 0.17 0.21 0.17 0.15 0.16 0.21 0.18 0.17 0.19 0.15 0.20 0.19 0.20 0.15 0.20 0.16 0.18 0.18 0.15 0.19 0.19 0.22 0.18 0.19
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[55] 0.15 0.17 0.20 0.18 0.18 0.18 0.17 0.17 0.14 0.19 0.20 0.16 0.20 0.19 0.15 0.16 0.16 0.19 0.18 0.18 0.18 0.15 0.17 0.17 0.18 0.18 0.19 0.14 0.20 0.17 0.17 0.17 0.19 0.12 0.18 0.19 0.16 0.18 0.18 0.18 0.17 0.20
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[56] 0.13 0.22 0.20 0.17 0.21 0.18 0.18 0.17 0.13 0.21 0.18 0.14 0.21 0.21 0.20 0.19 0.20 0.21 0.19 0.20 0.18 0.12 0.21 0.21 0.20 0.19 0.18 0.12 0.22 0.16 0.20 0.14 0.16 0.17 0.22 0.19 0.18 0.17 0.19 0.21 0.18 0.20
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[57] 0.14 0.19 0.18 0.17 0.18 0.17 0.17 0.14 0.15 0.19 0.18 0.15 0.20 0.18 0.18 0.16 0.18 0.19 0.19 0.20 0.17 0.11 0.18 0.19 0.19 0.15 0.21 0.12 0.21 0.16 0.18 0.04 0.16 0.17 0.18 0.17 0.18 0.18 0.18 0.19 0.17 0.18
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[58] 0.13 0.20 0.21 0.17 0.21 0.17 0.18 0.16 0.13 0.18 0.17 0.17 0.19 0.21 0.20 0.15 0.19 0.20 0.18 0.21 0.20 0.12 0.18 0.19 0.19 0.18 0.19 0.11 0.21 0.16 0.18 0.12 0.18 0.15 0.19 0.19 0.17 0.19 0.18 0.19 0.16 0.18
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[59] 0.14 0.20 0.20 0.15 0.18 0.15 0.18 0.16 0.14 0.18 0.19 0.14 0.18 0.18 0.18 0.17 0.16 0.17 0.18 0.18 0.18 0.11 0.18 0.19 0.18 0.16 0.18 0.11 0.21 0.15 0.18 0.14 0.17 0.16 0.17 0.20 0.15 0.17 0.17 0.18 0.15 0.18
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[60] 0.15 0.19 0.23 0.19 0.19 0.17 0.18 0.18 0.15 0.19 0.22 0.16 0.18 0.20 0.18 0.20 0.18 0.18 0.18 0.20 0.19 0.16 0.19 0.19 0.19 0.17 0.19 0.15 0.23 0.17 0.20 0.18 0.18 0.16 0.19 0.19 0.16 0.18 0.19 0.20 0.18 0.19
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[61] 0.15 0.19 0.21 0.17 0.16 0.16 0.18 0.16 0.13 0.18 0.20 0.15 0.20 0.20 0.17 0.17 0.18 0.17 0.19 0.18 0.17 0.13 0.19 0.19 0.16 0.15 0.17 0.13 0.21 0.14 0.17 0.16 0.19 0.14 0.18 0.15 0.13 0.16 0.17 0.18 0.16 0.17
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[62] 0.12 0.17 0.21 0.18 0.16 0.16 0.18 0.14 0.09 0.18 0.18 0.13 0.19 0.20 0.18 0.15 0.17 0.20 0.18 0.20 0.16 0.08 0.18 0.16 0.16 0.15 0.19 0.08 0.21 0.16 0.19 0.12 0.16 0.13 0.19 0.16 0.15 0.18 0.14 0.18 0.16 0.19
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[63] 0.14 0.19 0.21 0.19 0.18 0.17 0.18 0.15 0.08 0.18 0.20 0.13 0.19 0.19 0.18 0.16 0.19 0.20 0.19 0.21 0.18 0.07 0.18 0.18 0.17 0.16 0.19 0.08 0.20 0.18 0.19 0.13 0.17 0.16 0.20 0.17 0.16 0.18 0.15 0.19 0.16 0.21
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[64] 0.17 0.16 0.16 0.16 0.18 0.18 0.20 0.19 0.18 0.15 0.17 0.16 0.16 0.17 0.18 0.21 0.16 0.17 0.17 0.19 0.19 0.16 0.19 0.17 0.14 0.14 0.20 0.17 0.16 0.14 0.17 0.17 0.18 0.19 0.17 0.15 0.18 0.16 0.18 0.17 0.19 0.18
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[65] 0.18 0.18 0.14 0.15 0.18 0.19 0.23 0.20 0.22 0.19 0.15 0.19 0.23 0.13 0.20 0.17 0.18 0.18 0.16 0.19 0.17 0.18 0.20 0.19 0.17 0.17 0.18 0.20 0.20 0.16 0.17 0.19 0.21 0.22 0.19 0.20 0.24 0.17 0.19 0.14 0.19 0.16
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[66] 0.17 0.17 0.13 0.12 0.17 0.17 0.21 0.18 0.18 0.16 0.15 0.17 0.20 0.15 0.16 0.20 0.16 0.17 0.08 0.18 0.18 0.17 0.19 0.17 0.16 0.15 0.17 0.16 0.16 0.16 0.16 0.19 0.17 0.19 0.19 0.19 0.19 0.16 0.18 0.13 0.17 0.16
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[67] 0.19 0.18 0.12 0.11 0.19 0.17 0.21 0.20 0.19 0.18 0.17 0.21 0.18 0.11 0.16 0.19 0.17 0.17 0.15 0.19 0.20 0.16 0.20 0.18 0.18 0.16 0.18 0.19 0.18 0.15 0.16 0.20 0.19 0.20 0.18 0.19 0.21 0.18 0.17 0.11 0.18 0.16
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[68] 0.19 0.17 0.12 0.13 0.19 0.16 0.20 0.18 0.17 0.19 0.15 0.20 0.19 0.12 0.16 0.19 0.16 0.17 0.16 0.20 0.16 0.17 0.21 0.18 0.19 0.18 0.17 0.17 0.19 0.15 0.16 0.18 0.17 0.19 0.18 0.19 0.21 0.15 0.18 0.12 0.17 0.16
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[69] 0.19 0.19 0.14 0.13 0.20 0.18 0.21 0.19 0.18 0.19 0.15 0.20 0.20 0.13 0.16 0.20 0.16 0.18 0.16 0.19 0.19 0.16 0.21 0.20 0.19 0.18 0.17 0.19 0.19 0.15 0.16 0.19 0.19 0.18 0.18 0.19 0.21 0.17 0.20 0.14 0.18 0.17
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[70] 0.21 0.19 0.15 0.18 0.21 0.21 0.21 0.22 0.20 0.19 0.16 0.21 0.21 0.20 0.19 0.21 0.20 0.19 0.18 0.20 0.20 0.18 0.21 0.21 0.20 0.19 0.18 0.19 0.20 0.16 0.17 0.20 0.21 0.17 0.20 0.20 0.20 0.17 0.21 0.20 0.20 0.20
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[71] 0.20 0.19 0.16 0.18 0.20 0.19 0.20 0.23 0.19 0.19 0.17 0.21 0.21 0.18 0.17 0.19 0.19 0.20 0.17 0.18 0.19 0.17 0.20 0.20 0.18 0.17 0.17 0.19 0.20 0.15 0.16 0.20 0.19 0.18 0.19 0.19 0.21 0.16 0.20 0.20 0.18 0.19
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[72] 0.21 0.20 0.21 0.23 0.22 0.18 0.21 0.24 0.21 0.22 0.22 0.21 0.23 0.22 0.20 0.23 0.19 0.19 0.20 0.23 0.20 0.19 0.21 0.19 0.22 0.22 0.20 0.20 0.22 0.18 0.23 0.22 0.20 0.22 0.23 0.22 0.20 0.19 0.21 0.24 0.23 0.21
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[73] 0.20 0.23 0.21 0.22 0.22 0.22 0.23 0.20 0.21 0.25 0.22 0.21 0.23 0.23 0.19 0.24 0.19 0.19 0.21 0.23 0.23 0.19 0.20 0.23 0.21 0.21 0.20 0.22 0.21 0.18 0.21 0.22 0.21 0.21 0.23 0.20 0.20 0.19 0.22 0.25 0.22 0.21
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[74] 0.20 0.18 0.19 0.17 0.22 0.18 0.20 0.20 0.21 0.19 0.18 0.22 0.19 0.21 0.17 0.16 0.17 0.19 0.19 0.21 0.21 0.18 0.21 0.19 0.19 0.20 0.19 0.20 0.22 0.16 0.20 0.18 0.21 0.18 0.18 0.18 0.19 0.19 0.19 0.20 0.19 0.20
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[75] 0.19 0.19 0.18 0.20 0.23 0.17 0.20 0.21 0.22 0.17 0.18 0.21 0.22 0.23 0.16 0.18 0.15 0.19 0.17 0.19 0.20 0.18 0.18 0.19 0.18 0.19 0.20 0.19 0.20 0.17 0.20 0.19 0.20 0.20 0.19 0.20 0.20 0.19 0.19 0.21 0.20 0.19
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[76] 0.19 0.17 0.21 0.18 0.20 0.19 0.21 0.19 0.20 0.18 0.19 0.20 0.18 0.20 0.18 0.20 0.17 0.19 0.18 0.22 0.19 0.19 0.23 0.18 0.19 0.19 0.20 0.19 0.20 0.18 0.19 0.19 0.19 0.22 0.19 0.17 0.22 0.17 0.20 0.20 0.18 0.17
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[77] 0.20 0.19 0.20 0.18 0.20 0.20 0.18 0.19 0.21 0.18 0.19 0.21 0.18 0.19 0.17 0.21 0.17 0.18 0.18 0.21 0.20 0.21 0.22 0.19 0.18 0.18 0.17 0.20 0.21 0.15 0.17 0.19 0.20 0.22 0.20 0.17 0.20 0.19 0.19 0.21 0.21 0.18
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[78] 0.19 0.20 0.20 0.19 0.20 0.21 0.19 0.20 0.21 0.18 0.18 0.19 0.18 0.20 0.19 0.20 0.18 0.19 0.19 0.22 0.21 0.20 0.20 0.21 0.21 0.19 0.17 0.22 0.21 0.16 0.18 0.20 0.20 0.20 0.19 0.20 0.22 0.18 0.21 0.20 0.20 0.17
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[79] 0.24 0.22 0.18 0.17 0.21 0.19 0.22 0.21 0.20 0.22 0.19 0.24 0.23 0.19 0.18 0.23 0.17 0.19 0.17 0.23 0.21 0.21 0.21 0.22 0.22 0.20 0.20 0.20 0.20 0.20 0.18 0.20 0.21 0.21 0.19 0.20 0.25 0.19 0.18 0.18 0.22 0.19
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[80] 0.20 0.21 0.17 0.18 0.17 0.18 0.23 0.21 0.19 0.18 0.19 0.19 0.20 0.17 0.17 0.22 0.17 0.14 0.19 0.19 0.16 0.18 0.21 0.22 0.15 0.15 0.18 0.18 0.20 0.15 0.18 0.19 0.20 0.19 0.19 0.16 0.17 0.15 0.18 0.17 0.18 0.17
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[81] 0.18 0.19 0.22 0.16 0.21 0.19 0.20 0.20 0.18 0.20 0.21 0.18 0.21 0.19 0.17 0.19 0.19 0.19 0.17 0.21 0.20 0.20 0.21 0.20 0.19 0.18 0.20 0.18 0.19 0.16 0.17 0.21 0.21 0.17 0.20 0.19 0.22 0.19 0.20 0.18 0.20 0.19
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[82] 0.20 0.17 0.16 0.15 0.17 0.16 0.20 0.19 0.19 0.16 0.15 0.22 0.19 0.17 0.15 0.18 0.17 0.14 0.16 0.17 0.17 0.18 0.19 0.18 0.17 0.15 0.17 0.17 0.15 0.14 0.13 0.19 0.17 0.20 0.16 0.14 0.20 0.16 0.18 0.16 0.16 0.15
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[83] 0.18 0.17 0.15 0.14 0.16 0.16 0.19 0.16 0.19 0.15 0.15 0.19 0.18 0.15 0.15 0.17 0.16 0.13 0.15 0.18 0.18 0.17 0.18 0.18 0.15 0.14 0.17 0.18 0.15 0.12 0.12 0.16 0.16 0.18 0.14 0.14 0.18 0.15 0.17 0.14 0.16 0.15
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[84] 0.20 0.18 0.15 0.15 0.18 0.16 0.19 0.21 0.21 0.18 0.17 0.22 0.20 0.17 0.15 0.20 0.16 0.15 0.17 0.17 0.20 0.19 0.20 0.19 0.19 0.16 0.17 0.18 0.18 0.14 0.11 0.17 0.19 0.20 0.17 0.19 0.23 0.15 0.17 0.16 0.17 0.16
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[85] 0.18 0.18 0.16 0.17 0.16 0.17 0.21 0.18 0.18 0.17 0.16 0.21 0.20 0.19 0.16 0.19 0.17 0.17 0.16 0.19 0.19 0.18 0.20 0.18 0.18 0.17 0.19 0.17 0.16 0.15 0.13 0.18 0.19 0.17 0.16 0.19 0.20 0.16 0.16 0.17 0.18 0.16
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[86] 0.18 0.17 0.14 0.17 0.17 0.16 0.19 0.22 0.19 0.18 0.14 0.20 0.18 0.17 0.14 0.16 0.16 0.16 0.16 0.19 0.20 0.19 0.19 0.17 0.17 0.15 0.16 0.18 0.16 0.14 0.05 0.21 0.18 0.22 0.14 0.17 0.19 0.16 0.15 0.16 0.13 0.19
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[87] 0.22 0.22 0.16 0.18 0.20 0.17 0.21 0.19 0.22 0.18 0.18 0.22 0.21 0.18 0.18 0.21 0.17 0.16 0.20 0.20 0.20 0.21 0.19 0.22 0.17 0.14 0.17 0.21 0.19 0.16 0.15 0.20 0.19 0.20 0.17 0.18 0.23 0.17 0.19 0.19 0.19 0.16
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[88] 0.23 0.21 0.21 0.22 0.22 0.21 0.23 0.21 0.20 0.20 0.19 0.22 0.23 0.20 0.23 0.20 0.24 0.22 0.20 0.23 0.23 0.19 0.21 0.22 0.19 0.19 0.20 0.20 0.21 0.17 0.20 0.21 0.23 0.21 0.21 0.20 0.21 0.21 0.23 0.22 0.20 0.21
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[89] 0.18 0.18 0.17 0.17 0.18 0.17 0.20 0.17 0.20 0.17 0.19 0.19 0.16 0.19 0.17 0.19 0.15 0.17 0.17 0.19 0.20 0.17 0.20 0.18 0.15 0.17 0.16 0.17 0.18 0.13 0.16 0.18 0.17 0.18 0.16 0.20 0.19 0.16 0.19 0.19 0.15 0.17
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[90] 0.22 0.20 0.18 0.19 0.19 0.17 0.19 0.18 0.18 0.19 0.16 0.24 0.22 0.19 0.18 0.18 0.15 0.16 0.18 0.19 0.20 0.18 0.18 0.20 0.19 0.18 0.17 0.17 0.19 0.15 0.16 0.20 0.20 0.18 0.16 0.17 0.18 0.17 0.18 0.18 0.17 0.16
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0.15 0.17 0.19 0.14

[91] 0.20 0.19 0.17 0.16 0.21 0.21 0.22 0.18 0.19 0.20 0.20 0.22 0.21 0.18 0.19 0.23 0.17 0.17 0.19 0.20 0.22 0.19 0.24 0.20 0.17 0.16 0.20 0.19 0.19 0.16 0.20 0.20 0.21 0.22 0.20 0.20 0.20 0.19 0.20 0.17 0.18 0.20
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[92] 0.21 0.20 0.18 0.19 0.20 0.20 0.23 0.19 0.21 0.19 0.19 0.21 0.21 0.21 0.18 0.20 0.17 0.17 0.19 0.21 0.20 0.19 0.18 0.21 0.20 0.19 0.17 0.20 0.18 0.15 0.18 0.20 0.20 0.20 0.20 0.18 0.18 0.17 0.18 0.19 0.20 0.18
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[93] 0.20 0.20 0.20 0.17 0.20 0.17 0.24 0.21 0.20 0.18 0.19 0.21 0.23 0.18 0.17 0.22 0.19 0.15 0.19 0.20 0.20 0.20 0.23 0.21 0.18 0.16 0.18 0.21 0.19 0.16 0.20 0.19 0.21 0.21 0.18 0.20 0.21 0.19 0.19 0.19 0.20 0.16
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[94] 0.21 0.19 0.18 0.17 0.20 0.18 0.22 0.19 0.20 0.16 0.20 0.22 0.19 0.20 0.17 0.21 0.17 0.17 0.18 0.18 0.22 0.19 0.21 0.19 0.18 0.15 0.19 0.18 0.17 0.17 0.15 0.18 0.21 0.22 0.18 0.19 0.20 0.16 0.19 0.18 0.19 0.16
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[95] 0.19 0.19 0.19 0.17 0.18 0.17 0.20 0.22 0.19 0.17 0.19 0.21 0.20 0.19 0.17 0.16 0.15 0.16 0.19 0.19 0.19 0.20 0.18 0.19 0.17 0.18 0.20 0.19 0.17 0.17 0.18 0.21 0.20 0.20 0.15 0.17 0.20 0.18 0.18 0.18 0.17 0.16
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[96] 0.19 0.18 0.17 0.18 0.17 0.16 0.20 0.20 0.20 0.19 0.19 0.21 0.19 0.17 0.16 0.18 0.15 0.17 0.19 0.18 0.19 0.20 0.18 0.18 0.16 0.19 0.19 0.20 0.17 0.16 0.18 0.20 0.20 0.18 0.17 0.17 0.21 0.18 0.17 0.16 0.16 0.17
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[97] 0.17 0.19 0.15 0.16 0.16 0.18 0.20 0.18 0.18 0.16 0.16 0.19 0.19 0.18 0.16 0.19 0.16 0.16 0.16 0.18 0.19 0.17 0.15 0.19 0.16 0.09 0.19 0.17 0.16 0.13 0.16 0.17 0.17 0.18 0.18 0.16 0.18 0.15 0.16 0.16 0.16 0.14
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[98] 0.17 0.19 0.16 0.17 0.17 0.18 0.18 0.20 0.16 0.17 0.16 0.18 0.19 0.18 0.16 0.21 0.16 0.16 0.18 0.20 0.21 0.16 0.15 0.19 0.18 0.10 0.19 0.15 0.19 0.14 0.17 0.19 0.16 0.17 0.17 0.17 0.19 0.19 0.18 0.17 0.17 0.16
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[99] 0.21 0.18 0.20 0.19 0.21 0.20 0.23 0.21 0.19 0.21 0.18 0.20 0.21 0.20 0.18 0.20 0.20 0.21 0.19 0.22 0.20 0.18 0.22 0.19 0.20 0.21 0.21 0.18 0.21 0.20 0.20 0.21 0.23 0.21 0.20 0.19 0.21 0.20 0.20 0.18 0.20 0.18
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[100] 0.19 0.20 0.18 0.15 0.21 0.18 0.22 0.19 0.18 0.18 0.17 0.21 0.21 0.19 0.18 0.21 0.21 0.17 0.19 0.20 0.21 0.18 0.21 0.21 0.20 0.18 0.17 0.17 0.19 0.16 0.17 0.18 0.18 0.21 0.19 0.19 0.20 0.18 0.21 0.16 0.18 0.19
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[101] 0.20 0.19 0.18 0.17 0.19 0.20 0.20 0.22 0.19 0.18 0.16 0.21 0.21 0.17 0.17 0.19 0.16 0.15 0.18 0.20 0.20 0.18 0.17 0.20 0.17 0.16 0.18 0.18 0.17 0.15 0.17 0.21 0.18 0.20 0.18 0.16 0.21 0.16 0.18 0.16 0.18 0.17
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[102] 0.20 0.20 0.17 0.17 0.20 0.19 0.21 0.19 0.21 0.17 0.17 0.19 0.19 0.20 0.17 0.21 0.18 0.17 0.17 0.17 0.21 0.18 0.17 0.19 0.17 0.15 0.15 0.19 0.19 0.15 0.16 0.18 0.20 0.22 0.18 0.18 0.19 0.17 0.18 0.18 0.18 0.18
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[103] 0.22 0.20 0.19 0.19 0.20 0.19 0.21 0.22 0.21 0.19 0.20 0.23 0.22 0.19 0.17 0.18 0.19 0.18 0.17 0.21 0.19 0.19 0.20 0.21 0.20 0.17 0.18 0.20 0.21 0.17 0.16 0.20 0.21 0.20 0.18 0.18 0.21 0.17 0.18 0.16 0.20 0.19
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[104] 0.21 0.19 0.18 0.20 0.20 0.17 0.21 0.20 0.21 0.16 0.18 0.21 0.20 0.19 0.16 0.20 0.18 0.17 0.17 0.20 0.20 0.20 0.18 0.20 0.17 0.18 0.13 0.20 0.17 0.15 0.18 0.19 0.21 0.20 0.18 0.19 0.20 0.16 0.21 0.17 0.18 0.18
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[105] 0.20 0.18 0.17 0.19 0.20 0.17 0.20 0.22 0.19 0.18 0.17 0.21 0.19 0.16 0.16 0.22 0.15 0.16 0.19 0.18 0.20 0.20 0.17 0.19 0.15 0.17 0.13 0.19 0.18 0.14 0.17 0.20 0.21 0.17 0.19 0.17 0.20 0.16 0.18 0.17 0.18 0.20
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[106] 0.22 0.19 0.17 0.17 0.20 0.19 0.20 0.18 0.20 0.17 0.18 0.22 0.20 0.16 0.15 0.22 0.15 0.15 0.17 0.19 0.20 0.18 0.19 0.19 0.16 0.16 0.13 0.19 0.19 0.14 0.17 0.20 0.18 0.20 0.18 0.17 0.21 0.16 0.18 0.16 0.20 0.17
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[107] 0.20 0.20 0.20 0.18 0.18 0.17 0.21 0.22 0.19 0.18 0.21 0.21 0.21 0.18 0.18 0.19 0.17 0.19 0.21 0.19 0.20 0.20 0.19 0.20 0.21 0.20 0.19 0.20 0.21 0.17 0.18 0.19 0.19 0.19 0.17 0.19 0.18 0.17 0.18 0.18 0.19 0.19
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[108] 0.21 0.20 0.20 0.18 0.18 0.17 0.22 0.22 0.19 0.18 0.21 0.21 0.21 0.18 0.18 0.19 0.17 0.18 0.21 0.19 0.21 0.19 0.20 0.20 0.21 0.19 0.19 0.21 0.21 0.18 0.19 0.19 0.19 0.19 0.17 0.19 0.18 0.17 0.18 0.18 0.19 0.19
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[109] 0.24 0.22 0.18 0.19 0.21 0.20 0.22 0.22 0.21 0.19 0.19 0.23 0.23 0.17 0.18 0.20 0.16 0.19 0.20 0.23 0.23 0.20 0.20 0.22 0.20 0.19 0.21 0.22 0.19 0.17 0.17 0.21 0.20 0.21 0.20 0.18 0.21 0.20 0.23 0.19 0.21 0.20
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[110] 0.19 0.20 0.16 0.15 0.20 0.16 0.18 0.20 0.21 0.16 0.17 0.21 0.17 0.16 0.16 0.17 0.15 0.16 0.17 0.18 0.19 0.19 0.18 0.19 0.17 0.17 0.18 0.21 0.19 0.13 0.16 0.19 0.17 0.20 0.17 0.16 0.19 0.16 0.19 0.16 0.17 0.17
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[111] 0.20 0.23 0.19 0.20 0.23 0.19 0.23 0.22 0.22 0.17 0.18 0.19 0.24 0.18 0.21 0.19 0.16 0.19 0.18 0.20 0.21 0.21 0.19 0.23 0.20 0.19 0.18 0.21 0.22 0.17 0.19 0.21 0.18 0.21 0.19 0.18 0.23 0.19 0.22 0.18 0.22 0.18
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[112] 0.20 0.21 0.17 0.18 0.21 0.17 0.20 0.21 0.21 0.17 0.17 0.18 0.21 0.16 0.18 0.18 0.15 0.17 0.19 0.17 0.20 0.20 0.18 0.21 0.20 0.18 0.17 0.20 0.22 0.15 0.17 0.20 0.18 0.20 0.17 0.18 0.21 0.17 0.20 0.18 0.19 0.18
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[113] 0.22 0.18 0.20 0.17 0.20 0.19 0.20 0.21 0.20 0.19 0.18 0.20 0.21 0.18 0.18 0.17 0.17 0.19 0.19 0.19 0.20 0.19 0.21 0.20 0.22 0.18 0.19 0.18 0.21 0.17 0.18 0.22 0.16 0.21 0.19 0.20 0.20 0.20 0.22 0.18 0.19 0.18
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[114] 0.22 0.19 0.19 0.17 0.20 0.17 0.21 0.22 0.20 0.19 0.17 0.22 0.24 0.17 0.19 0.19 0.19 0.18 0.17 0.21 0.20 0.20 0.21 0.21 0.20 0.19 0.19 0.20 0.20 0.16 0.16 0.20 0.18 0.21 0.18 0.18 0.24 0.18 0.21 0.18 0.21 0.19
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[115] 0.23 0.24 0.21 0.19 0.22 0.21 0.20 0.24 0.22 0.19 0.18 0.24 0.25 0.20 0.21 0.21 0.20 0.20 0.21 0.22 0.23 0.20 0.21 0.24 0.20 0.19 0.22 0.21 0.22 0.17 0.21 0.19 0.20 0.23 0.22 0.19 0.22 0.20 0.22 0.22 0.24 0.21
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[116] 0.24 0.23 0.20 0.18 0.22 0.19 0.22 0.24 0.22 0.20 0.19 0.24 0.23 0.19 0.20 0.21 0.20 0.20 0.22 0.21 0.23 0.22 0.23 0.24 0.21 0.20 0.20 0.21 0.24 0.19 0.19 0.23 0.20 0.22 0.20 0.22 0.21 0.19 0.23 0.20 0.21 0.21
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[117] 0.19 0.23 0.20 0.19 0.20 0.17 0.20 0.21 0.19 0.18 0.19 0.20 0.23 0.20 0.18 0.18 0.17 0.21 0.21 0.22 0.20 0.19 0.18 0.22 0.17 0.19 0.21 0.19 0.19 0.17 0.17 0.22 0.20 0.20 0.19 0.21 0.21 0.18 0.22 0.20 0.17 0.19
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[118] 0.19 0.17 0.18 0.18 0.18 0.16 0.19 0.22 0.18 0.16 0.18 0.19 0.19 0.17 0.16 0.17 0.16 0.17 0.16 0.19 0.18 0.17 0.17 0.17 0.18 0.17 0.19 0.17 0.17 0.17 0.17 0.17 0.19 0.19 0.15 0.18 0.19 0.18 0.17 0.16 0.17 0.16
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[119] 0.19 0.12 0.22 0.19 0.21 0.19 0.21 0.23 0.21 0.20 0.19 0.21 0.16 0.17 0.20 0.17 0.18 0.21 0.21 0.21 0.24 0.18 0.19 0.13 0.21 0.19 0.22 0.19 0.21 0.18 0.21 0.20 0.20 0.22 0.19 0.19 0.22 0.22 0.20 0.19 0.16 0.21
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[120] 0.23 0.15 0.20 0.18 0.21 0.20 0.22 0.23 0.23 0.21 0.19 0.22 0.12 0.18 0.17 0.18 0.18 0.18 0.22 0.21 0.20 0.22 0.21 0.17 0.19 0.18 0.20 0.22 0.19 0.18 0.20 0.21 0.19 0.21 0.18 0.18 0.23 0.17 0.22 0.19 0.16 0.18
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[121] 0.20 0.14 0.19 0.19 0.22 0.20 0.21 0.21 0.19 0.20 0.18 0.21 0.08 0.20 0.19 0.18 0.18 0.17 0.20 0.23 0.23 0.18 0.21 0.14 0.17 0.19 0.21 0.18 0.19 0.17 0.18 0.20 0.20 0.20 0.20 0.18 0.22 0.18 0.20 0.20 0.15 0.21
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[122] 0.24 0.16 0.22 0.19 0.22 0.22 0.21 0.22 0.20 0.24 0.22 0.24 0.14 0.19 0.20 0.17 0.17 0.19 0.20 0.24 0.24 0.17 0.20 0.16 0.21 0.21 0.23 0.18 0.21 0.18 0.21 0.21 0.22 0.19 0.23 0.19 0.20 0.22 0.22 0.20 0.15 0.21
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[123] 0.17 0.20 0.19 0.16 0.17 0.13 0.20 0.16 0.18 0.16 0.16 0.19 0.22 0.18 0.17 0.17 0.16 0.15 0.16 0.19 0.17 0.17 0.18 0.19 0.14 0.15 0.18 0.16 0.19 0.14 0.18 0.17 0.16 0.17 0.17 0.17 0.18 0.18 0.17 0.17 0.17 0.16
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[124] 0.19 0.17 0.18 0.16 0.17 0.10 0.20 0.19 0.18 0.16 0.16 0.19 0.20 0.16 0.15 0.17 0.16 0.16 0.16 0.17 0.16 0.17 0.15 0.17 0.17 0.15 0.18 0.17 0.17 0.15 0.16 0.18 0.16 0.18 0.15 0.17 0.17 0.19 0.16 0.16 0.16 0.18
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[125] 0.17 0.17 0.16 0.16 0.16 0.08 0.21 0.16 0.16 0.17 0.17 0.18 0.19 0.15 0.15 0.17 0.16 0.17 0.15 0.18 0.15 0.16 0.17 0.17 0.14 0.14 0.17 0.15 0.19 0.15 0.15 0.16 0.17 0.16 0.16 0.17 0.17 0.17 0.16 0.17 0.17 0.17
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[126] 0.19 0.18 0.18 0.17 0.17 0.12 0.23 0.16 0.18 0.18 0.18 0.20 0.18 0.17 0.18 0.18 0.17 0.19 0.18 0.21 0.17 0.17 0.19 0.18 0.14 0.15 0.17 0.17 0.19 0.17 0.16 0.16 0.19 0.19 0.19 0.17 0.20 0.18 0.17 0.17 0.18 0.16
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[127] 0.18 0.20 0.20 0.17 0.21 0.13 0.22 0.17 0.17 0.19 0.17 0.22 0.20 0.17 0.17 0.17 0.18 0.21 0.20 0.21 0.19 0.16 0.21 0.20 0.15 0.17 0.17 0.16 0.18 0.14 0.17 0.16 0.19 0.19 0.20 0.16 0.19 0.18 0.20 0.20 0.19 0.19
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[128] 0.20 0.19 0.18 0.17 0.19 0.19 0.19 0.22 0.20 0.18 0.19 0.21 0.19 0.18 0.17 0.20 0.17 0.17 0.17 0.20 0.20 0.21 0.20 0.19 0.17 0.16 0.18 0.20 0.18 0.13 0.19 0.18 0.16 0.21 0.17 0.17 0.21 0.17 0.20 0.17 0.18 0.16
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[129] 0.23 0.20 0.23 0.23 0.20 0.19 0.24 0.22 0.19 0.21 0.19 0.22 0.25 0.20 0.20 0.20 0.19 0.19 0.22 0.21 0.23 0.20 0.21 0.20 0.18 0.21 0.23 0.18 0.24 0.16 0.18 0.21 0.20 0.23 0.22 0.22 0.20 0.21 0.21 0.21 0.18 0.20
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[130] 0.19 0.20 0.16 0.19 0.16 0.18 0.22 0.18 0.22 0.18 0.17 0.19 0.20 0.17 0.17 0.17 0.16 0.16 0.17 0.19 0.19 0.18 0.16 0.21 0.18 0.15 0.19 0.19 0.16 0.12 0.17 0.17 0.19 0.20 0.17 0.18 0.20 0.19 0.18 0.17 0.18 0.17
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[131] 0.17 0.16 0.16 0.16 0.16 0.17 0.19 0.18 0.16 0.20 0.18 0.16 0.16 0.16 0.17 0.18 0.13 0.15 0.15 0.18 0.17 0.16 0.18 0.16 0.16 0.16 0.19 0.17 0.16 0.14 0.18 0.18 0.18 0.17 0.16 0.17 0.18 0.16 0.16 0.14 0.16 0.18
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[132] 0.18 0.17 0.16 0.16 0.16 0.16 0.21 0.16 0.18 0.17 0.17 0.18 0.19 0.18 0.17 0.17 0.18 0.18 0.17 0.20 0.18 0.16 0.18 0.18 0.14 0.15 0.17 0.18 0.17 0.11 0.14 0.16 0.18 0.18 0.17 0.16 0.19 0.18 0.17 0.17 0.16 0.18
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[133] 0.22 0.19 0.19 0.20 0.19 0.18 0.21 0.20 0.20 0.17 0.18 0.22 0.23 0.19 0.18 0.18 0.16 0.18 0.21 0.22 0.21 0.21 0.19 0.20 0.17 0.16 0.17 0.20 0.18 0.10 0.16 0.21 0.21 0.21 0.17 0.19 0.20 0.20 0.22 0.19 0.19 0.17
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[134] 0.21 0.19 0.20 0.20 0.17 0.19 0.23 0.18 0.17 0.19 0.15 0.22 0.21 0.18 0.18 0.15 0.15 0.17 0.18 0.21 0.19 0.17 0.18 0.20 0.16 0.17 0.20 0.17 0.20 0.11 0.18 0.17 0.19 0.19 0.20 0.18 0.21 0.19 0.19 0.18 0.20 0.19
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[135] 0.20 0.16 0.17 0.16 0.20 0.17 0.17 0.18 0.19 0.18 0.14 0.20 0.19 0.16 0.16 0.18 0.15 0.16 0.17 0.19 0.19 0.18 0.17 0.18 0.17 0.14 0.18 0.17 0.20 0.12 0.16 0.18 0.17 0.18 0.18 0.18 0.19 0.18 0.17 0.18 0.18 0.17
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[136] 0.20 0.22 0.19 0.17 0.18 0.18 0.18 0.19 0.19 0.15 0.19 0.19 0.20 0.17 0.15 0.22 0.13 0.18 0.17 0.17 0.19 0.16 0.19 0.21 0.16 0.16 0.19 0.17 0.16 0.15 0.19 0.20 0.19 0.17 0.15 0.18 0.19 0.18 0.16 0.18 0.22 0.14
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0.18 0.17 0.23 0.17 0.17 0.21 0.19 0.18 0.17 0.17 0.19 0.16 0.15 0.18 0.19 0.18 0.19 0.19 0.18 0.16 0.16 0.19 0.19 0.19 0.16 0.20 0.19 0.21 0.19 0.20 0.23 0.20 0.18 0.20 0.21 0.23 0.21 0.18 0.17 0.16 0.20 0.18 0.18
0.23 0.19 0.16 0.19 0.22 0.19 0.18

[137] 0.19 0.19 0.17 0.16 0.16 0.15 0.17 0.17 0.18 0.17 0.16 0.20 0.20 0.16 0.15 0.16 0.17 0.16 0.18 0.20 0.18 0.18 0.15 0.19 0.15 0.14 0.18 0.18 0.15 0.11 0.17 0.18 0.17 0.18 0.17 0.18 0.16 0.18 0.16 0.17 0.18 0.16
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0.17 0.13 0.14 0.11 0.13 0.13 0.11 0.16

[138] 0.20 0.19 0.19 0.17 0.20 0.18 0.18 0.20 0.20 0.14 0.19 0.20 0.18 0.17 0.14 0.21 0.15 0.18 0.15 0.21 0.20 0.19 0.19 0.19 0.18 0.18 0.17 0.19 0.16 0.15 0.17 0.22 0.19 0.20 0.15 0.18 0.21 0.18 0.20 0.18 0.20 0.14
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0.25 0.17 0.19 0.18 0.18 0.19 0.19 0.11 0.18

[139] 0.22 0.21 0.20 0.18 0.21 0.20 0.20 0.20 0.21 0.17 0.18 0.20 0.19 0.20 0.14 0.25 0.17 0.16 0.19 0.21 0.21 0.20 0.20 0.20 0.18 0.18 0.21 0.20 0.17 0.15 0.18 0.20 0.19 0.18 0.18 0.19 0.20 0.18 0.21 0.20 0.21 0.16
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0.22 0.18 0.17 0.21 0.20 0.19 0.18 0.11 0.17 0.13

[140] 0.21 0.23 0.21 0.21 0.22 0.20 0.22 0.22 0.20 0.18 0.21 0.21 0.22 0.22 0.17 0.20 0.18 0.19 0.21 0.22 0.19 0.17 0.19 0.23 0.19 0.19 0.20 0.20 0.17 0.17 0.21 0.19 0.21 0.19 0.18 0.17 0.21 0.21 0.21 0.21 0.21 0.20
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[141] 0.19 0.19 0.17 0.17 0.19 0.16 0.21 0.20 0.17 0.15 0.17 0.20 0.20 0.18 0.15 0.19 0.16 0.16 0.17 0.18 0.17 0.17 0.19 0.19 0.17 0.16 0.17 0.18 0.13 0.15 0.16 0.19 0.19 0.18 0.15 0.16 0.19 0.15 0.20 0.19 0.17 0.15
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[142] 0.20 0.20 0.18 0.17 0.18 0.18 0.21 0.20 0.18 0.18 0.20 0.19 0.20 0.18 0.17 0.20 0.16 0.18 0.21 0.22 0.20 0.17 0.20 0.21 0.18 0.14 0.19 0.19 0.19 0.16 0.19 0.18 0.20 0.16 0.17 0.20 0.21 0.18 0.20 0.18 0.19 0.17
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[143] 0.18 0.22 0.17 0.17 0.17 0.18 0.21 0.21 0.17 0.15 0.18 0.21 0.21 0.19 0.15 0.21 0.13 0.15 0.16 0.19 0.18 0.19 0.19 0.22 0.19 0.16 0.18 0.16 0.19 0.17 0.16 0.18 0.17 0.19 0.14 0.19 0.21 0.15 0.17 0.19 0.18 0.14
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[144] 0.21 0.18 0.19 0.17 0.18 0.17 0.18 0.21 0.20 0.14 0.18 0.22 0.18 0.19 0.15 0.22 0.14 0.15 0.18 0.19 0.19 0.19 0.17 0.18 0.19 0.16 0.17 0.18 0.17 0.16 0.15 0.19 0.18 0.18 0.14 0.16 0.21 0.17 0.18 0.17 0.19 0.14
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[145] 0.20 0.22 0.16 0.17 0.20 0.19 0.19 0.20 0.18 0.14 0.16 0.21 0.20 0.19 0.17 0.20 0.16 0.16 0.18 0.19 0.19 0.18 0.18 0.21 0.19 0.17 0.21 0.20 0.15 0.16 0.17 0.19 0.19 0.18 0.15 0.15 0.19 0.17 0.19 0.19 0.20 0.15
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[146] 0.21 0.21 0.15 0.19 0.20 0.17 0.20 0.19 0.19 0.15 0.15 0.22 0.23 0.18 0.15 0.22 0.17 0.15 0.18 0.18 0.21 0.18 0.20 0.21 0.18 0.16 0.18 0.16 0.16 0.17 0.16 0.19 0.20 0.21 0.15 0.15 0.19 0.19 0.19 0.18 0.18 0.14
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[147] 0.19 0.18 0.16 0.15 0.18 0.17 0.19 0.17 0.18 0.11 0.15 0.19 0.16 0.17 0.15 0.17 0.12 0.15 0.16 0.17 0.18 0.17 0.16 0.18 0.16 0.15 0.17 0.19 0.14 0.15 0.15 0.17 0.16 0.17 0.13 0.18 0.19 0.16 0.16 0.16 0.18 0.12
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[148] 0.20 0.19 0.15 0.16 0.18 0.16 0.17 0.18 0.20 0.13 0.16 0.21 0.17 0.17 0.15 0.17 0.13 0.15 0.17 0.19 0.17 0.17 0.16 0.19 0.17 0.14 0.19 0.19 0.13 0.13 0.16 0.19 0.18 0.16 0.15 0.17 0.22 0.17 0.18 0.15 0.17 0.11
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[149] 0.19 0.18 0.18 0.18 0.19 0.16 0.18 0.18 0.20 0.14 0.16 0.22 0.19 0.17 0.14 0.19 0.14 0.13 0.18 0.18 0.20 0.19 0.19 0.18 0.17 0.14 0.18 0.19 0.15 0.15 0.15 0.17 0.17 0.18 0.15 0.15 0.19 0.16 0.16 0.16 0.19 0.12
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[150] 0.19 0.17 0.19 0.17 0.19 0.18 0.22 0.22 0.19 0.16 0.18 0.18 0.16 0.18 0.17 0.20 0.13 0.17 0.20 0.17 0.19 0.18 0.18 0.17 0.16 0.14 0.19 0.18 0.15 0.16 0.16 0.18 0.19 0.17 0.16 0.17 0.19 0.17 0.18 0.19 0.19 0.14
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[151] 0.17 0.18 0.16 0.16 0.18 0.15 0.18 0.19 0.17 0.12 0.16 0.19 0.18 0.17 0.16 0.17 0.13 0.15 0.15 0.16 0.16 0.18 0.18 0.19 0.14 0.16 0.17 0.17 0.16 0.16 0.15 0.17 0.16 0.17 0.14 0.16 0.18 0.15 0.16 0.16 0.18 0.13
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[152] 0.18 0.18 0.13 0.17 0.19 0.17 0.20 0.20 0.19 0.12 0.16 0.19 0.19 0.16 0.17 0.18 0.16 0.15 0.15 0.20 0.18 0.17 0.17 0.18 0.16 0.13 0.18 0.17 0.13 0.15 0.14 0.16 0.17 0.18 0.17 0.15 0.19 0.16 0.18 0.14 0.15 0.12
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[153] 0.18 0.21 0.16 0.19 0.21 0.20 0.18 0.20 0.20 0.13 0.17 0.20 0.21 0.17 0.17 0.22 0.17 0.17 0.17 0.20 0.19 0.16 0.16 0.21 0.17 0.17 0.20 0.19 0.16 0.14 0.17 0.18 0.17 0.18 0.15 0.18 0.20 0.17 0.20 0.18 0.17 0.14
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[154] 0.20 0.19 0.16 0.19 0.21 0.18 0.20 0.20 0.18 0.08 0.18 0.20 0.20 0.18 0.16 0.21 0.16 0.15 0.17 0.22 0.20 0.17 0.19 0.20 0.14 0.16 0.17 0.18 0.13 0.16 0.18 0.19 0.19 0.19 0.15 0.19 0.19 0.17 0.18 0.18 0.18 0.11
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[155] 0.19 0.18 0.16 0.15 0.18 0.17 0.18 0.17 0.17 0.07 0.15 0.19 0.17 0.18 0.16 0.19 0.15 0.15 0.16 0.20 0.18 0.15 0.18 0.18 0.13 0.15 0.17 0.17 0.14 0.14 0.15 0.17 0.19 0.18 0.13 0.16 0.19 0.15 0.15 0.17 0.17 0.11
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[156] 0.20 0.19 0.19 0.17 0.17 0.17 0.19 0.18 0.19 0.11 0.19 0.22 0.18 0.18 0.15 0.20 0.15 0.14 0.19 0.18 0.20 0.19 0.19 0.19 0.17 0.12 0.18 0.19 0.17 0.15 0.16 0.17 0.17 0.21 0.13 0.16 0.18 0.18 0.17 0.16 0.17 0.12
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[157] 0.21 0.19 0.17 0.17 0.18 0.16 0.20 0.20 0.20 0.12 0.19 0.21 0.18 0.20 0.16 0.22 0.15 0.13 0.19 0.19 0.19 0.19 0.18 0.19 0.18 0.12 0.19 0.19 0.17 0.16 0.16 0.17 0.17 0.20 0.15 0.16 0.19 0.18 0.16 0.17 0.17 0.12
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[158] 0.22 0.22 0.18 0.17 0.18 0.18 0.22 0.21 0.21 0.14 0.19 0.22 0.21 0.20 0.16 0.20 0.16 0.18 0.20 0.20 0.19 0.18 0.21 0.21 0.19 0.15 0.21 0.21 0.17 0.18 0.19 0.16 0.19 0.23 0.13 0.18 0.21 0.19 0.17 0.20 0.18 0.12
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[159] 0.23 0.21 0.18 0.19 0.18 0.19 0.22 0.20 0.23 0.15 0.19 0.24 0.21 0.20 0.16 0.21 0.15 0.17 0.19 0.22 0.21 0.22 0.20 0.21 0.20 0.15 0.20 0.21 0.18 0.17 0.17 0.18 0.21 0.21 0.12 0.19 0.22 0.18 0.16 0.19 0.19 0.13
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[160] 0.20 0.22 0.18 0.18 0.18 0.19 0.20 0.20 0.21 0.13 0.17 0.22 0.23 0.18 0.15 0.21 0.13 0.17 0.18 0.21 0.20 0.21 0.20 0.22 0.18 0.15 0.20 0.19 0.15 0.15 0.17 0.18 0.19 0.19 0.13 0.18 0.21 0.17 0.18 0.18 0.17 0.11
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[161] 0.20 0.19 0.17 0.16 0.19 0.20 0.20 0.21 0.21 0.14 0.17 0.21 0.21 0.16 0.14 0.20 0.14 0.15 0.16 0.20 0.19 0.18 0.21 0.20 0.17 0.15 0.19 0.20 0.16 0.15 0.17 0.18 0.19 0.21 0.14 0.19 0.22 0.15 0.21 0.17 0.18 0.07
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[162] 0.22 0.19 0.17 0.17 0.16 0.17 0.23 0.20 0.22 0.12 0.18 0.22 0.19 0.19 0.14 0.18 0.14 0.14 0.15 0.19 0.20 0.18 0.20 0.19 0.16 0.17 0.19 0.20 0.14 0.14 0.17 0.17 0.18 0.20 0.14 0.18 0.20 0.16 0.16 0.17 0.17 0.12
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[163] 0.21 0.21 0.16 0.19 0.21 0.22 0.24 0.22 0.21 0.13 0.18 0.22 0.21 0.17 0.16 0.21 0.15 0.15 0.17 0.21 0.21 0.18 0.21 0.22 0.17 0.15 0.17 0.20 0.18 0.16 0.18 0.20 0.21 0.22 0.16 0.17 0.21 0.17 0.22 0.18 0.19 0.07
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[164] 0.21 0.19 0.15 0.17 0.19 0.20 0.23 0.22 0.21 0.13 0.18 0.21 0.20 0.15 0.15 0.19 0.13 0.14 0.16 0.21 0.18 0.18 0.20 0.20 0.15 0.14 0.17 0.19 0.17 0.15 0.17 0.18 0.19 0.21 0.15 0.18 0.21 0.16 0.20 0.15 0.18 0.06
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[165] 0.21 0.22 0.19 0.19 0.19 0.21 0.22 0.21 0.22 0.14 0.18 0.22 0.20 0.19 0.17 0.22 0.17 0.15 0.18 0.20 0.20 0.22 0.21 0.22 0.18 0.16 0.19 0.22 0.18 0.17 0.18 0.19 0.20 0.20 0.16 0.21 0.21 0.17 0.19 0.19 0.17 0.12
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[166] 0.20 0.20 0.16 0.17 0.19 0.20 0.22 0.21 0.19 0.12 0.17 0.21 0.21 0.18 0.15 0.21 0.15 0.15 0.17 0.21 0.20 0.17 0.20 0.20 0.16 0.15 0.19 0.19 0.16 0.16 0.18 0.20 0.19 0.21 0.15 0.18 0.21 0.16 0.20 0.17 0.18 0.08
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[167] 0.17 0.17 0.16 0.17 0.16 0.15 0.19 0.19 0.16 0.12 0.18 0.18 0.17 0.17 0.15 0.18 0.13 0.16 0.15 0.18 0.17 0.16 0.19 0.17 0.15 0.16 0.19 0.15 0.17 0.16 0.14 0.16 0.17 0.17 0.13 0.16 0.18 0.15 0.14 0.17 0.17 0.14
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[168] 0.21 0.19 0.16 0.19 0.20 0.20 0.22 0.20 0.21 0.14 0.17 0.22 0.21 0.19 0.13 0.19 0.13 0.15 0.17 0.21 0.20 0.18 0.19 0.20 0.18 0.15 0.18 0.19 0.15 0.13 0.16 0.18 0.19 0.17 0.15 0.18 0.20 0.17 0.17 0.17 0.17 0.12
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[169] 0.20 0.18 0.17 0.15 0.16 0.17 0.21 0.19 0.20 0.12 0.16 0.20 0.19 0.17 0.13 0.17 0.13 0.16 0.15 0.18 0.20 0.18 0.19 0.18 0.15 0.15 0.18 0.20 0.14 0.14 0.16 0.17 0.18 0.19 0.13 0.18 0.19 0.15 0.16 0.16 0.17 0.10
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[170] 0.21 0.21 0.17 0.18 0.20 0.18 0.20 0.20 0.17 0.15 0.16 0.23 0.18 0.18 0.16 0.20 0.16 0.17 0.19 0.19 0.19 0.17 0.21 0.20 0.18 0.16 0.16 0.19 0.16 0.16 0.15 0.20 0.17 0.19 0.16 0.17 0.21 0.17 0.19 0.18 0.20 0.15
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[171] 0.21 0.17 0.17 0.17 0.18 0.16 0.20 0.17 0.18 0.13 0.17 0.21 0.18 0.18 0.13 0.19 0.16 0.18 0.16 0.21 0.17 0.18 0.20 0.17 0.16 0.17 0.19 0.18 0.14 0.15 0.14 0.17 0.18 0.16 0.14 0.17 0.19 0.16 0.16 0.16 0.16 0.15
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[172] 0.22 0.19 0.16 0.18 0.22 0.20 0.21 0.18 0.21 0.16 0.17 0.22 0.21 0.18 0.17 0.19 0.15 0.17 0.18 0.19 0.21 0.19 0.19 0.19 0.17 0.18 0.16 0.20 0.14 0.14 0.17 0.18 0.19 0.20 0.18 0.17 0.21 0.16 0.20 0.18 0.19 0.15
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[173] 0.20 0.21 0.18 0.18 0.21 0.16 0.19 0.18 0.19 0.17 0.18 0.21 0.21 0.17 0.16 0.20 0.16 0.17 0.19 0.20 0.20 0.19 0.20 0.20 0.19 0.18 0.16 0.19 0.15 0.14 0.15 0.19 0.18 0.18 0.16 0.16 0.19 0.17 0.19 0.17 0.16 0.16
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0.07

[174] 0.19 0.20 0.18 0.18 0.21 0.16 0.20 0.20 0.21 0.17 0.17 0.20 0.21 0.19 0.17 0.19 0.15 0.18 0.20 0.19 0.20 0.19 0.19 0.20 0.18 0.18 0.16 0.20 0.14 0.15 0.16 0.16 0.17 0.19 0.15 0.15 0.20 0.16 0.19 0.19 0.18 0.16
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0.07 0.08

[175] 0.19 0.16 0.16 0.16 0.17 0.16 0.20 0.19 0.19 0.15 0.17 0.19 0.17 0.15 0.08 0.16 0.09 0.16 0.16 0.19 0.17 0.18 0.18 0.16 0.16 0.17 0.17 0.17 0.17 0.14 0.15 0.18 0.16 0.16 0.14 0.17 0.19 0.14 0.15 0.16 0.15 0.16
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[176] 0.18 0.17 0.18 0.14 0.16 0.16 0.18 0.19 0.20 0.15 0.18 0.19 0.18 0.17 0.07 0.17 0.09 0.14 0.16 0.18 0.19 0.18 0.18 0.17 0.15 0.15 0.19 0.17 0.15 0.15 0.16 0.17 0.16 0.19 0.14 0.15 0.18 0.14 0.14 0.16 0.15 0.16
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[177] 0.19 0.16 0.17 0.15 0.17 0.16 0.18 0.21 0.21 0.16 0.18 0.20 0.17 0.18 0.08 0.17 0.09 0.14 0.17 0.17 0.20 0.19 0.17 0.16 0.17 0.16 0.19 0.19 0.15 0.15 0.15 0.18 0.17 0.18 0.14 0.17 0.17 0.13 0.14 0.17 0.15 0.18
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[178] 0.21 0.17 0.17 0.15 0.18 0.15 0.16 0.20 0.19 0.16 0.17 0.20 0.18 0.16 0.02 0.17 0.10 0.16 0.17 0.17 0.19 0.18 0.19 0.17 0.18 0.16 0.17 0.18 0.14 0.15 0.14 0.19 0.18 0.18 0.14 0.16 0.19 0.15 0.16 0.16 0.16 0.17
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[179] 0.22 0.16 0.17 0.13 0.16 0.16 0.17 0.19 0.17 0.15 0.17 0.20 0.19 0.17 0.04 0.18 0.10 0.15 0.17 0.18 0.17 0.18 0.18 0.16 0.17 0.17 0.18 0.16 0.15 0.15 0.16 0.19 0.17 0.18 0.14 0.15 0.16 0.15 0.15 0.15 0.16 0.15
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[180] 0.22 0.21 0.20 0.20 0.17 0.19 0.25 0.22 0.22 0.18 0.20 0.22 0.21 0.21 0.19 0.22 0.20 0.14 0.20 0.18 0.22 0.23 0.20 0.22 0.17 0.18 0.19 0.22 0.19 0.18 0.19 0.22 0.20 0.23 0.17 0.16 0.22 0.19 0.19 0.20 0.19 0.18
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[181] 0.22 0.20 0.20 0.19 0.19 0.20 0.21 0.21 0.20 0.18 0.17 0.23 0.21 0.20 0.19 0.22 0.16 0.15 0.18 0.19 0.20 0.19 0.18 0.20 0.17 0.20 0.18 0.20 0.19 0.19 0.18 0.23 0.20 0.21 0.17 0.20 0.20 0.16 0.18 0.18 0.21 0.15
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[182] 0.19 0.20 0.19 0.19 0.17 0.22 0.22 0.19 0.19 0.18 0.18 0.20 0.20 0.19 0.20 0.19 0.19 0.16 0.21 0.17 0.21 0.18 0.21 0.21 0.18 0.18 0.18 0.18 0.19 0.15 0.19 0.18 0.21 0.19 0.18 0.20 0.19 0.16 0.17 0.19 0.19 0.18
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[183] 0.20 0.19 0.20 0.17 0.16 0.19 0.23 0.19 0.18 0.17 0.18 0.21 0.19 0.20 0.18 0.19 0.17 0.14 0.18 0.18 0.21 0.19 0.20 0.19 0.19 0.17 0.18 0.16 0.20 0.16 0.18 0.18 0.19 0.18 0.17 0.19 0.20 0.16 0.16 0.20 0.16 0.18
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[184] 0.21 0.22 0.21 0.20 0.20 0.21 0.24 0.22 0.21 0.19 0.21 0.23 0.21 0.22 0.20 0.21 0.20 0.15 0.20 0.16 0.23 0.21 0.20 0.21 0.20 0.18 0.18 0.20 0.21 0.18 0.19 0.23 0.20 0.19 0.20 0.21 0.21 0.17 0.18 0.19 0.18 0.19
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[185] 0.19 0.22 0.20 0.20 0.20 0.22 0.22 0.21 0.20 0.16 0.20 0.20 0.23 0.22 0.17 0.20 0.21 0.15 0.20 0.18 0.20 0.20 0.21 0.21 0.21 0.17 0.18 0.20 0.20 0.19 0.19 0.21 0.20 0.21 0.17 0.18 0.22 0.16 0.18 0.19 0.19 0.18
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[186] 0.18 0.18 0.17 0.19 0.16 0.18 0.21 0.18 0.17 0.19 0.15 0.19 0.18 0.19 0.17 0.19 0.17 0.14 0.20 0.16 0.21 0.18 0.18 0.18 0.19 0.16 0.18 0.18 0.20 0.16 0.17 0.19 0.19 0.18 0.19 0.17 0.18 0.15 0.17 0.19 0.17 0.20
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[187] 0.17 0.18 0.17 0.18 0.16 0.18 0.21 0.19 0.16 0.17 0.15 0.18 0.18 0.16 0.16 0.19 0.14 0.13 0.17 0.14 0.19 0.18 0.18 0.18 0.17 0.15 0.16 0.17 0.19 0.16 0.15 0.19 0.17 0.18 0.17 0.16 0.18 0.13 0.15 0.17 0.16 0.18
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[188] 0.16 0.18 0.16 0.18 0.16 0.18 0.21 0.19 0.17 0.18 0.16 0.17 0.17 0.21 0.18 0.20 0.17 0.13 0.19 0.16 0.19 0.17 0.17 0.18 0.16 0.16 0.18 0.17 0.20 0.15 0.17 0.18 0.18 0.17 0.18 0.16 0.16 0.14 0.16 0.19 0.16 0.20
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[189] 0.18 0.19 0.16 0.19 0.15 0.17 0.22 0.19 0.17 0.18 0.16 0.19 0.17 0.19 0.18 0.21 0.17 0.13 0.20 0.15 0.20 0.18 0.18 0.19 0.17 0.16 0.17 0.18 0.21 0.15 0.16 0.19 0.18 0.18 0.19 0.17 0.17 0.14 0.16 0.19 0.16 0.18
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[190] 0.21 0.17 0.17 0.19 0.17 0.19 0.23 0.19 0.18 0.18 0.18 0.21 0.20 0.16 0.18 0.19 0.18 0.15 0.19 0.16 0.20 0.19 0.19 0.17 0.17 0.17 0.16 0.18 0.18 0.17 0.17 0.20 0.20 0.20 0.18 0.17 0.19 0.15 0.18 0.16 0.19 0.18
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[191] 0.18 0.19 0.16 0.17 0.17 0.19 0.20 0.18 0.17 0.16 0.15 0.21 0.18 0.19 0.16 0.19 0.18 0.13 0.17 0.16 0.20 0.18 0.18 0.18 0.18 0.18 0.17 0.18 0.17 0.16 0.18 0.19 0.19 0.19 0.18 0.18 0.20 0.15 0.17 0.17 0.18 0.18
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[192] 0.20 0.23 0.18 0.20 0.19 0.20 0.23 0.21 0.19 0.17 0.17 0.20 0.22 0.17 0.18 0.19 0.16 0.15 0.19 0.18 0.21 0.17 0.20 0.23 0.16 0.18 0.19 0.19 0.20 0.18 0.16 0.19 0.21 0.20 0.19 0.20 0.22 0.16 0.18 0.19 0.18 0.17
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[193] 0.20 0.21 0.14 0.18 0.18 0.19 0.21 0.18 0.19 0.16 0.15 0.20 0.19 0.17 0.18 0.21 0.17 0.15 0.17 0.15 0.21 0.17 0.19 0.21 0.16 0.17 0.18 0.19 0.20 0.16 0.16 0.18 0.19 0.20 0.18 0.18 0.19 0.14 0.18 0.17 0.19 0.17
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[194] 0.20 0.21 0.20 0.22 0.18 0.21 0.24 0.22 0.20 0.18 0.18 0.20 0.25 0.18 0.18 0.23 0.18 0.16 0.19 0.16 0.22 0.21 0.21 0.21 0.18 0.18 0.18 0.20 0.20 0.19 0.18 0.20 0.20 0.22 0.19 0.21 0.21 0.15 0.18 0.20 0.20 0.20
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[195] 0.19 0.21 0.18 0.18 0.18 0.17 0.20 0.21 0.19 0.18 0.17 0.20 0.22 0.16 0.17 0.18 0.17 0.16 0.18 0.17 0.20 0.18 0.20 0.21 0.17 0.17 0.17 0.19 0.20 0.17 0.16 0.18 0.20 0.21 0.18 0.19 0.22 0.16 0.17 0.18 0.18 0.18
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[196] 0.18 0.21 0.18 0.19 0.18 0.16 0.20 0.19 0.19 0.16 0.15 0.19 0.21 0.15 0.19 0.16 0.16 0.14 0.17 0.16 0.19 0.17 0.17 0.21 0.15 0.16 0.16 0.18 0.19 0.15 0.16 0.16 0.20 0.19 0.18 0.17 0.20 0.15 0.17 0.17 0.16 0.16
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[197] 0.23 0.21 0.22 0.23 0.20 0.24 0.22 0.21 0.19 0.21 0.19 0.22 0.24 0.22 0.18 0.22 0.20 0.20 0.22 0.21 0.19 0.17 0.19 0.22 0.20 0.16 0.22 0.18 0.19 0.19 0.21 0.19 0.20 0.17 0.20 0.19 0.20 0.22 0.19 0.20 0.21 0.19
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[198] 0.21 0.20 0.18 0.21 0.22 0.22 0.25 0.22 0.22 0.21 0.20 0.23 0.24 0.19 0.19 0.21 0.20 0.19 0.20 0.19 0.21 0.21 0.21 0.22 0.20 0.19 0.19 0.21 0.20 0.16 0.18 0.21 0.21 0.22 0.20 0.18 0.22 0.22 0.23 0.18 0.22 0.21
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[199] 0.19 0.22 0.21 0.20 0.18 0.20 0.23 0.19 0.19 0.18 0.18 0.18 0.21 0.21 0.17 0.20 0.19 0.16 0.19 0.16 0.20 0.20 0.21 0.21 0.17 0.18 0.19 0.19 0.22 0.16 0.19 0.19 0.21 0.21 0.16 0.20 0.19 0.17 0.18 0.20 0.20 0.19
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[200] 0.17 0.20 0.15 0.16 0.15 0.16 0.21 0.18 0.17 0.14 0.16 0.18 0.20 0.16 0.15 0.19 0.16 0.13 0.18 0.14 0.17 0.17 0.17 0.20 0.16 0.14 0.16 0.16 0.18 0.14 0.15 0.16 0.17 0.19 0.15 0.17 0.15 0.13 0.14 0.16 0.17 0.17
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[201] 0.15 0.19 0.18 0.17 0.17 0.18 0.20 0.19 0.17 0.17 0.17 0.16 0.19 0.19 0.18 0.19 0.17 0.14 0.16 0.15 0.18 0.18 0.18 0.19 0.15 0.16 0.20 0.15 0.20 0.16 0.17 0.16 0.17 0.17 0.19 0.17 0.18 0.14 0.15 0.17 0.17 0.19
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[202] 0.20 0.18 0.17 0.17 0.16 0.16 0.21 0.19 0.17 0.18 0.17 0.19 0.20 0.18 0.15 0.19 0.16 0.13 0.15 0.15 0.19 0.18 0.17 0.18 0.17 0.16 0.18 0.16 0.15 0.15 0.16 0.19 0.19 0.17 0.18 0.19 0.20 0.14 0.18 0.16 0.16 0.19
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[203] 0.19 0.20 0.18 0.16 0.17 0.19 0.23 0.21 0.17 0.19 0.18 0.19 0.19 0.19 0.17 0.21 0.19 0.14 0.19 0.16 0.19 0.19 0.21 0.20 0.19 0.18 0.19 0.18 0.21 0.16 0.17 0.19 0.21 0.19 0.20 0.19 0.19 0.14 0.16 0.20 0.16 0.20
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[204] 0.19 0.18 0.19 0.15 0.15 0.16 0.20 0.19 0.18 0.19 0.18 0.18 0.17 0.18 0.14 0.16 0.15 0.12 0.18 0.13 0.15 0.18 0.19 0.18 0.18 0.15 0.16 0.17 0.20 0.14 0.16 0.17 0.18 0.17 0.16 0.17 0.18 0.12 0.13 0.17 0.15 0.18
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[205] 0.18 0.20 0.18 0.19 0.16 0.19 0.21 0.21 0.19 0.20 0.20 0.18 0.22 0.20 0.16 0.19 0.16 0.16 0.18 0.16 0.19 0.18 0.19 0.20 0.18 0.16 0.19 0.18 0.21 0.17 0.18 0.18 0.20 0.19 0.17 0.17 0.21 0.13 0.15 0.17 0.19 0.19
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[206] 0.18 0.17 0.16 0.17 0.15 0.16 0.19 0.19 0.17 0.17 0.16 0.20 0.18 0.19 0.14 0.17 0.15 0.13 0.17 0.16 0.19 0.17 0.16 0.17 0.18 0.15 0.15 0.17 0.20 0.15 0.16 0.18 0.18 0.20 0.15 0.17 0.17 0.12 0.15 0.16 0.16 0.16
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[207] 0.18 0.24 0.19 0.20 0.18 0.20 0.22 0.19 0.20 0.22 0.20 0.22 0.25 0.20 0.17 0.19 0.20 0.15 0.21 0.18 0.20 0.20 0.20 0.24 0.20 0.18 0.19 0.20 0.21 0.18 0.19 0.21 0.20 0.18 0.20 0.19 0.21 0.15 0.19 0.20 0.18 0.21
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[208] 0.17 0.19 0.16 0.16 0.14 0.17 0.19 0.17 0.17 0.16 0.16 0.18 0.18 0.18 0.15 0.18 0.16 0.12 0.17 0.13 0.18 0.17 0.15 0.18 0.16 0.16 0.19 0.16 0.18 0.14 0.16 0.17 0.17 0.18 0.15 0.17 0.16 0.12 0.15 0.17 0.16 0.17
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[209] 0.18 0.21 0.16 0.17 0.15 0.17 0.19 0.18 0.18 0.15 0.15 0.20 0.20 0.19 0.16 0.20 0.16 0.13 0.18 0.14 0.18 0.18 0.17 0.20 0.17 0.16 0.19 0.17 0.18 0.15 0.17 0.17 0.18 0.17 0.17 0.18 0.17 0.13 0.16 0.19 0.18 0.16
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[210] 0.19 0.19 0.18 0.19 0.15 0.20 0.24 0.19 0.19 0.18 0.17 0.20 0.21 0.18 0.16 0.18 0.17 0.13 0.19 0.15 0.18 0.17 0.18 0.19 0.17 0.16 0.19 0.18 0.20 0.15 0.19 0.16 0.19 0.19 0.18 0.18 0.19 0.13 0.14 0.19 0.17 0.16
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[211] 0.20 0.20 0.17 0.16 0.14 0.16 0.19 0.19 0.18 0.17 0.18 0.20 0.19 0.18 0.15 0.18 0.16 0.11 0.18 0.14 0.17 0.18 0.15 0.19 0.16 0.16 0.19 0.16 0.16 0.14 0.17 0.16 0.19 0.16 0.16 0.16 0.18 0.11 0.16 0.17 0.16 0.17
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[212] 0.16 0.21 0.19 0.17 0.16 0.16 0.20 0.17 0.18 0.17 0.17 0.18 0.18 0.17 0.15 0.18 0.17 0.14 0.17 0.13 0.19 0.19 0.18 0.20 0.15 0.15 0.18 0.17 0.18 0.14 0.16 0.17 0.17 0.19 0.17 0.18 0.18 0.12 0.16 0.18 0.17 0.19
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[213] 0.17 0.20 0.18 0.17 0.15 0.17 0.20 0.19 0.17 0.16 0.16 0.18 0.18 0.18 0.17 0.19 0.17 0.13 0.17 0.14 0.18 0.19 0.17 0.20 0.17 0.18 0.18 0.18 0.18 0.14 0.17 0.17 0.18 0.18 0.17 0.18 0.18 0.10 0.17 0.18 0.17 0.18
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[214] 0.20 0.20 0.18 0.20 0.16 0.21 0.24 0.22 0.20 0.20 0.18 0.21 0.21 0.22 0.16 0.22 0.18 0.14 0.21 0.15 0.20 0.21 0.19 0.19 0.18 0.20 0.18 0.19 0.20 0.16 0.18 0.19 0.23 0.19 0.18 0.22 0.21 0.11 0.17 0.20 0.20 0.19
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[215] 0.19 0.19 0.18 0.18 0.15 0.18 0.21 0.18 0.19 0.19 0.18 0.20 0.20 0.19 0.15 0.20 0.14 0.13 0.18 0.17 0.16 0.20 0.18 0.19 0.15 0.18 0.18 0.18 0.17 0.15 0.16 0.19 0.18 0.19 0.19 0.19 0.21 0.09 0.18 0.18 0.18 0.18
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[216] 0.20 0.18 0.15 0.15 0.14 0.17 0.20 0.19 0.18 0.16 0.17 0.21 0.17 0.17 0.13 0.17 0.16 0.08 0.18 0.15 0.16 0.18 0.17 0.18 0.16 0.17 0.17 0.18 0.14 0.14 0.16 0.17 0.18 0.17 0.15 0.16 0.19 0.12 0.15 0.16 0.17 0.15
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[217] 0.21 0.19 0.17 0.17 0.15 0.17 0.21 0.20 0.19 0.18 0.17 0.21 0.19 0.17 0.14 0.19 0.18 0.11 0.16 0.15 0.18 0.19 0.17 0.20 0.19 0.17 0.18 0.18 0.18 0.15 0.17 0.19 0.18 0.21 0.17 0.18 0.22 0.12 0.15 0.17 0.17 0.17
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[218] 0.23 0.21 0.17 0.18 0.15 0.21 0.24 0.21 0.22 0.16 0.18 0.24 0.21 0.19 0.17 0.21 0.18 0.12 0.18 0.17 0.21 0.21 0.17 0.21 0.16 0.18 0.18 0.23 0.17 0.13 0.19 0.19 0.19 0.20 0.18 0.20 0.20 0.14 0.15 0.19 0.21 0.17
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[219] 0.21 0.20 0.15 0.17 0.14 0.19 0.23 0.20 0.20 0.16 0.18 0.22 0.19 0.18 0.16 0.20 0.17 0.10 0.19 0.16 0.19 0.19 0.17 0.21 0.17 0.17 0.17 0.21 0.17 0.13 0.17 0.19 0.19 0.20 0.16 0.19 0.19 0.13 0.15 0.18 0.19 0.17
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[220] 0.18 0.21 0.16 0.16 0.16 0.21 0.21 0.19 0.19 0.16 0.17 0.19 0.19 0.18 0.16 0.18 0.15 0.11 0.17 0.16 0.18 0.18 0.19 0.21 0.17 0.16 0.21 0.20 0.17 0.16 0.18 0.18 0.20 0.18 0.17 0.19 0.19 0.14 0.17 0.17 0.18 0.17
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[221] 0.20 0.20 0.16 0.16 0.15 0.17 0.21 0.20 0.19 0.17 0.15 0.21 0.19 0.17 0.15 0.17 0.16 0.09 0.18 0.14 0.18 0.19 0.17 0.20 0.17 0.15 0.19 0.20 0.16 0.15 0.16 0.20 0.18 0.18 0.18 0.18 0.19 0.14 0.15 0.16 0.17 0.17
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[222] 0.20 0.20 0.18 0.17 0.17 0.19 0.23 0.20 0.19 0.19 0.17 0.20 0.17 0.17 0.17 0.22 0.18 0.10 0.19 0.15 0.18 0.20 0.19 0.19 0.17 0.16 0.20 0.20 0.17 0.16 0.17 0.21 0.19 0.20 0.19 0.19 0.19 0.13 0.17 0.17 0.18 0.16
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[223] 0.17 0.19 0.16 0.17 0.14 0.18 0.21 0.19 0.19 0.17 0.19 0.18 0.17 0.18 0.15 0.22 0.16 0.10 0.18 0.14 0.17 0.19 0.18 0.18 0.17 0.15 0.18 0.19 0.18 0.16 0.17 0.18 0.17 0.18 0.18 0.18 0.19 0.10 0.15 0.17 0.16 0.17
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[224] 0.19 0.21 0.18 0.17 0.15 0.18 0.20 0.18 0.19 0.17 0.17 0.20 0.19 0.19 0.17 0.19 0.18 0.12 0.19 0.15 0.18 0.18 0.17 0.21 0.18 0.15 0.18 0.19 0.21 0.16 0.17 0.17 0.16 0.19 0.19 0.18 0.21 0.14 0.16 0.19 0.19 0.17
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[225] 0.19 0.19 0.17 0.18 0.13 0.17 0.21 0.20 0.18 0.15 0.17 0.19 0.20 0.19 0.17 0.20 0.16 0.12 0.18 0.15 0.17 0.19 0.17 0.19 0.18 0.16 0.19 0.18 0.19 0.16 0.17 0.18 0.17 0.18 0.19 0.21 0.20 0.12 0.14 0.18 0.19 0.17
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[226] 0.21 0.21 0.18 0.18 0.13 0.18 0.22 0.19 0.20 0.18 0.18 0.21 0.19 0.18 0.16 0.19 0.18 0.13 0.19 0.14 0.17 0.19 0.18 0.21 0.17 0.15 0.21 0.19 0.20 0.16 0.16 0.18 0.17 0.18 0.20 0.20 0.21 0.12 0.15 0.19 0.17 0.18
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[227] 0.21 0.19 0.20 0.17 0.03 0.19 0.24 0.22 0.19 0.18 0.20 0.21 0.21 0.20 0.16 0.19 0.17 0.15 0.17 0.18 0.20 0.18 0.19 0.18 0.16 0.16 0.21 0.18 0.20 0.15 0.17 0.18 0.19 0.20 0.17 0.18 0.21 0.13 0.10 0.19 0.17 0.18
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[228] 0.19 0.18 0.18 0.16 0.09 0.17 0.20 0.20 0.18 0.16 0.18 0.21 0.19 0.22 0.16 0.17 0.15 0.15 0.18 0.18 0.19 0.17 0.19 0.16 0.17 0.16 0.18 0.17 0.20 0.16 0.16 0.18 0.18 0.17 0.17 0.17 0.19 0.14 0.08 0.19 0.16 0.18
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[229] 0.20 0.19 0.19 0.17 0.08 0.18 0.22 0.21 0.18 0.21 0.19 0.21 0.20 0.19 0.16 0.17 0.14 0.14 0.17 0.18 0.19 0.16 0.20 0.18 0.16 0.16 0.19 0.17 0.19 0.15 0.16 0.18 0.19 0.17 0.17 0.19 0.19 0.15 0.10 0.19 0.16 0.19
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[230] 0.17 0.17 0.18 0.17 0.15 0.18 0.22 0.19 0.16 0.17 0.19 0.18 0.20 0.20 0.13 0.19 0.17 0.14 0.16 0.16 0.18 0.16 0.19 0.18 0.17 0.17 0.18 0.15 0.17 0.15 0.18 0.18 0.20 0.17 0.16 0.20 0.19 0.13 0.14 0.18 0.18 0.19
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[233] 0.17 0.19 0.20 0.18 0.16 0.17 0.18 0.18 0.16 0.16 0.19 0.18 0.16 0.16 0.15 0.17 0.13 0.14 0.17 0.16 0.16 0.15 0.15 0.19 0.16 0.15 0.18 0.15 0.19 0.14 0.17 0.16 0.15 0.16 0.17 0.16 0.18 0.16 0.15 0.17 0.16 0.16
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[234] 0.18 0.21 0.19 0.18 0.15 0.17 0.20 0.18 0.16 0.18 0.18 0.18 0.18 0.17 0.16 0.15 0.16 0.14 0.17 0.14 0.17 0.16 0.16 0.20 0.16 0.16 0.17 0.17 0.18 0.13 0.16 0.15 0.19 0.16 0.14 0.17 0.17 0.16 0.15 0.18 0.16 0.16
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0.17 0.17 0.20 0.14 0.16 0.17 0.17 0.19 0.19 0.16 0.16 0.15 0.17 0.20 0.20 0.16 0.17 0.17 0.17 0.16 0.18 0.18 0.18 0.18 0.18 0.18 0.15 0.18 0.20 0.22 0.20 0.20 0.15 0.20 0.19 0.19 0.19 0.15 0.16 0.14 0.16 0.19 0.16
0.19 0.16 0.16 0.15 0.15 0.17 0.19 0.17 0.15 0.17 0.18 0.20 0.18 0.17 0.16 0.15 0.16 0.16 0.15 0.16 0.15 0.16 0.16 0.16 0.16 0.17 0.14 0.15 0.15 0.16 0.17 0.16 0.16 0.16 0.17 0.15 0.17 0.17 0.15 0.15 0.15 0.16 0.16
0.17 0.17 0.16 0.16 0.16 0.17 0.16 0.16 0.16 0.16 0.16 0.15 0.18 0.17 0.15 0.14 0.16 0.15 0.16 0.17 0.17 0.14 0.18 0.17 0.15 0.18 0.20 0.16 0.13 0.15 0.16 0.17 0.13 0.15 0.13 0.15 0.13 0.15 0.14 0.12 0.13 0.14 0.17
0.15 0.13 0.15 0.16 0.15 0.14 0.14 0.14 0.14 0.15 0.15 0.16 0.16 0.16 0.15 0.15 0.14 0.18 0.09

[235] 0.09 0.18 0.23 0.20 0.21 0.22 0.21 0.18 0.18 0.21 0.21 0.09 0.21 0.26 0.21 0.19 0.20 0.21 0.20 0.21 0.20 0.18 0.17 0.19 0.19 0.19 0.21 0.18 0.23 0.18 0.23 0.19 0.22 0.18 0.20 0.20 0.19 0.20 0.23 0.22 0.18 0.23
0.22 0.21 0.19 0.12 0.12 0.11 0.10 0.11 0.19 0.18 0.21 0.17 0.15 0.19 0.19 0.17 0.17 0.16 0.15 0.16 0.18 0.16 0.19 0.20 0.21 0.21 0.21 0.24 0.23 0.25 0.23 0.20 0.20 0.22 0.20 0.22 0.22 0.21 0.21 0.21 0.19 0.22 0.23
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0.23 0.21 0.17 0.17 0.21 0.24 0.18 0.21 0.20 0.20 0.21 0.23 0.21 0.22 0.20 0.22 0.19 0.23 0.20 0.20 0.21 0.20 0.19 0.22 0.22 0.20 0.19 0.20 0.22 0.22 0.21 0.21 0.21 0.22 0.20 0.22 0.22 0.20 0.18 0.21 0.20 0.24 0.19
0.22 0.22 0.21 0.19 0.19 0.20 0.21 0.21 0.21 0.22 0.20 0.20 0.22 0.22 0.20 0.20 0.18 0.19 0.22 0.21 0.24 0.22 0.23 0.23 0.22 0.25 0.22 0.21 0.20 0.17 0.19 0.21 0.20 0.20 0.20 0.18 0.17 0.19 0.19 0.18 0.18 0.18 0.22
0.20 0.19 0.22 0.22 0.21 0.17 0.20 0.20 0.18 0.18 0.19 0.22 0.21 0.21 0.23 0.18 0.18 0.18 0.18 0.17

Table. Estimates of Evolutionary Divergence between Sequences

The number of base substitutions per site from between sequences are shown. Analyses were conducted using the Kimura 2-parameter model [1]. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 235 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding.

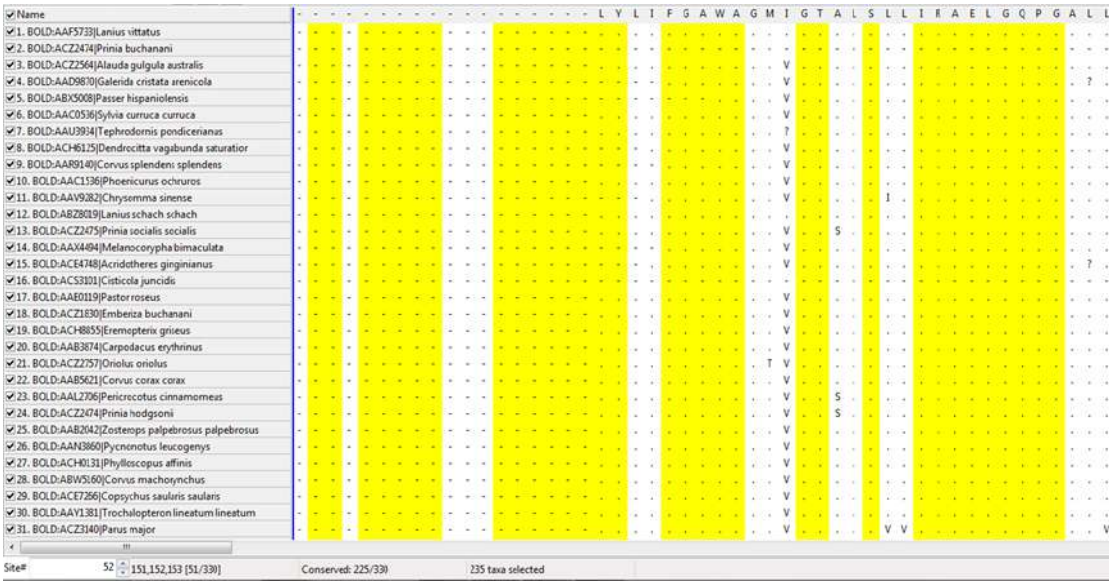
All ambiguous positions were removed for each sequence pair (pairwise deletion option). There were a total of 992 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [2]

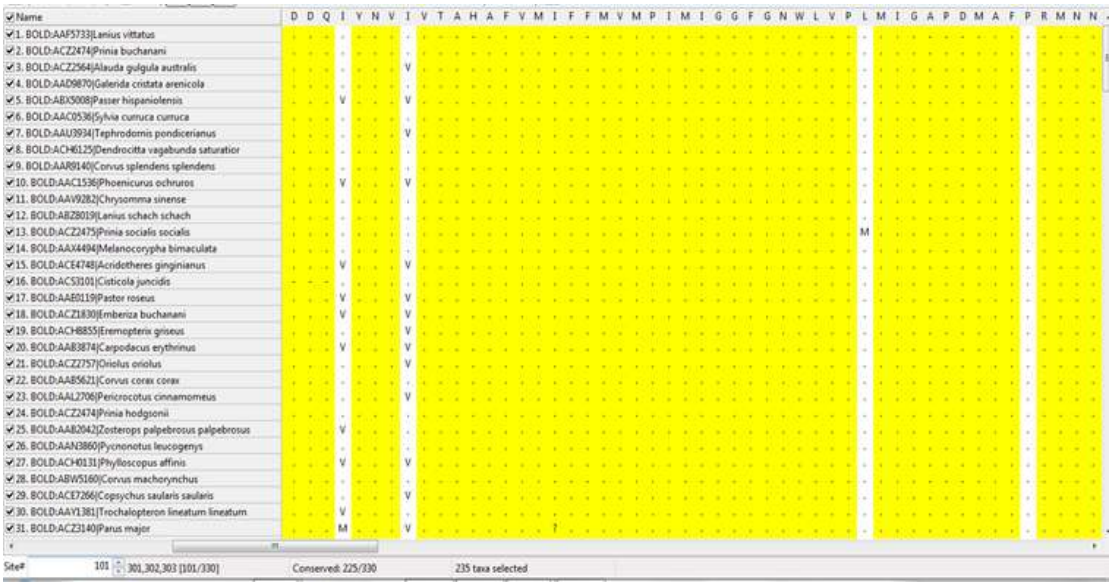
1. Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. Journal of Molecular Evolution 16:111-120.

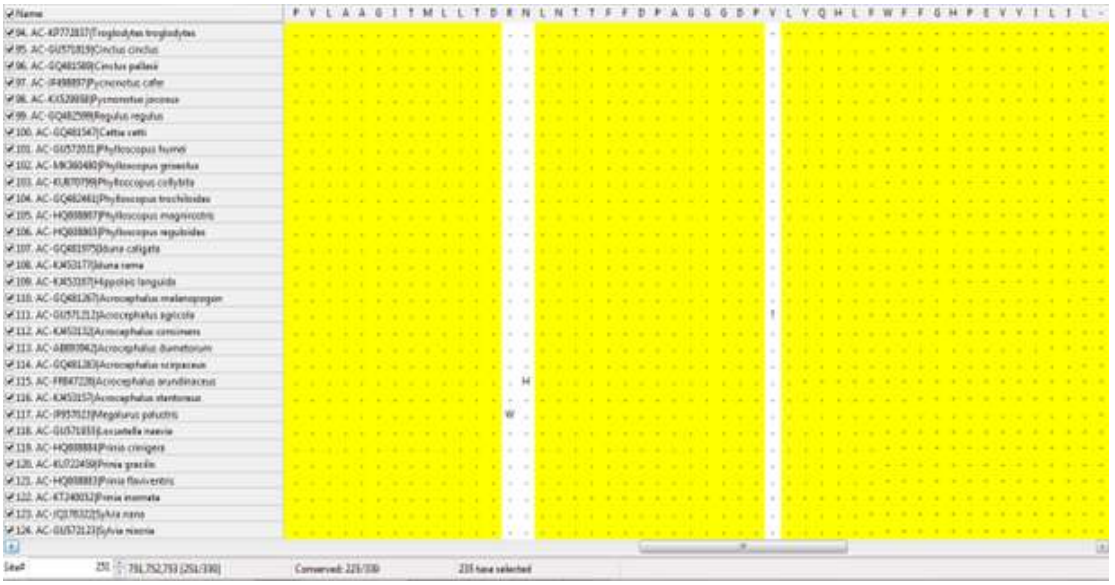
2. Kumar S., Stecher G., Li M., Knyaz C., and Tamura K. (2018). MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution 35:1547-1549.

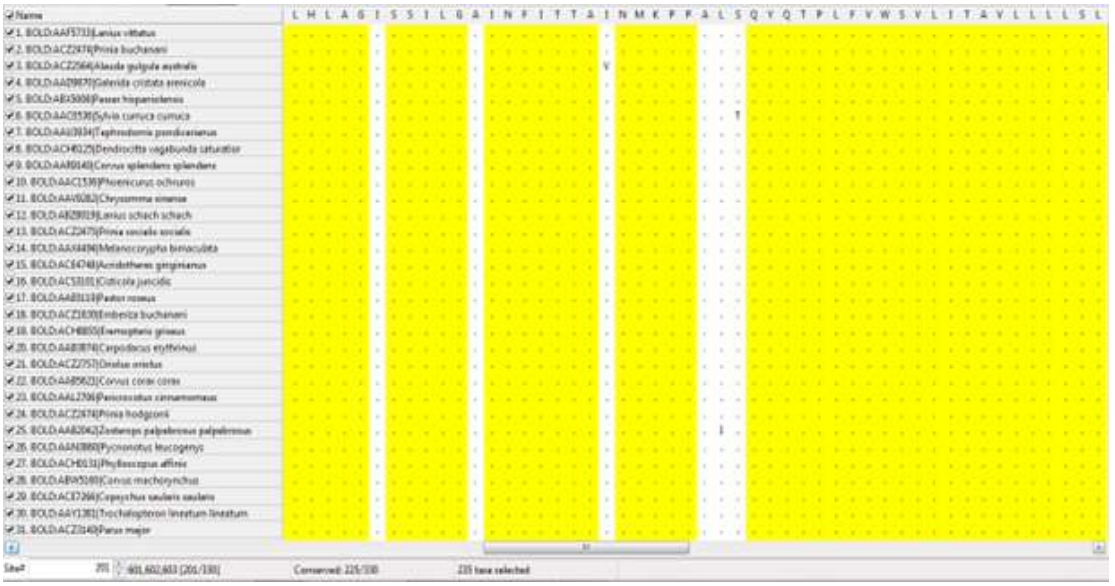
Appendix F

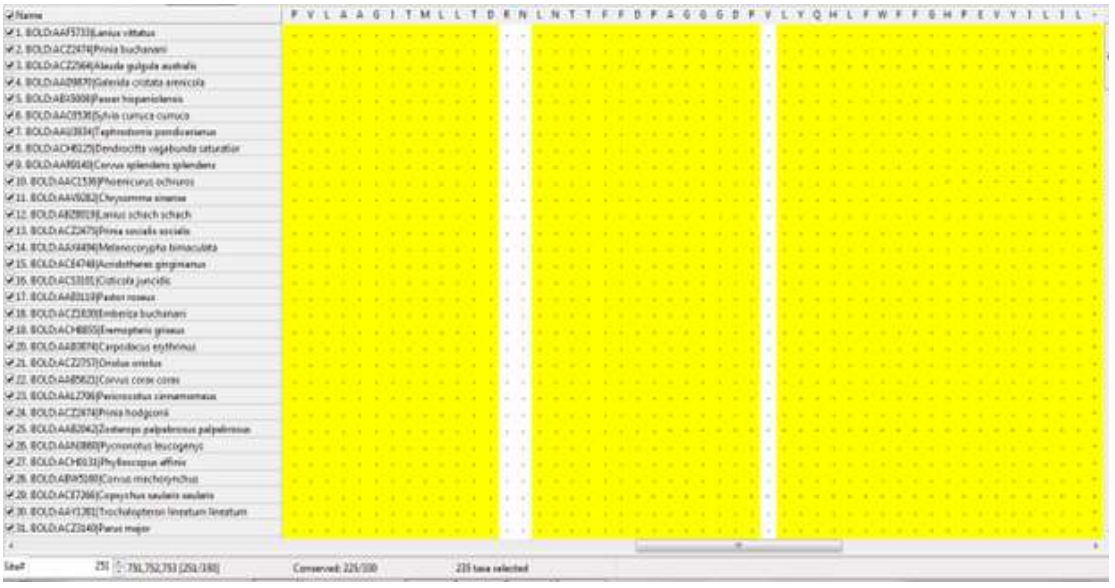
(i): Variation at Amino Acid Level (Conserved Sites) in Sequences of Passeriformes Computed by MEGAX

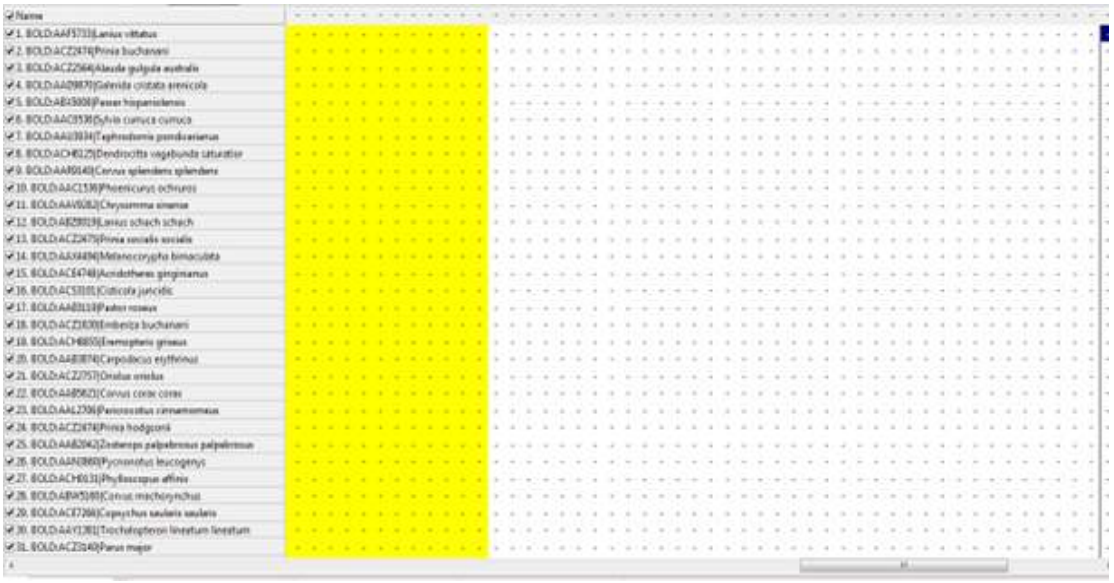


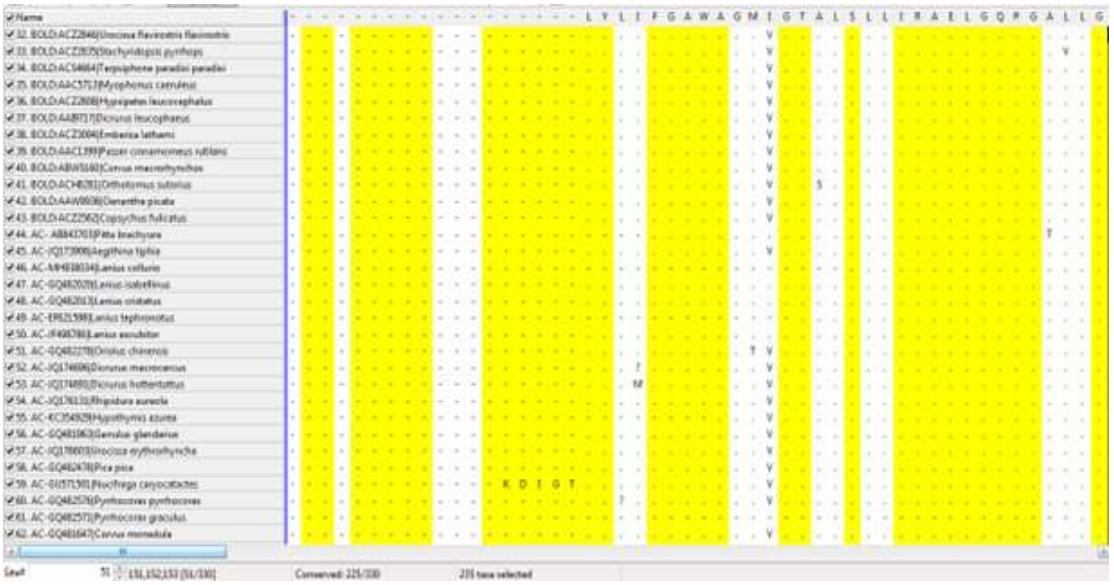


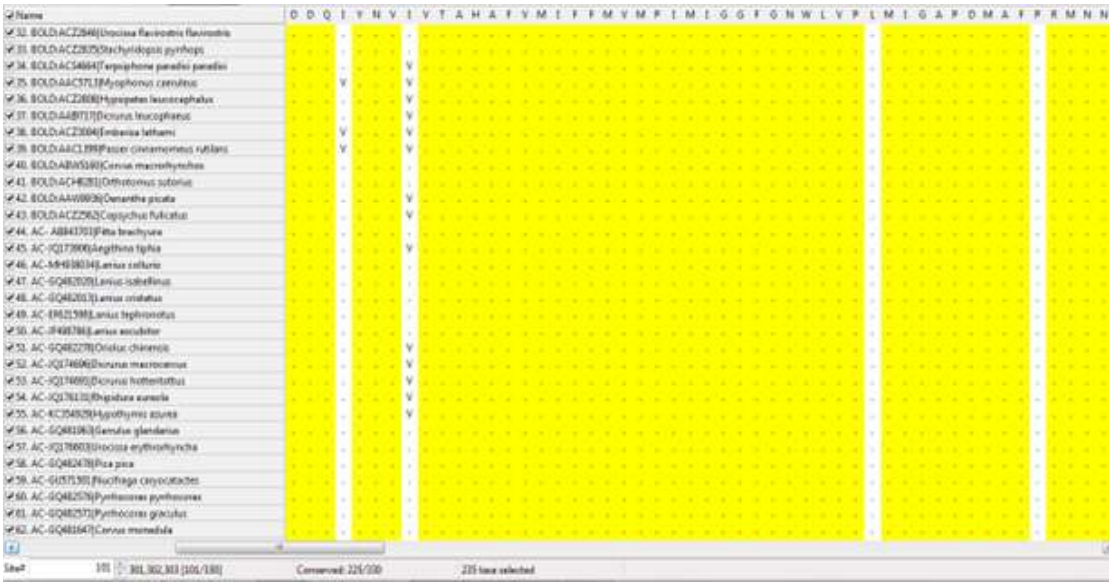


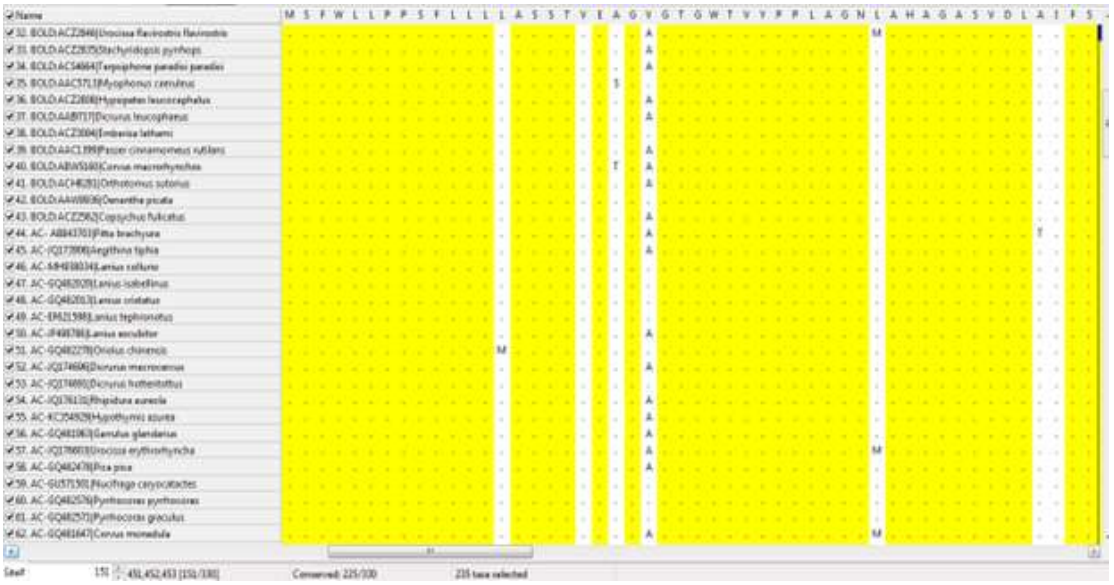


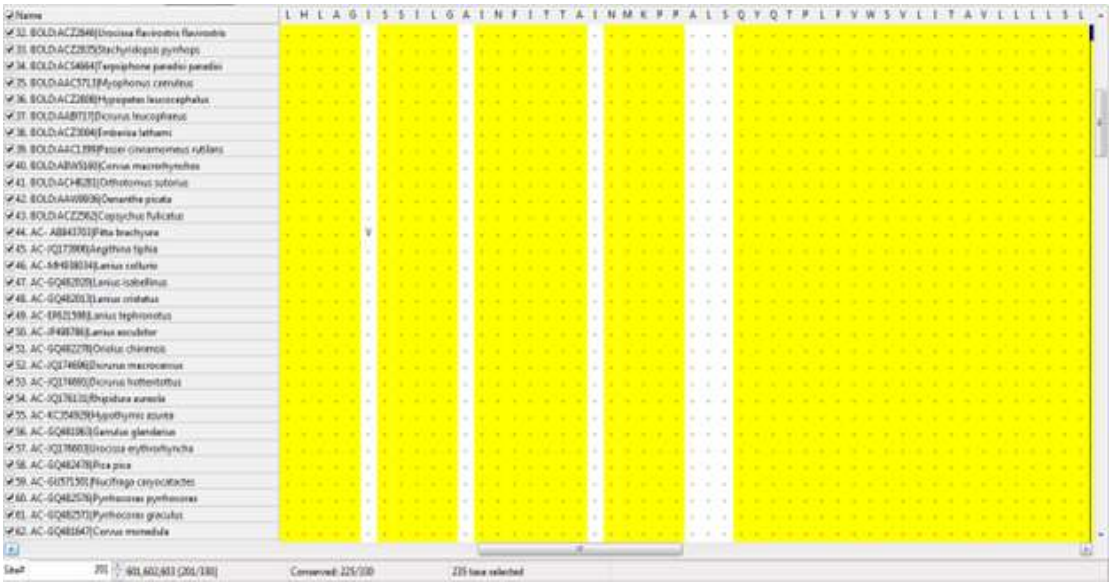


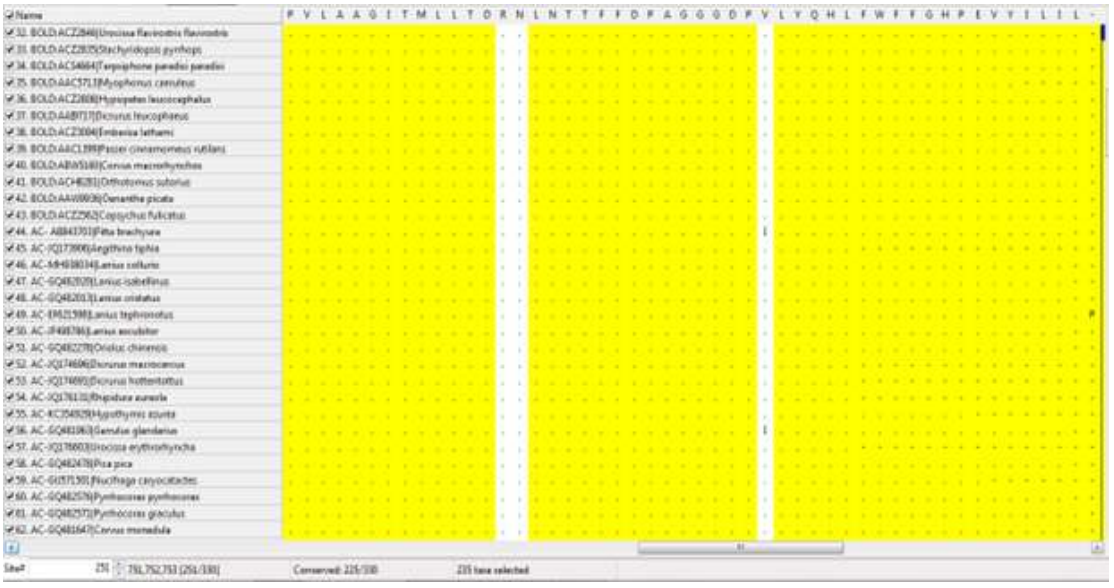


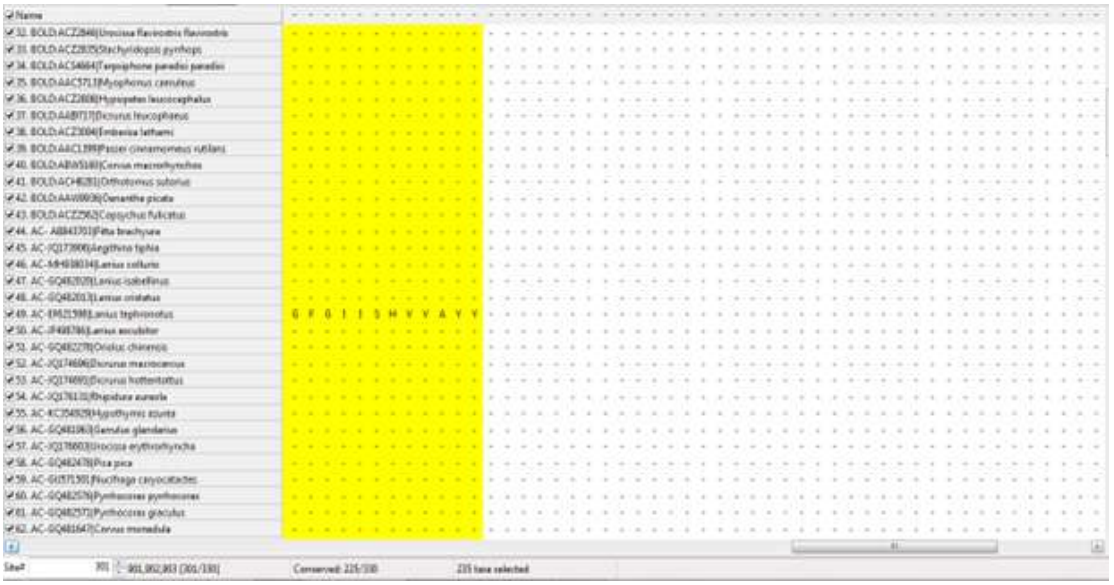


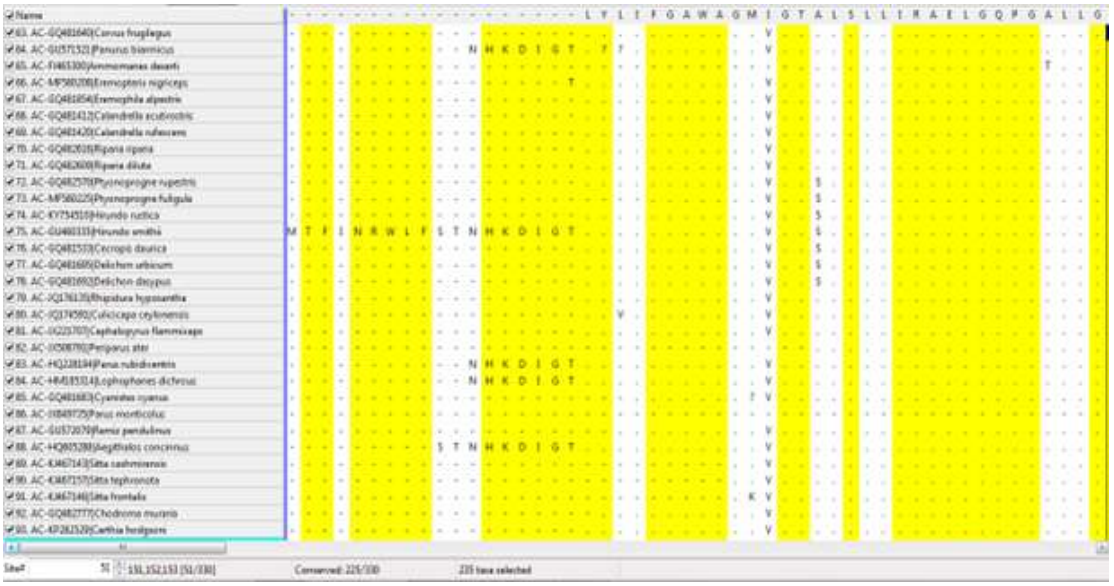


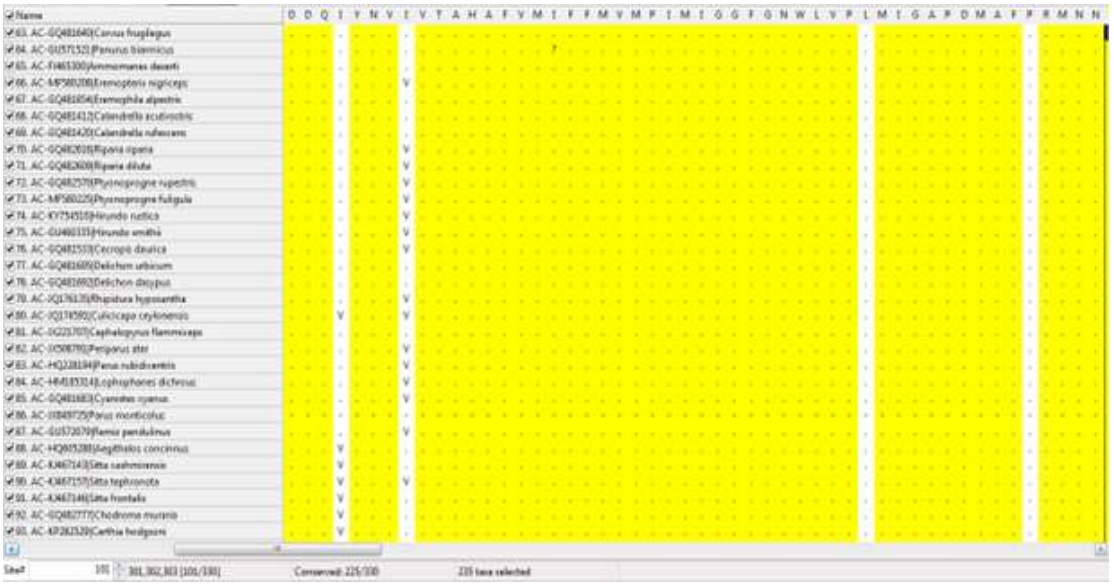


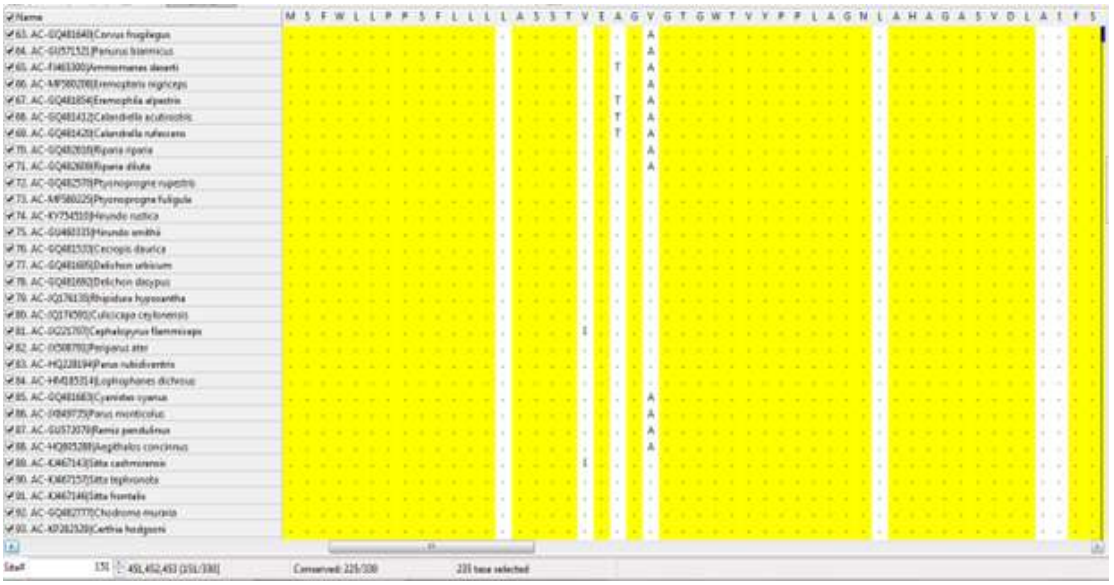


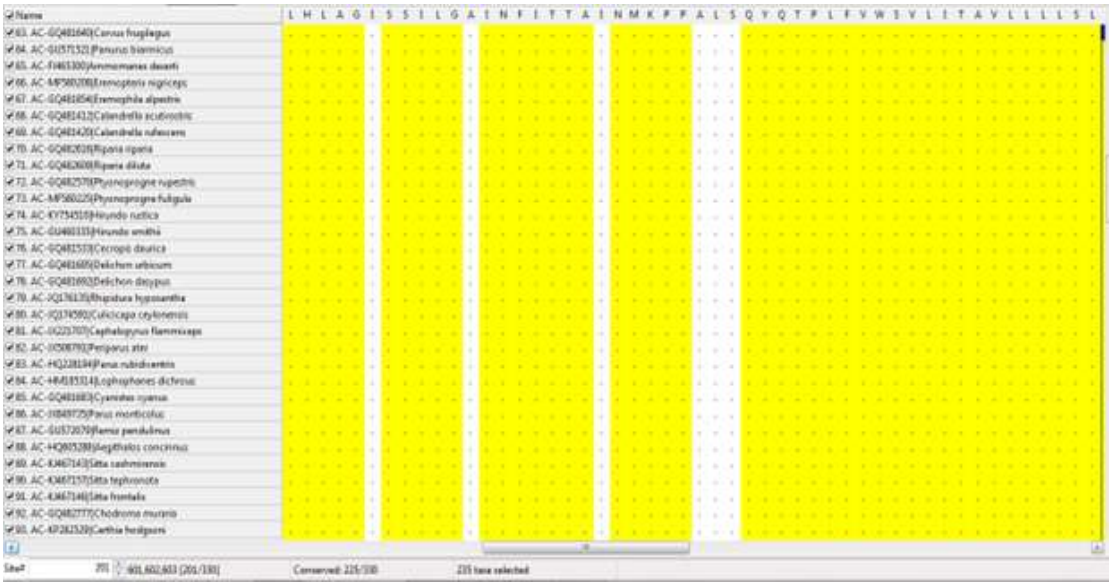


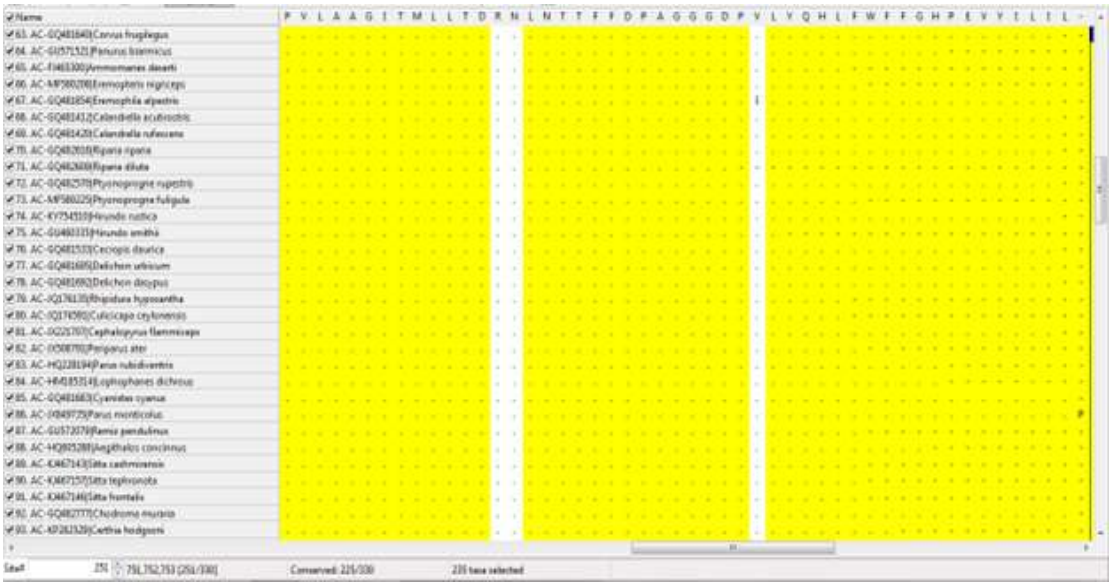


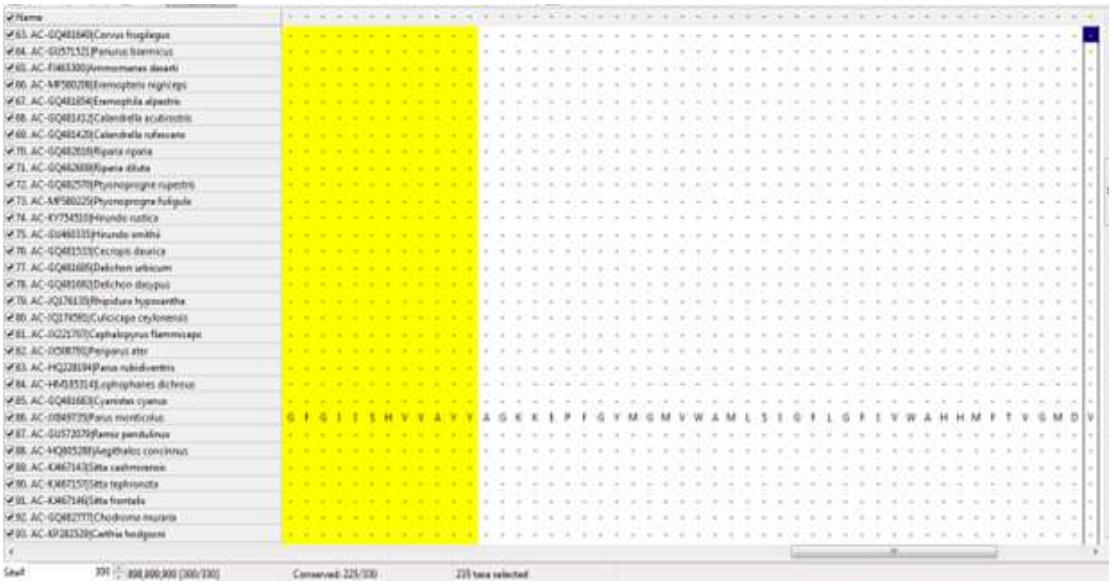


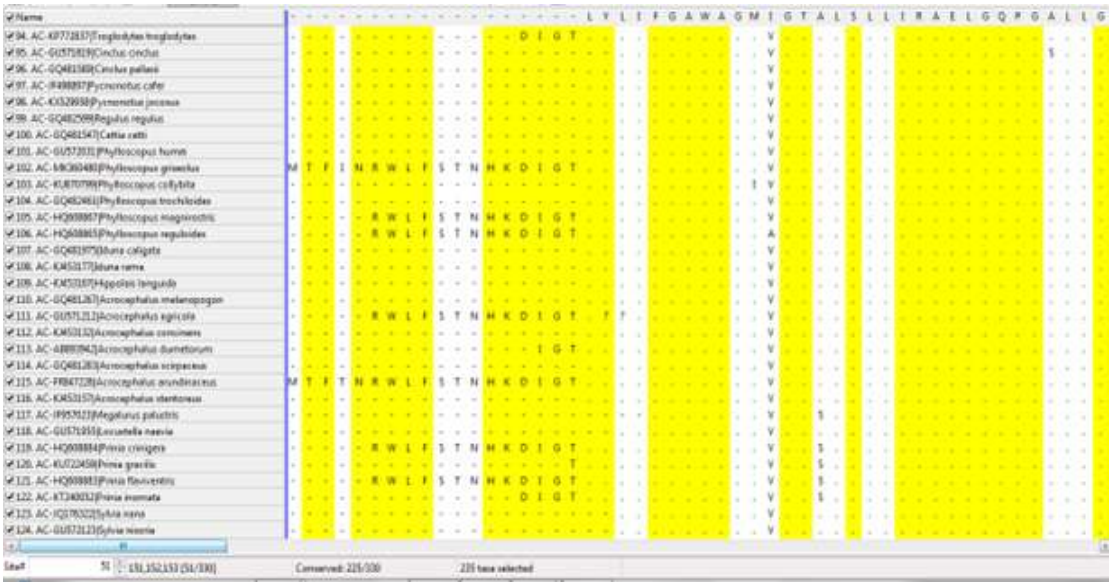


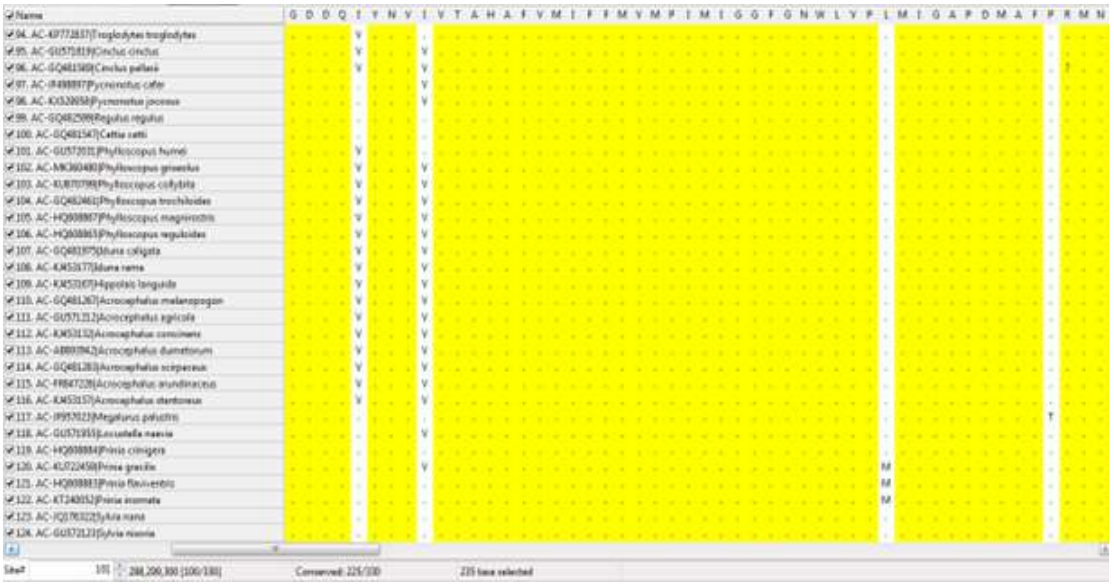


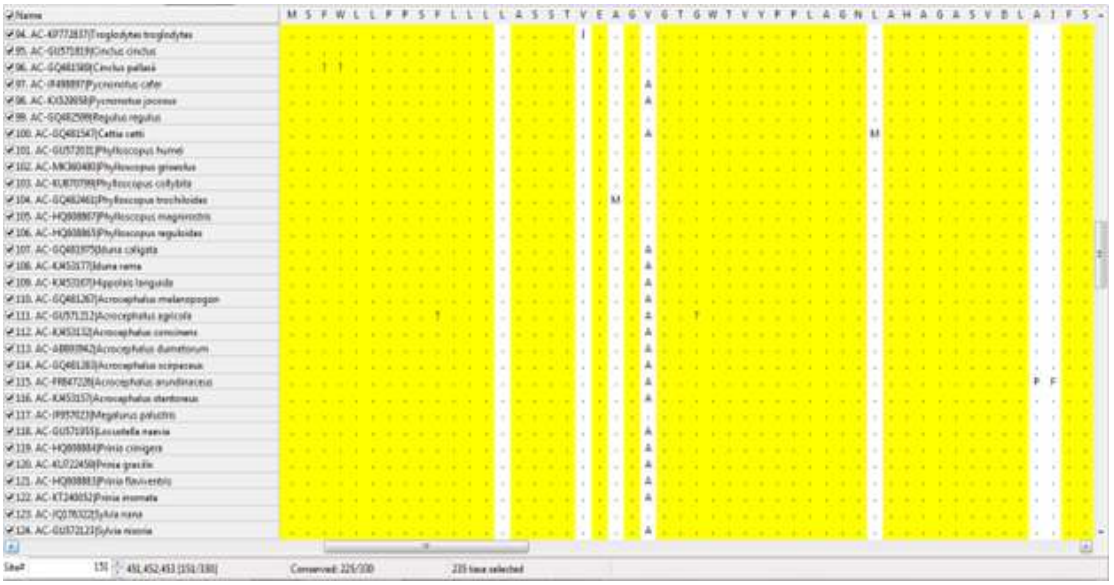


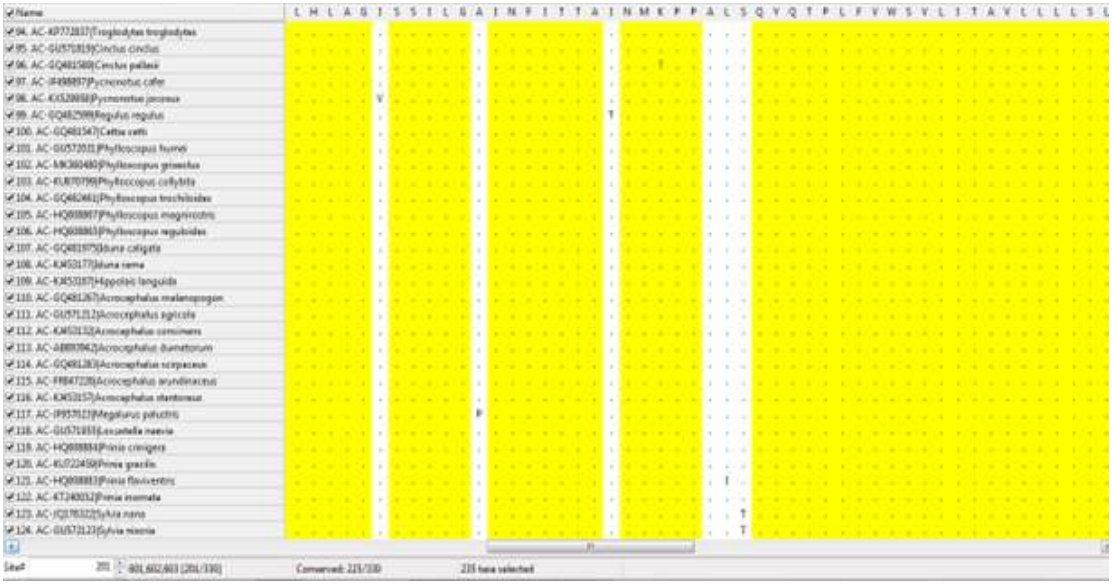


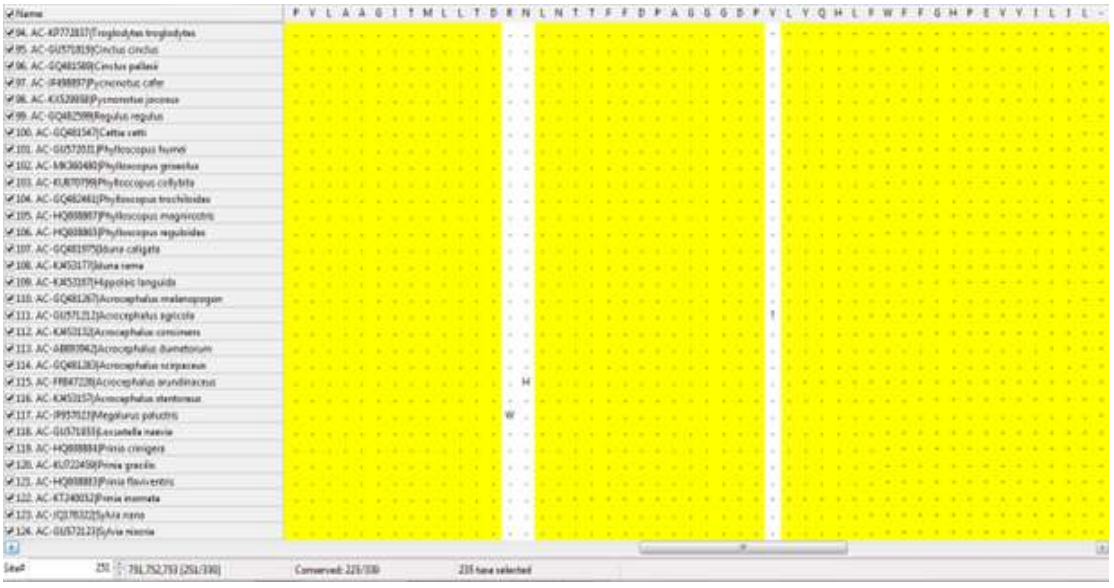


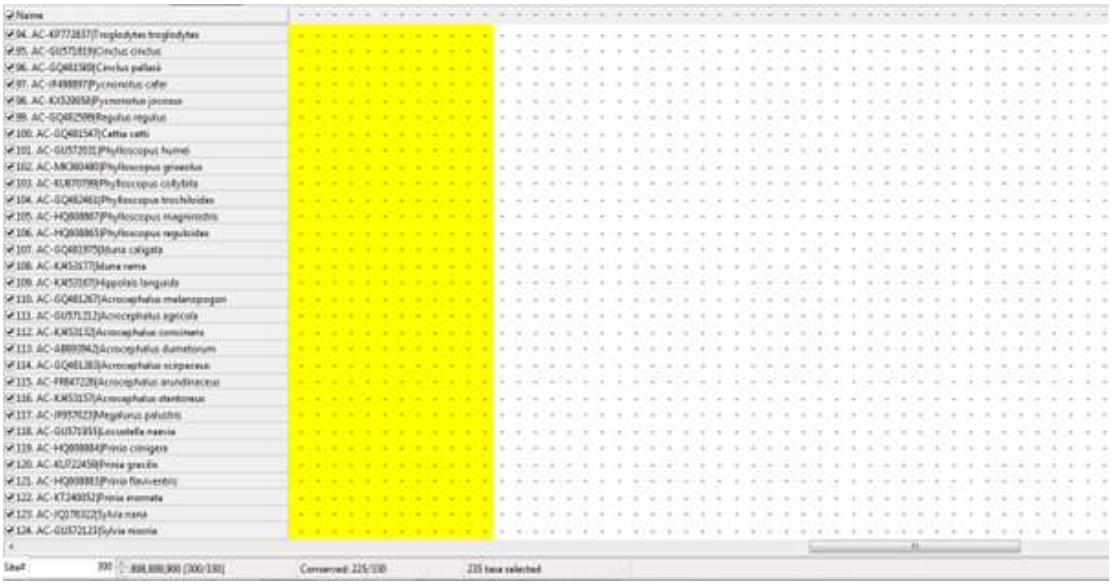


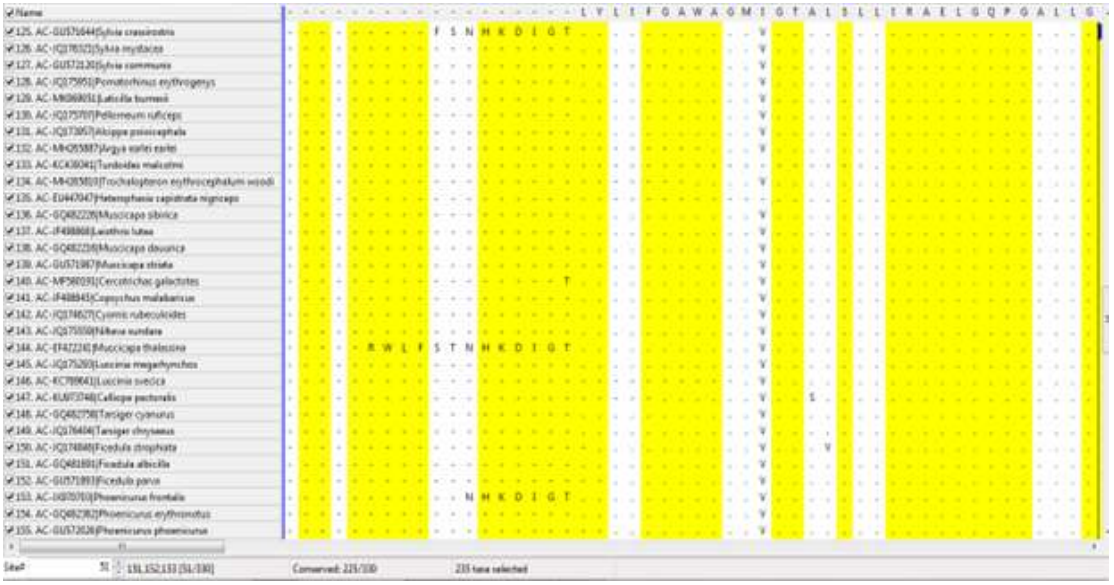


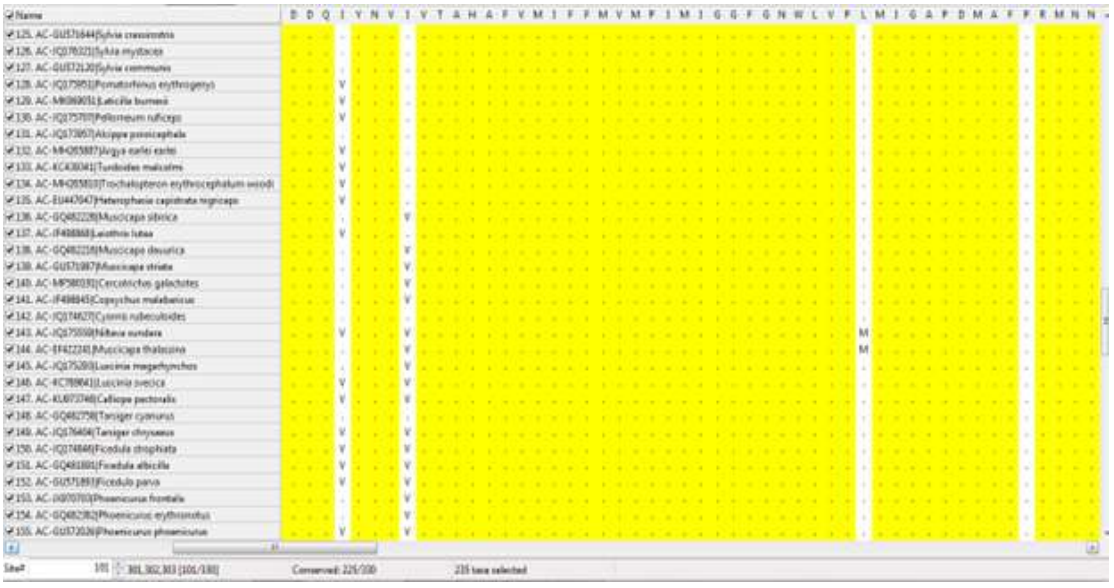


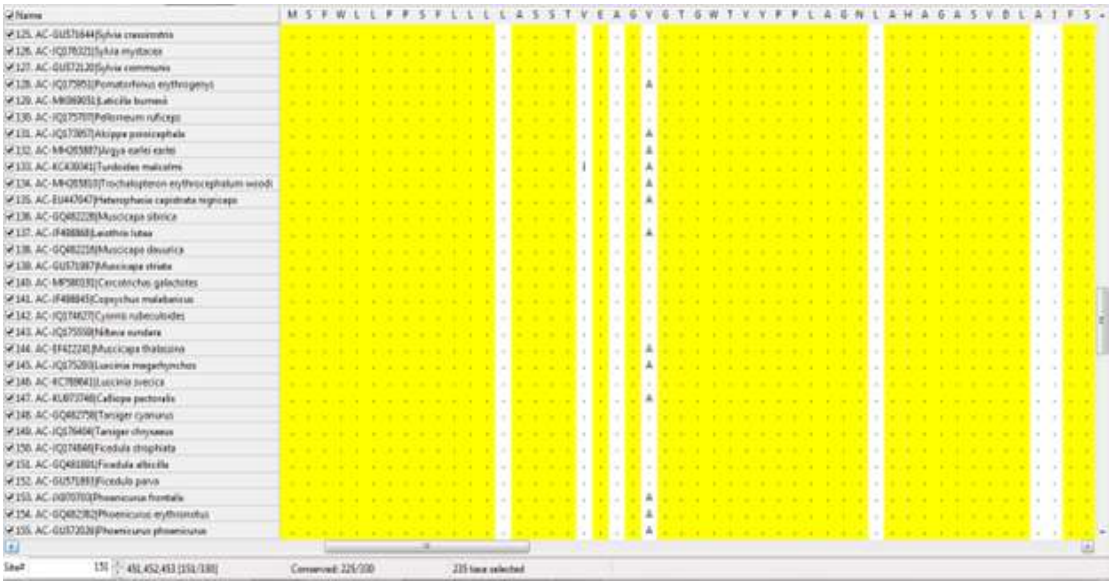


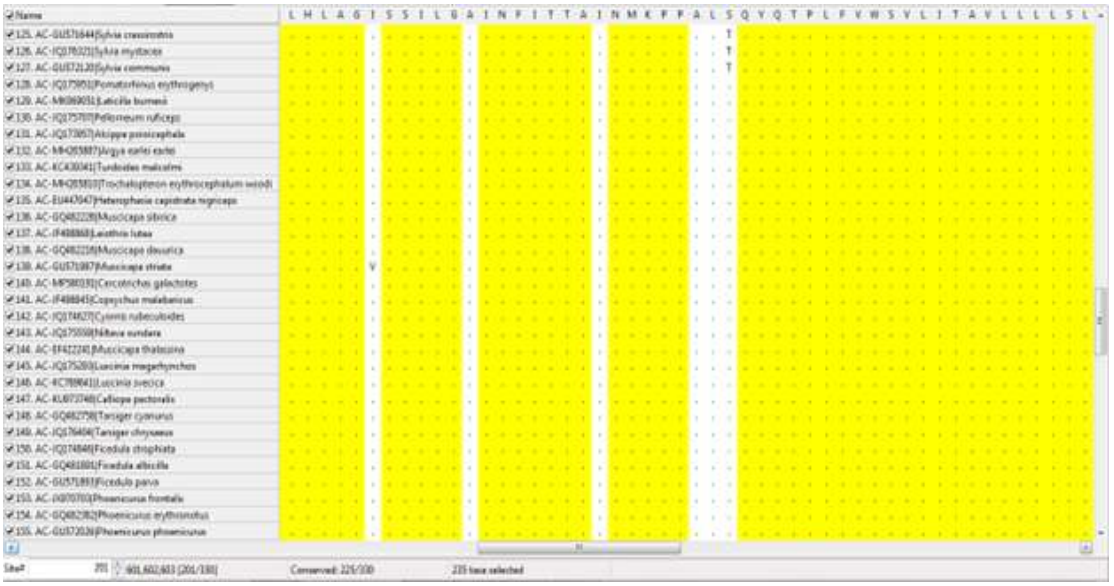


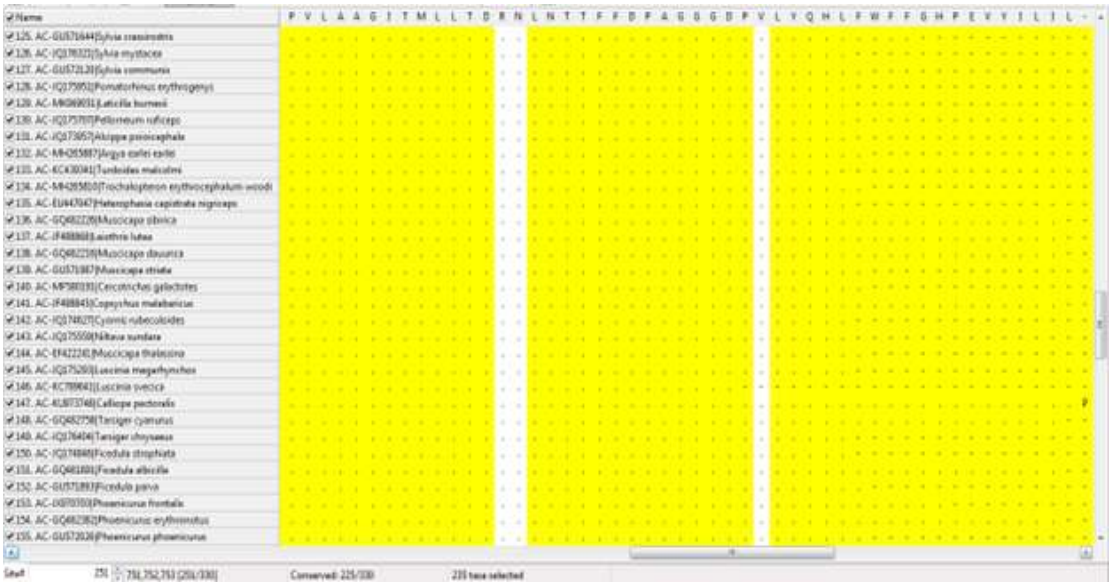


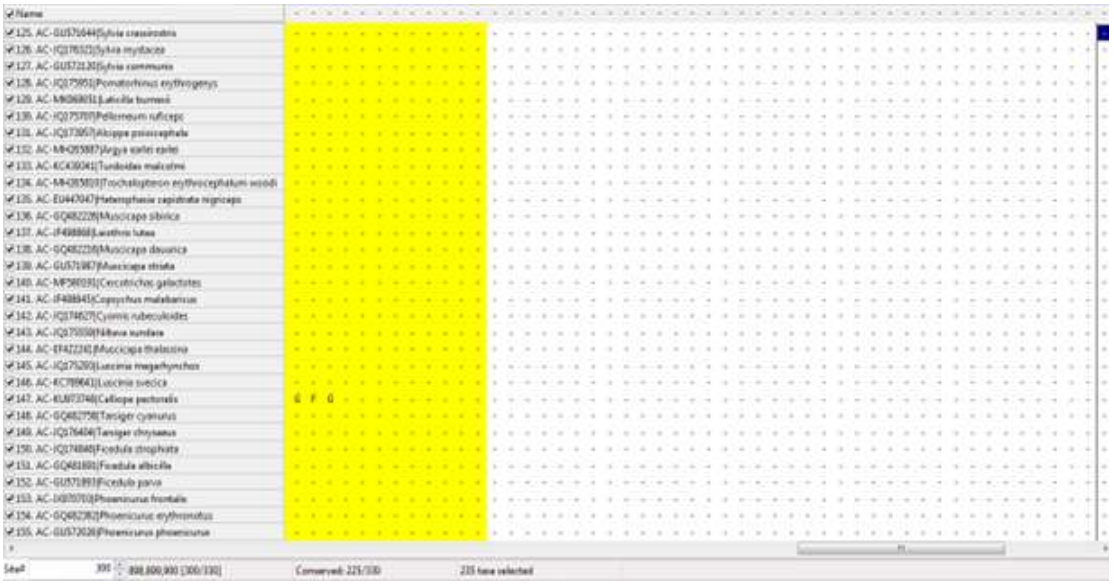


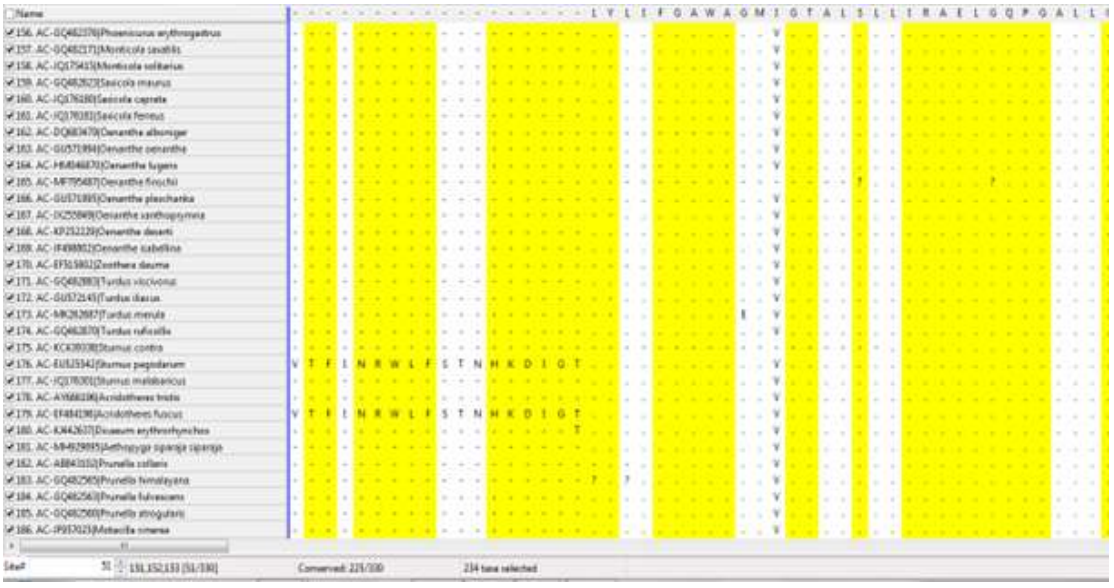


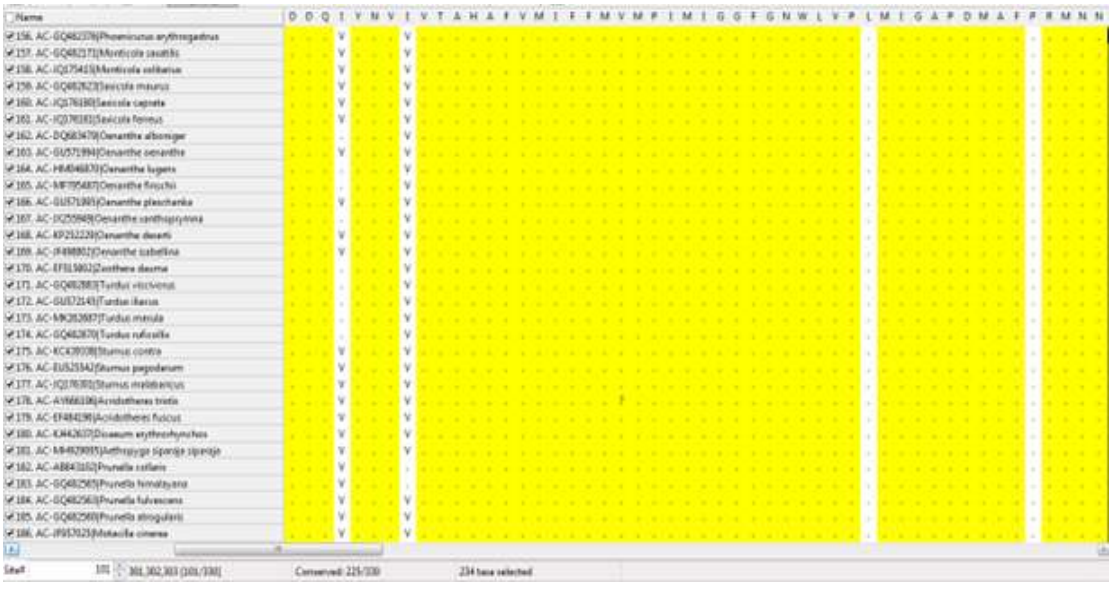


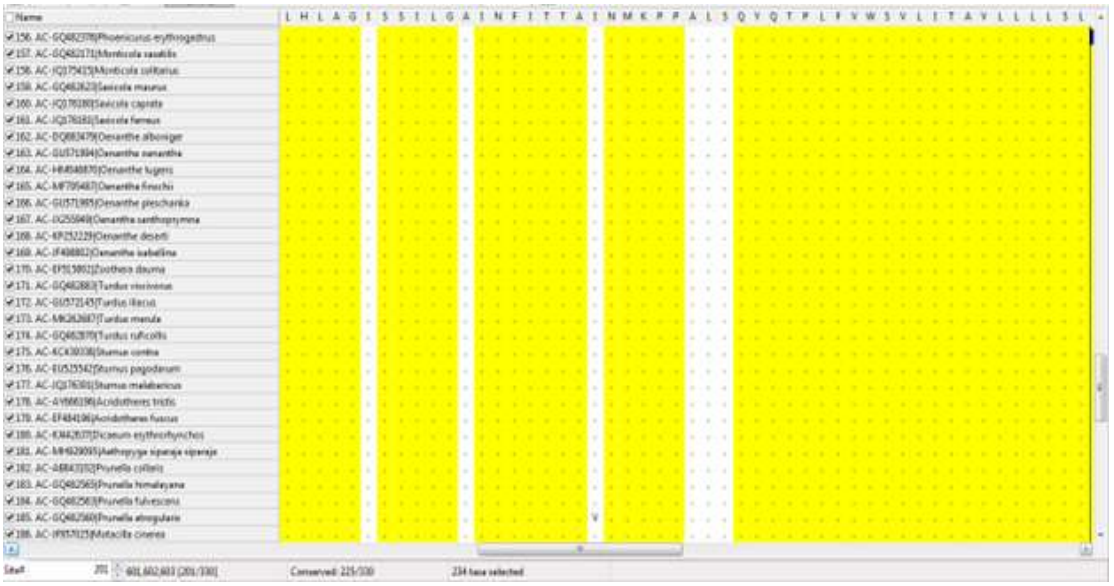


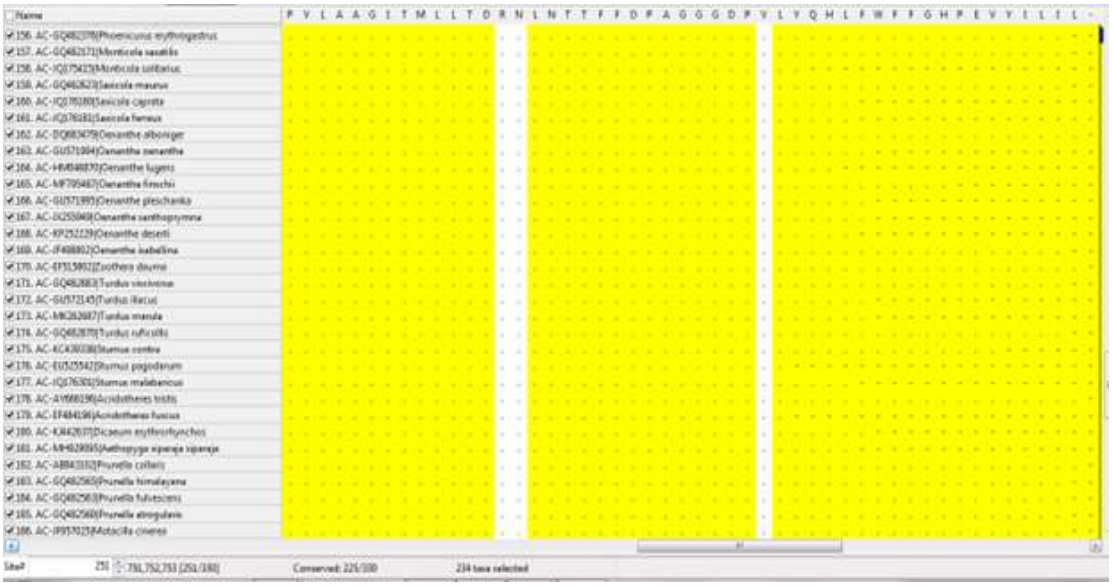


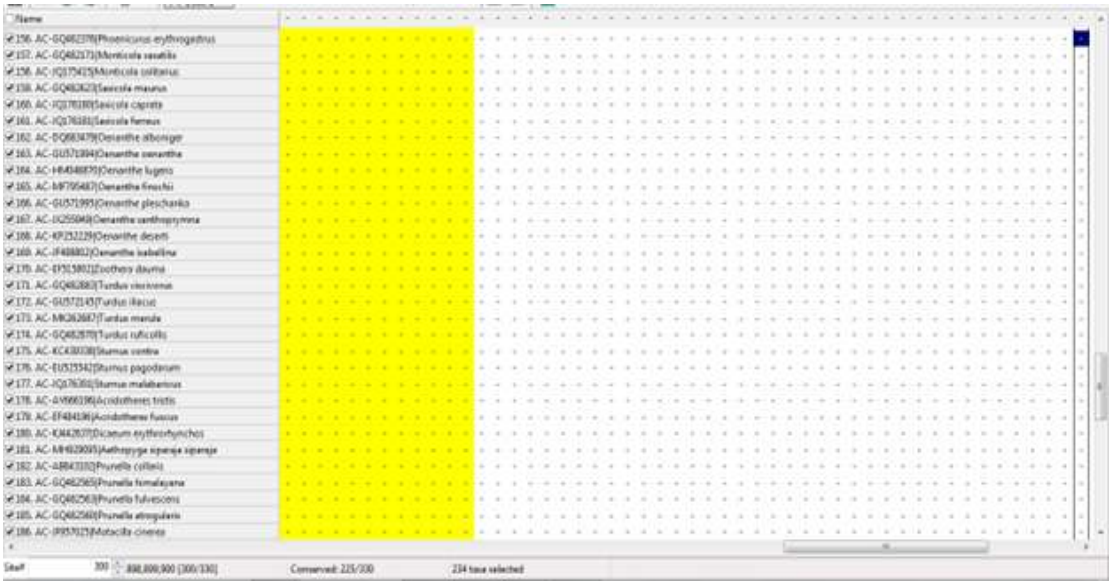


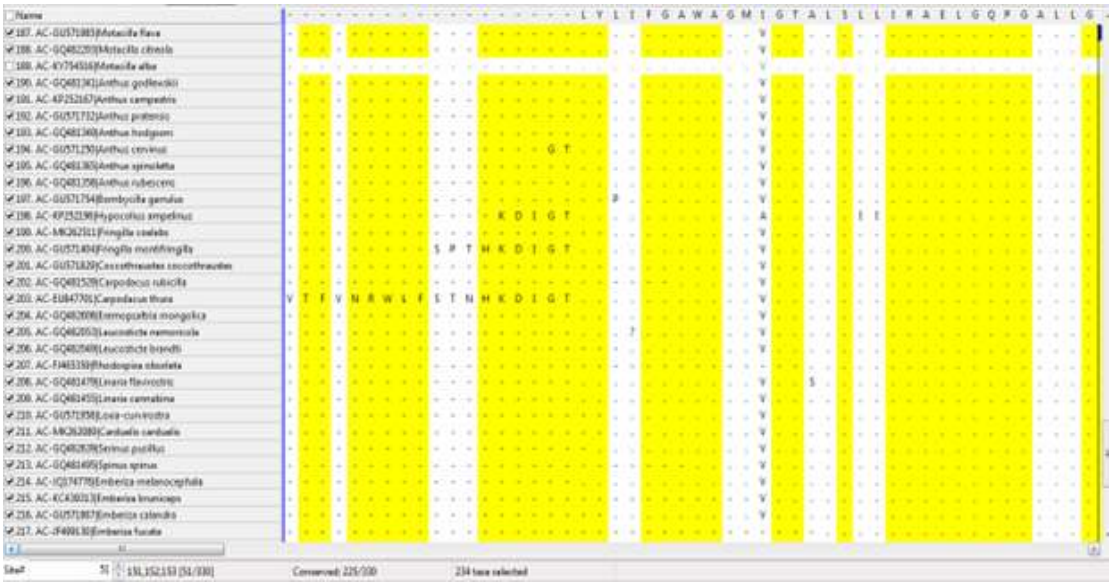


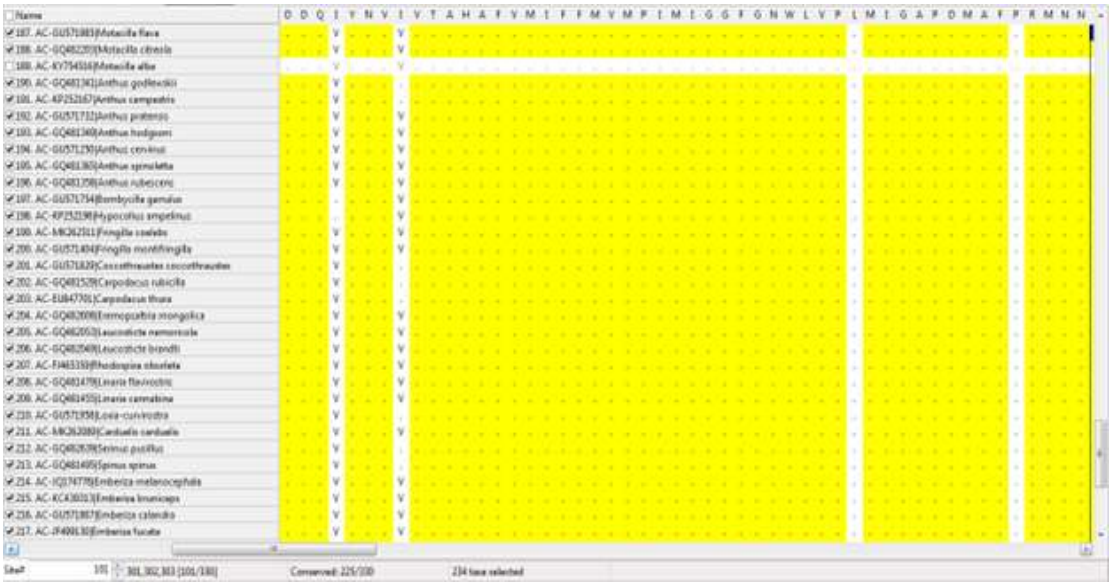


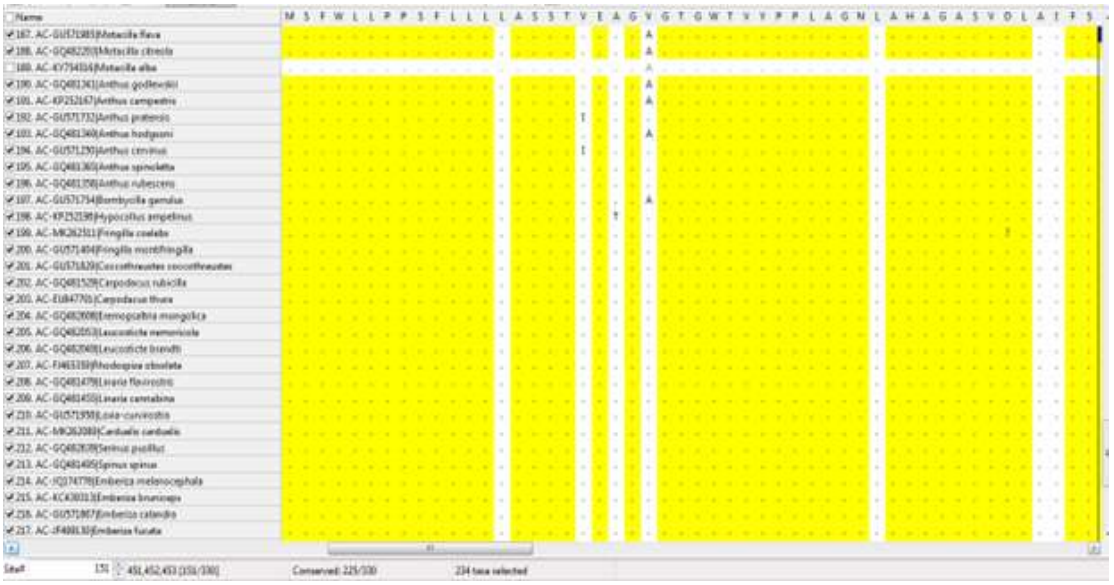


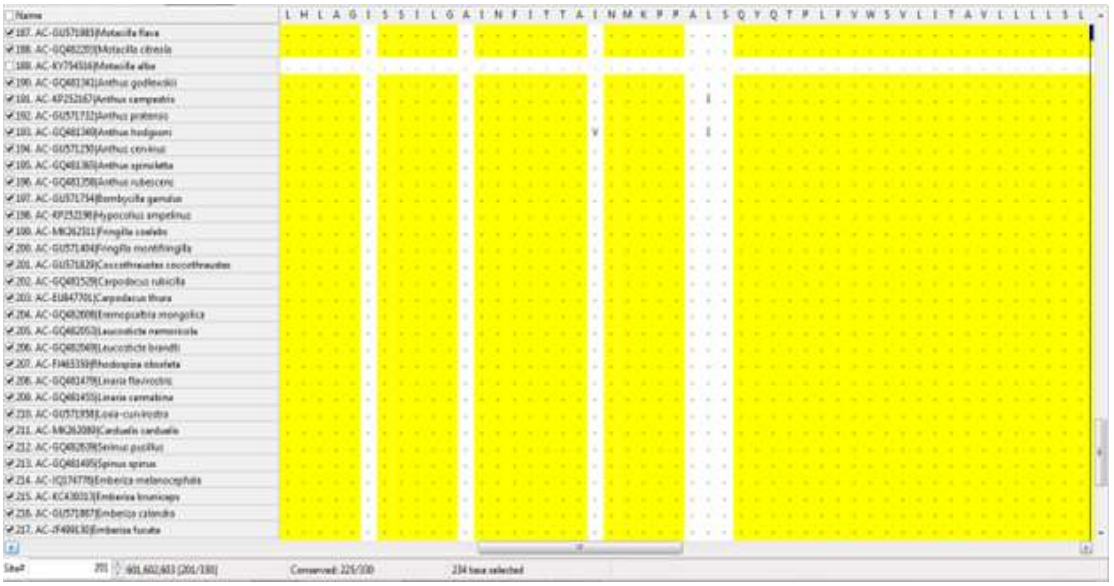


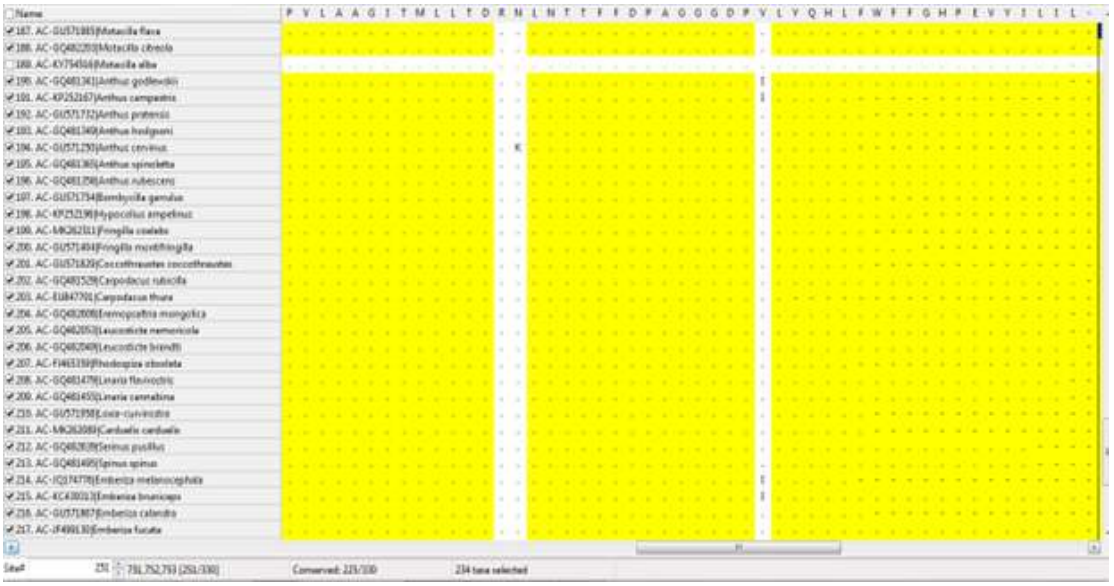


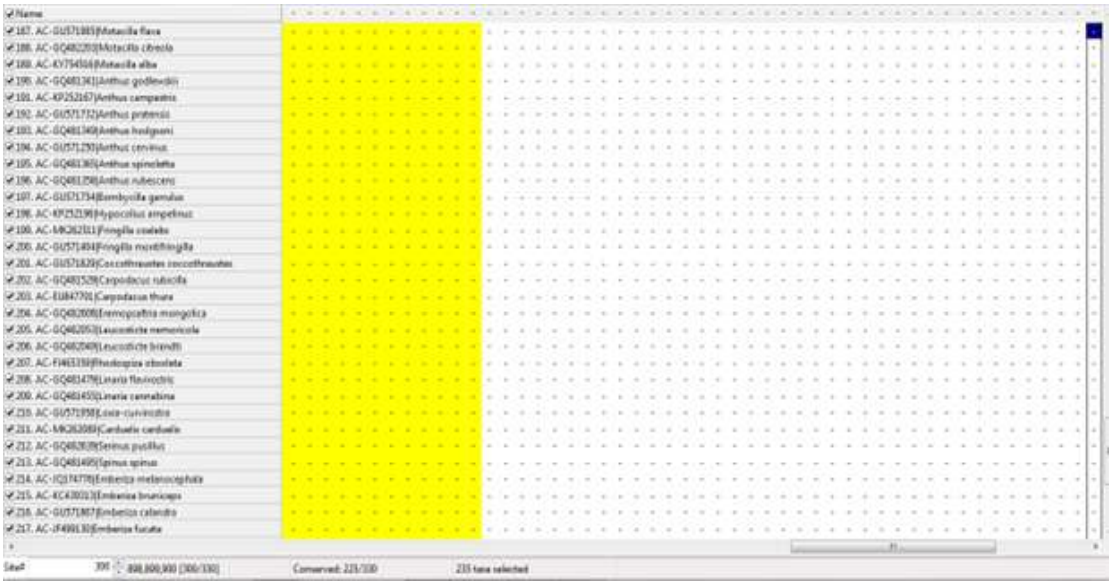


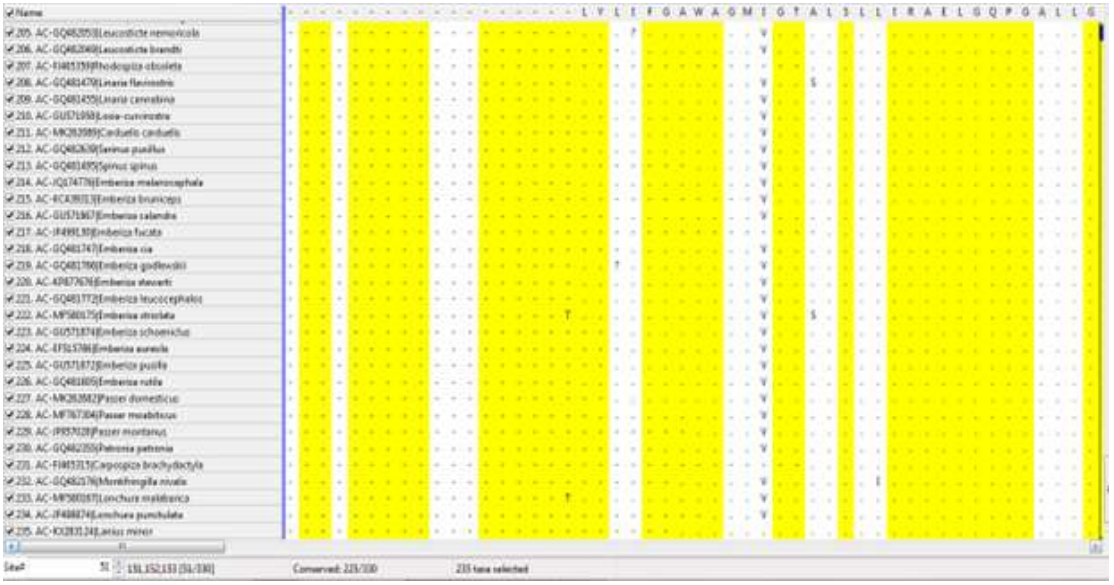


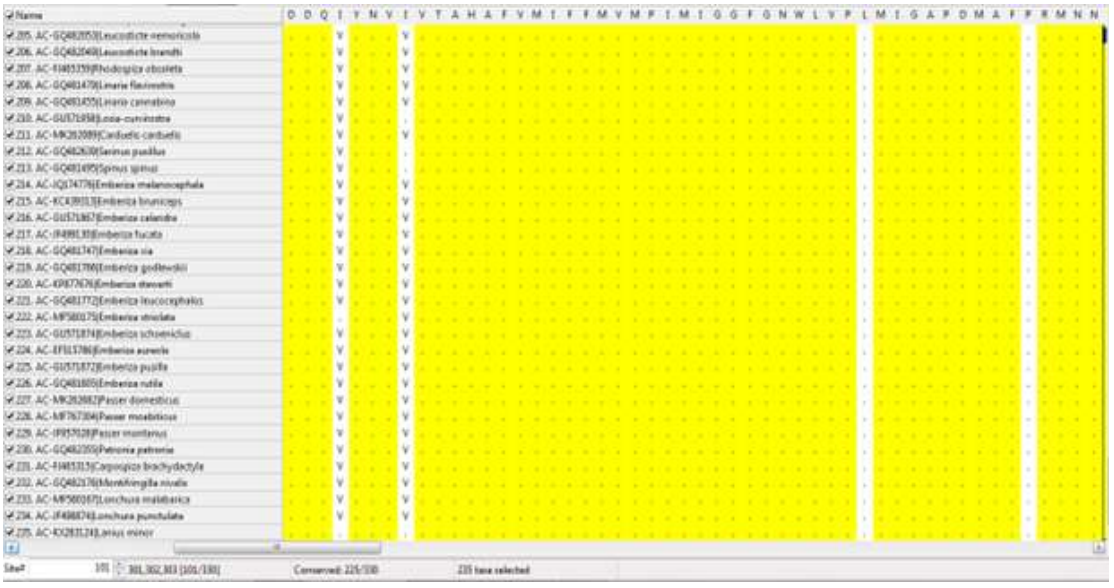


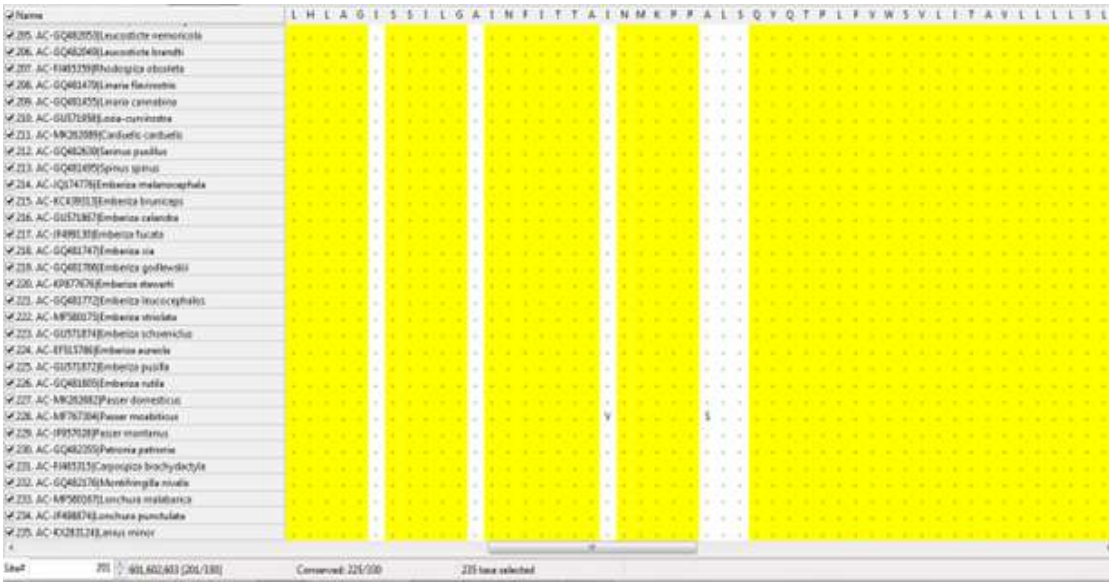


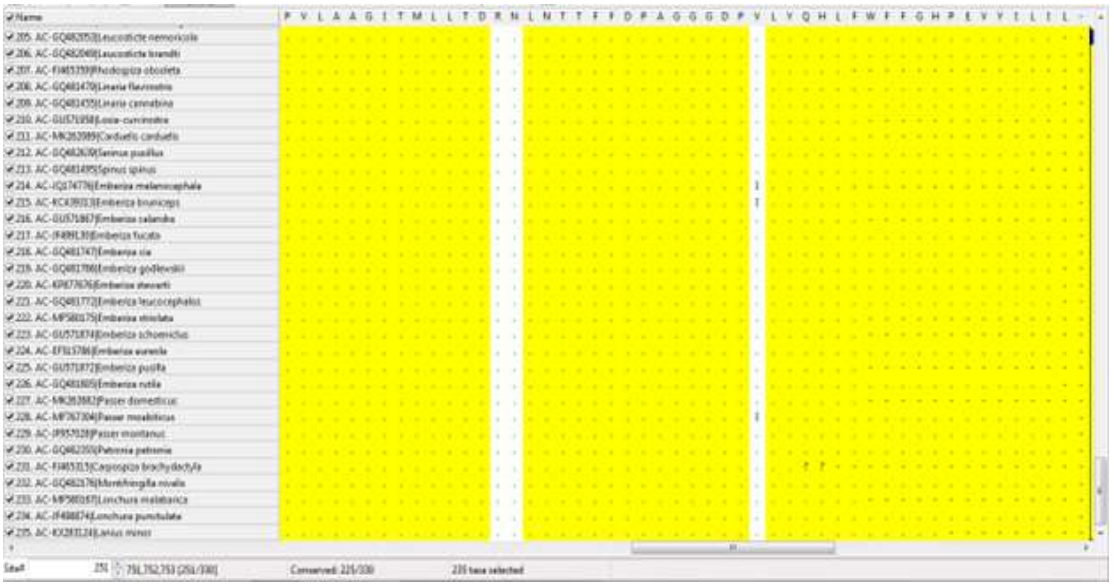


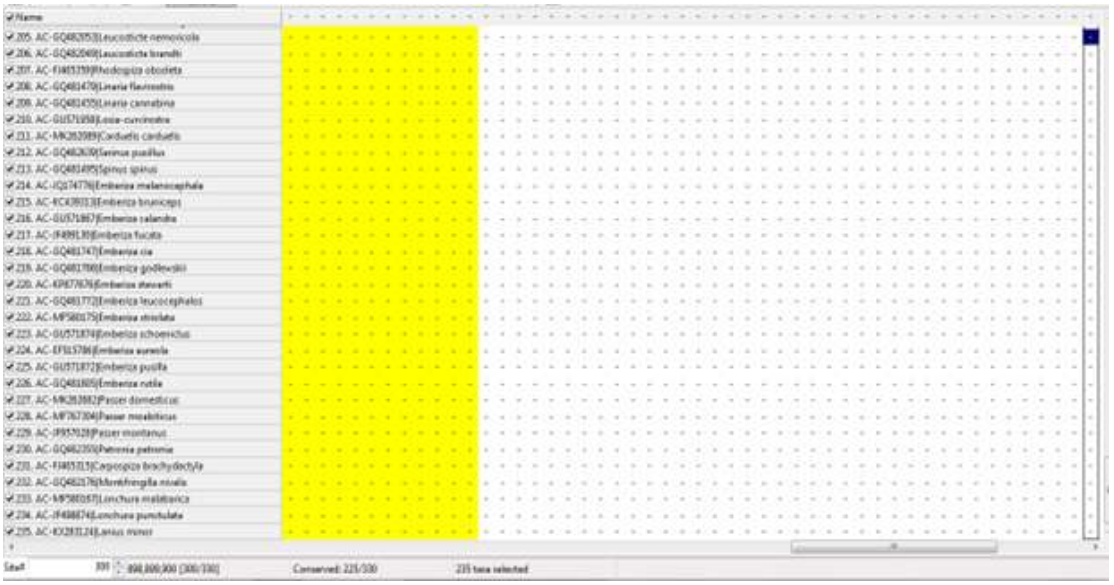




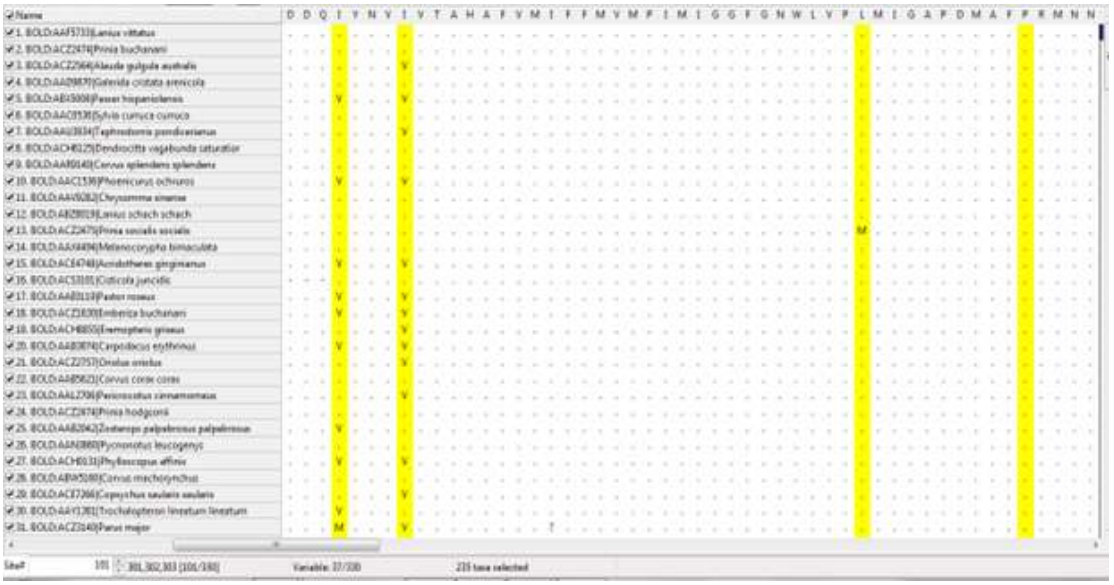


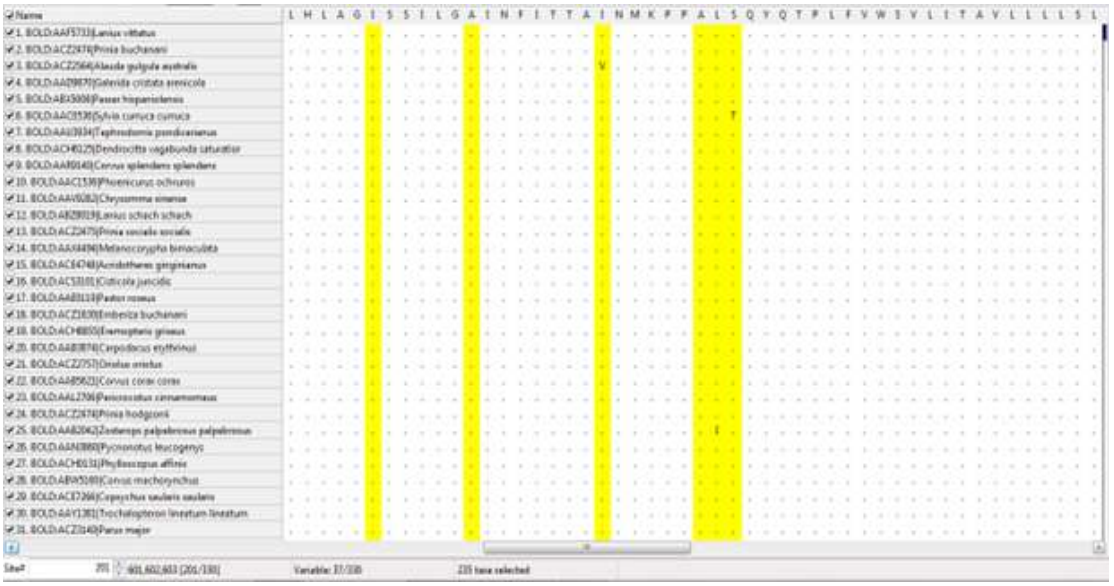


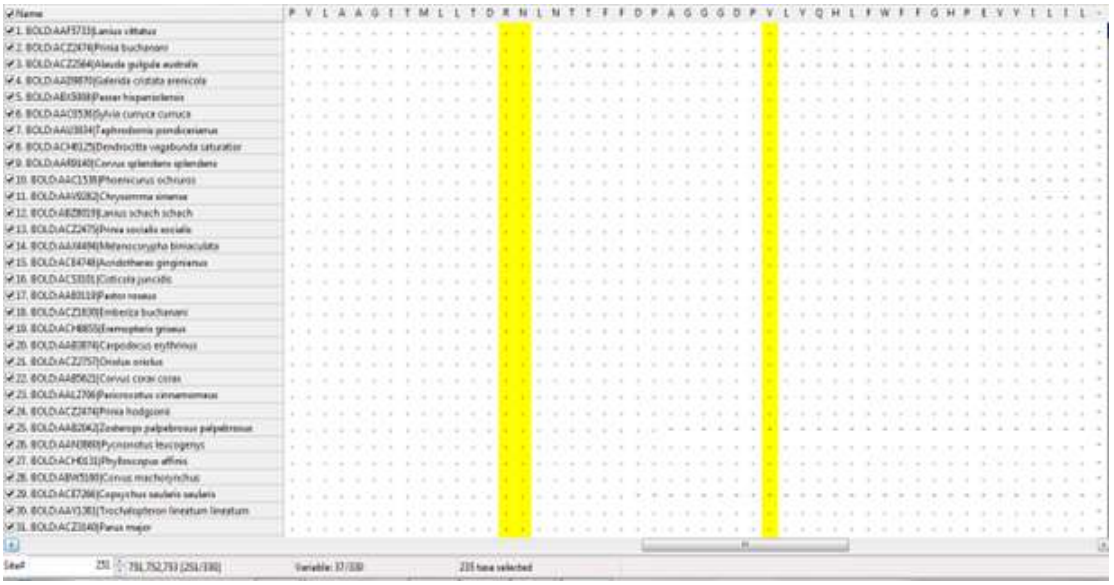


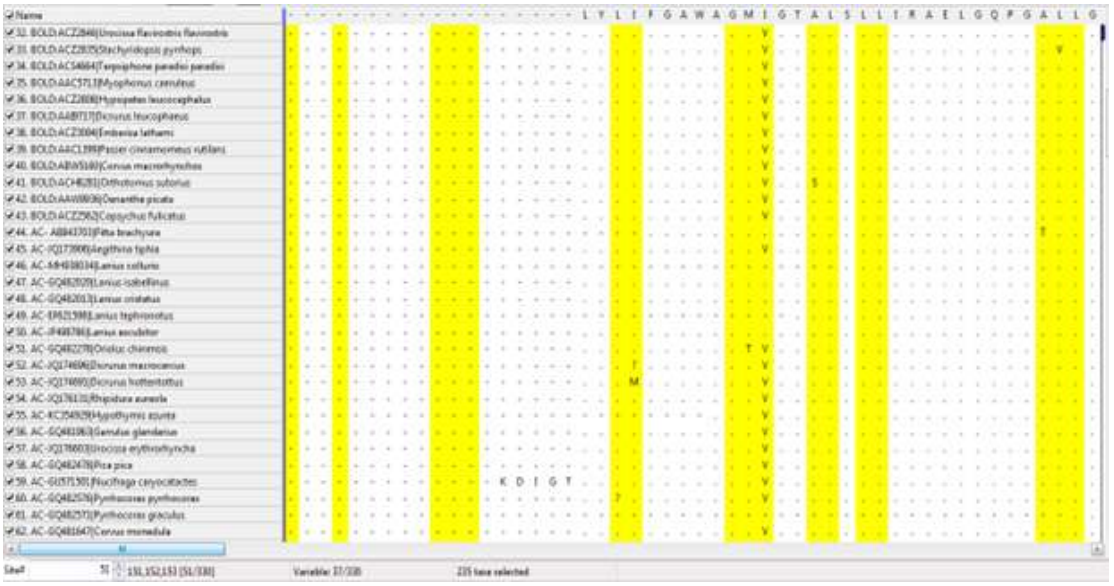


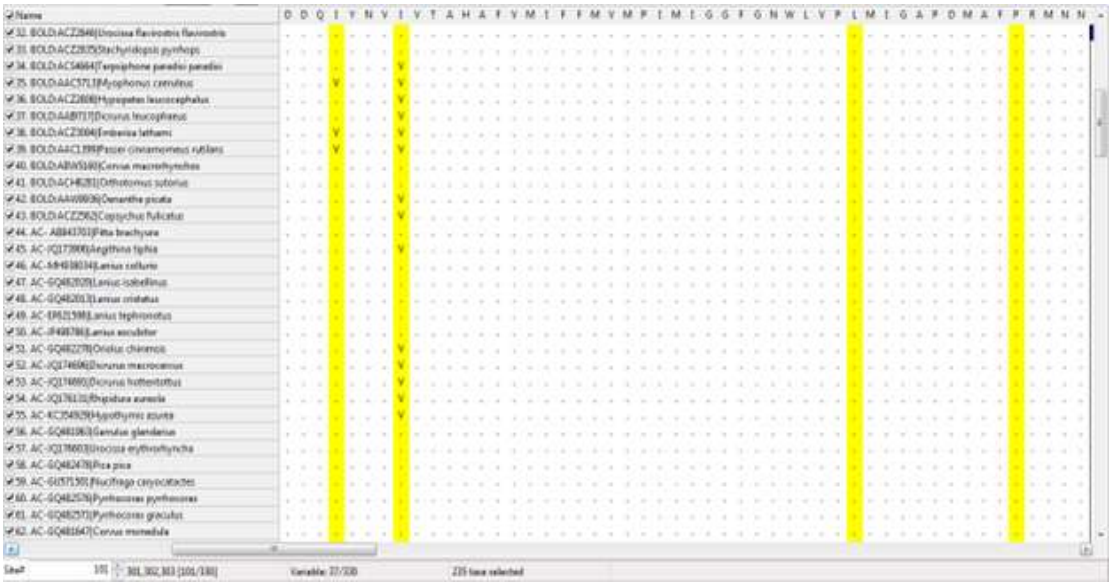
(ii): Variation at Amino Acid Level (Variable Sites) in Sequences of Passeriformes Computed by MEGAX

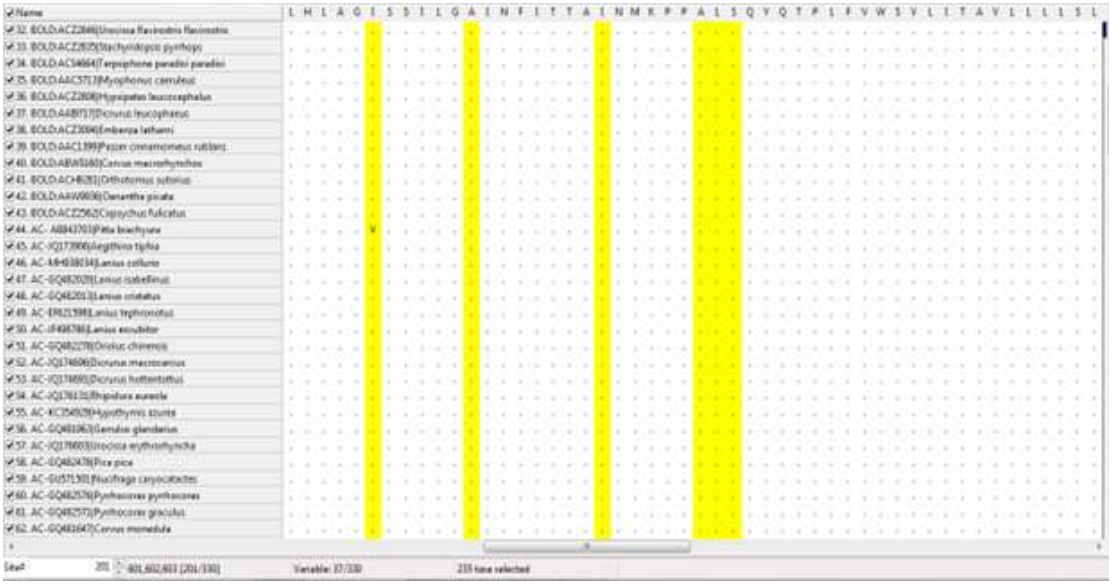


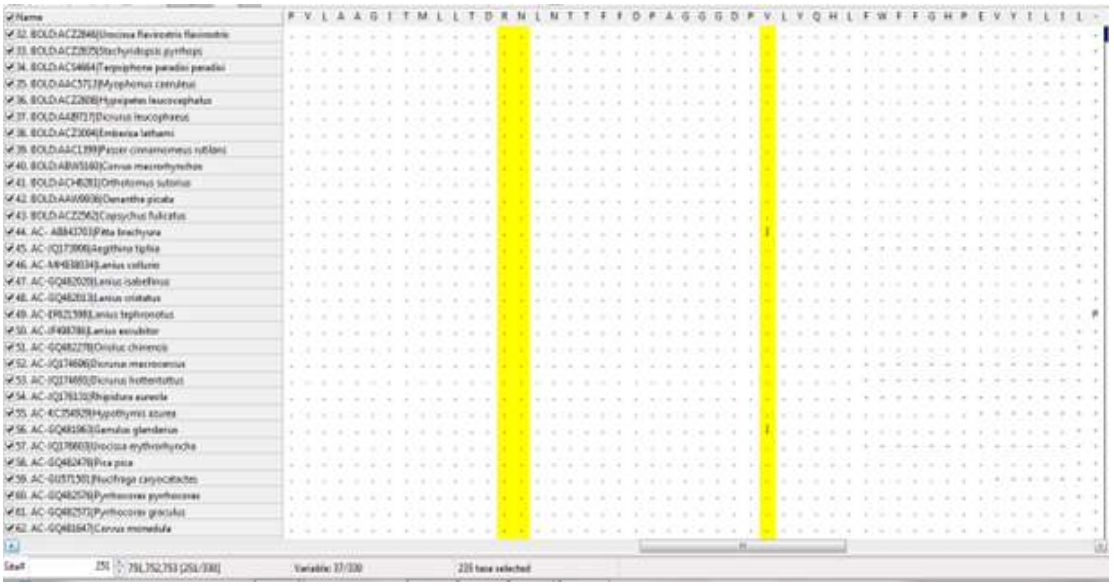


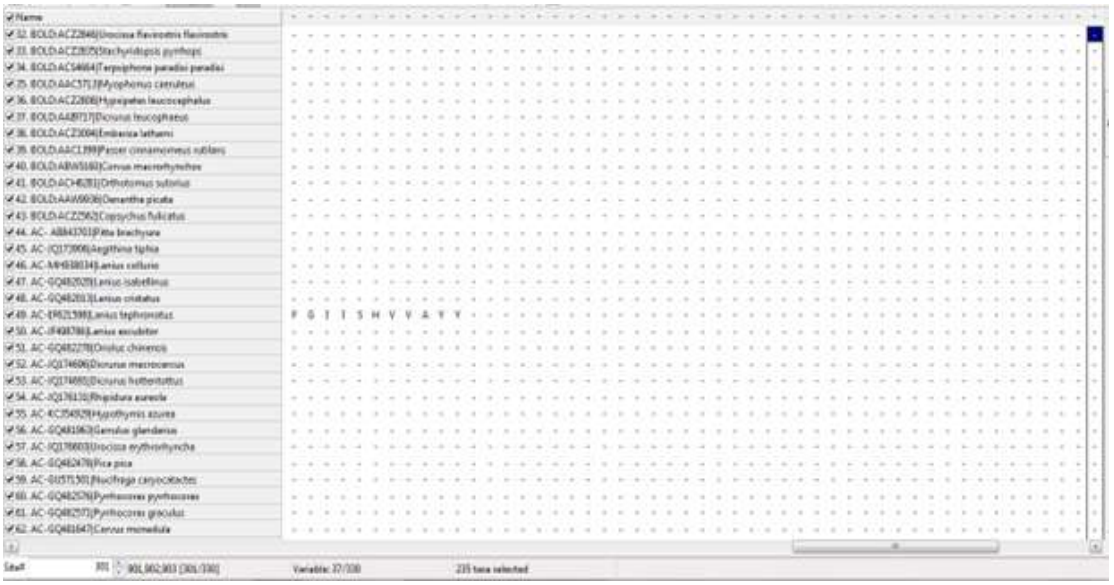


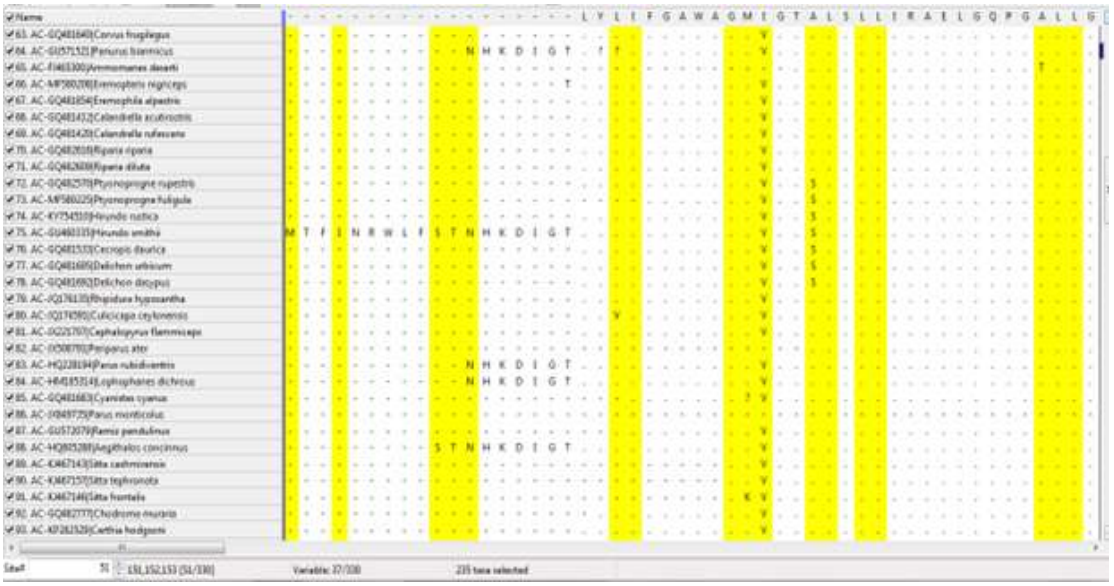


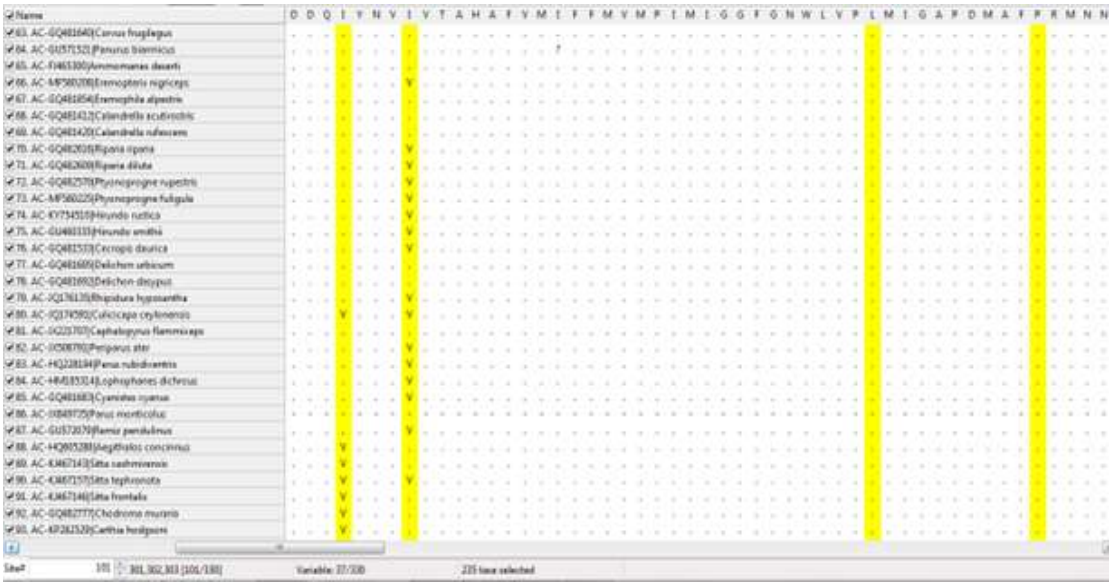


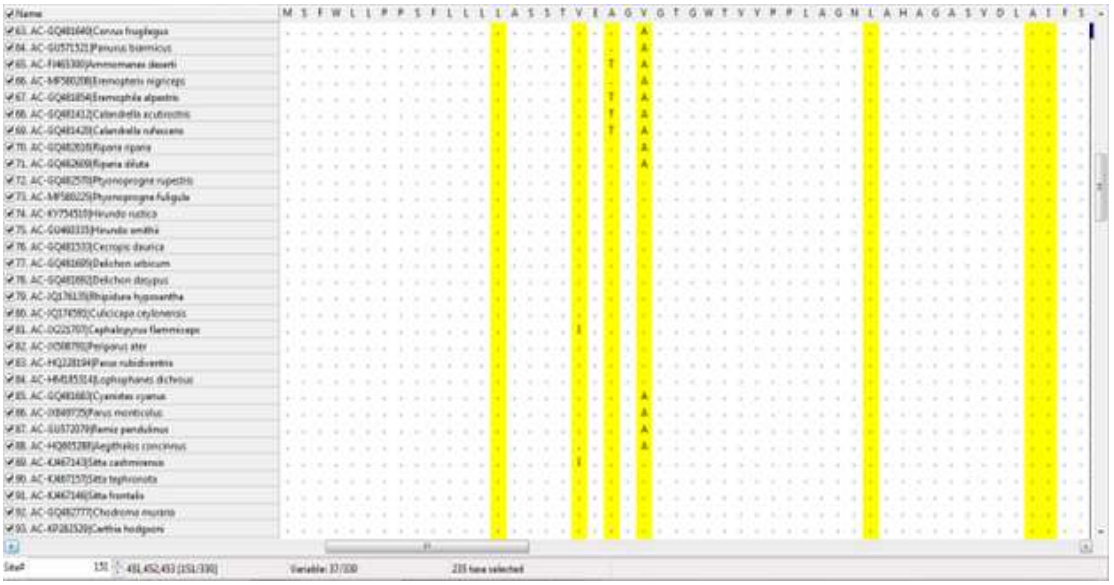


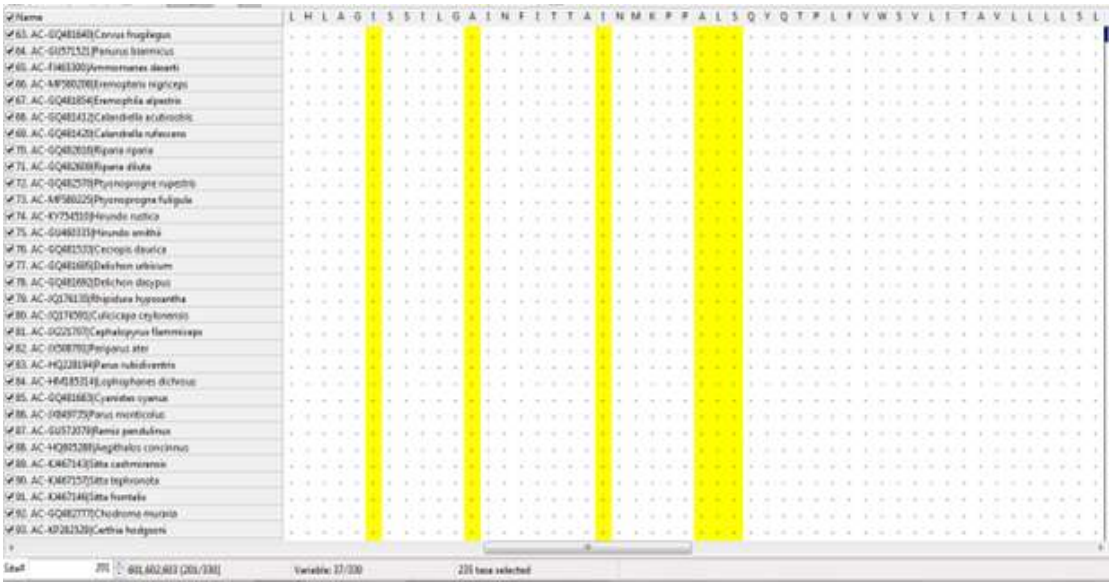


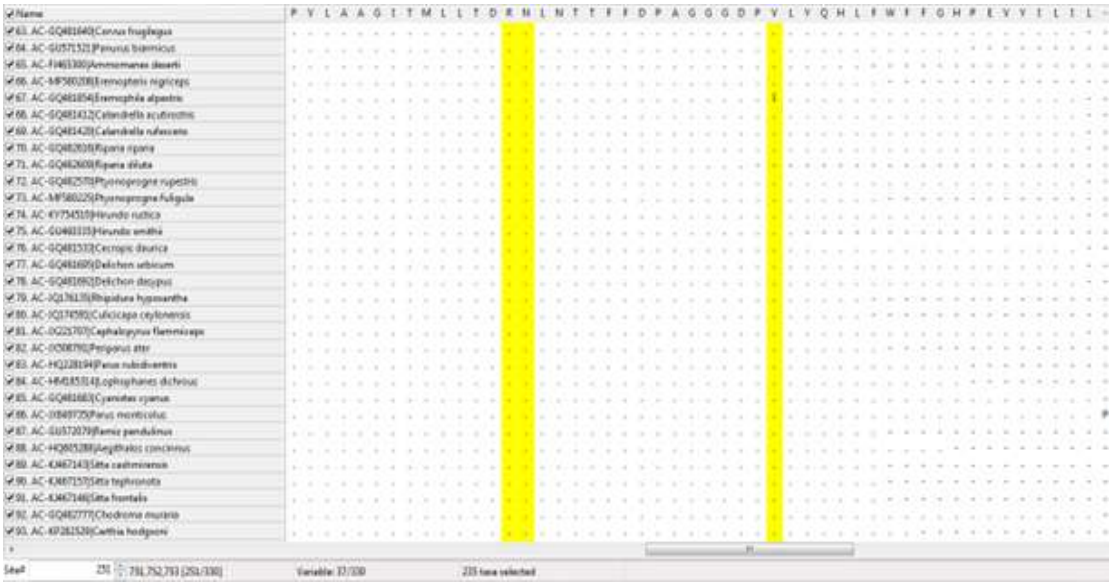


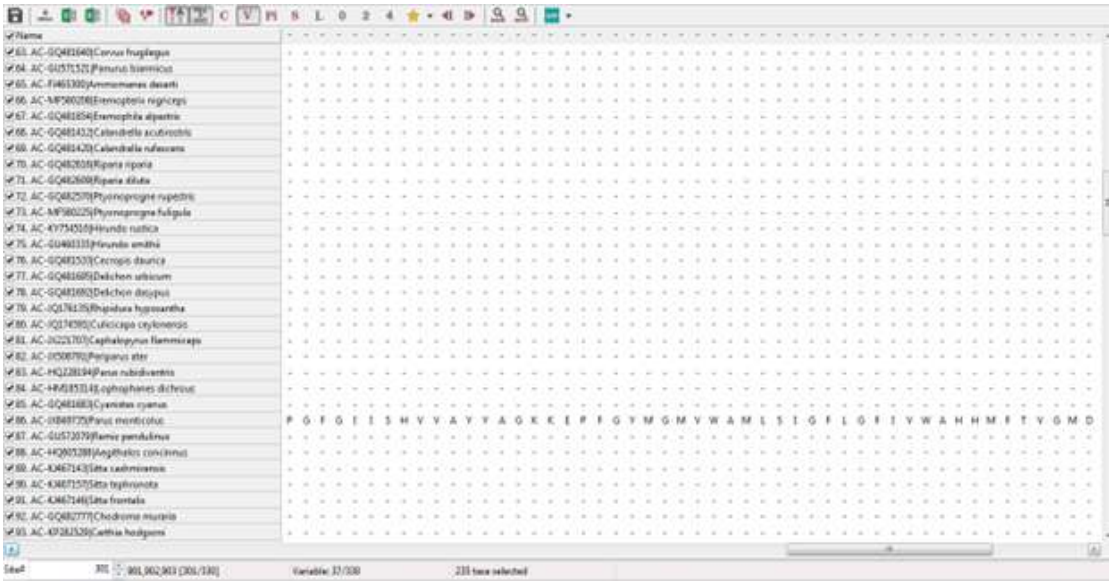


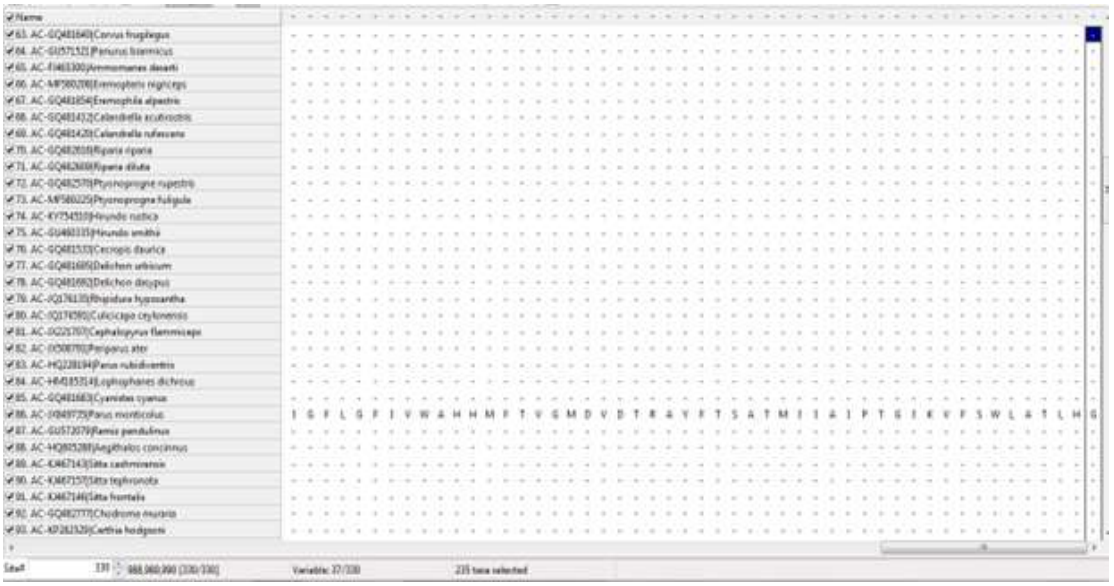


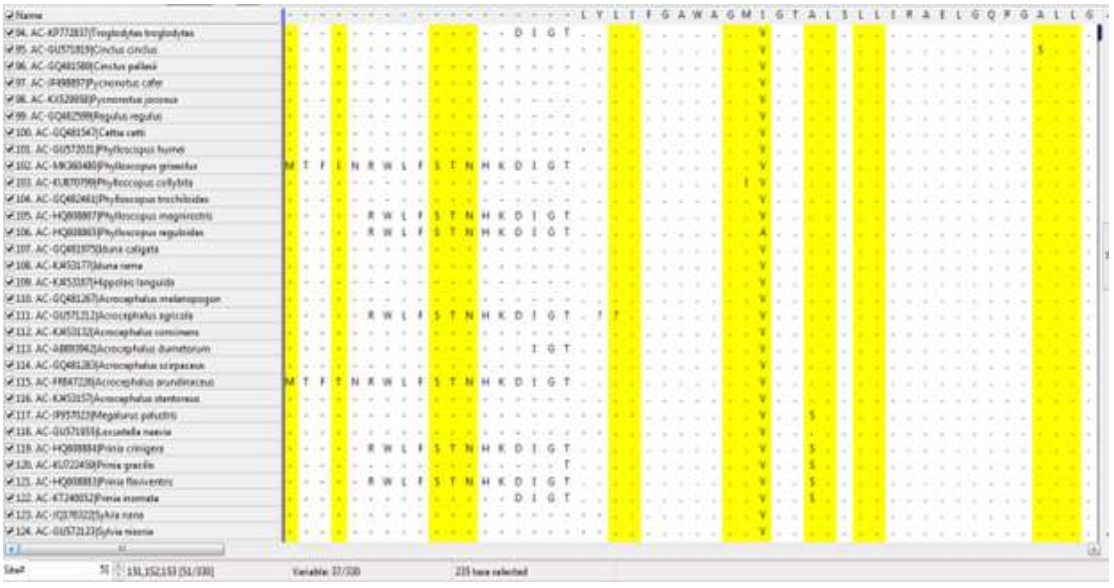


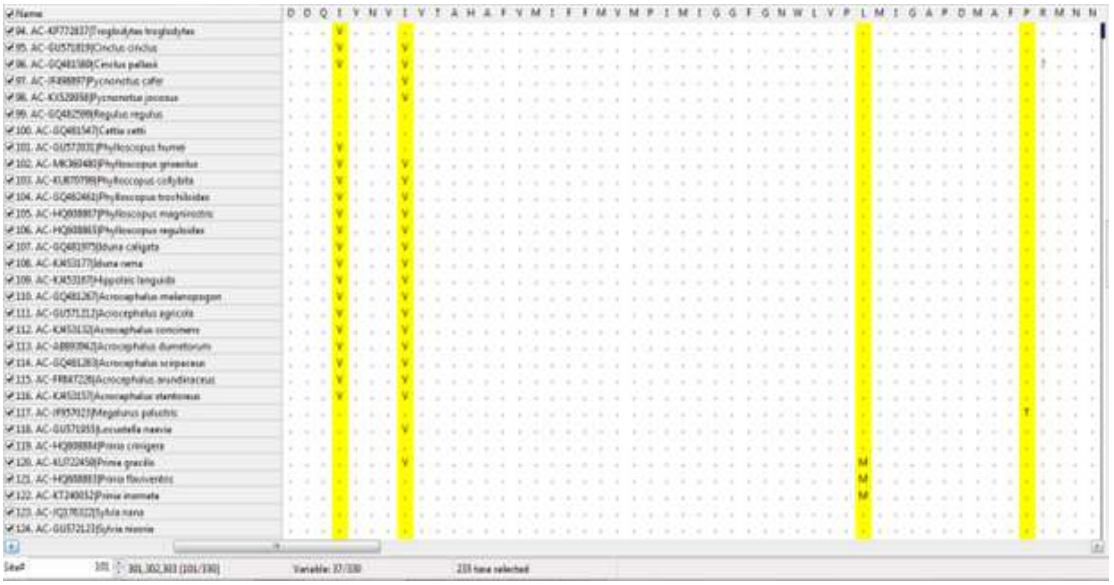


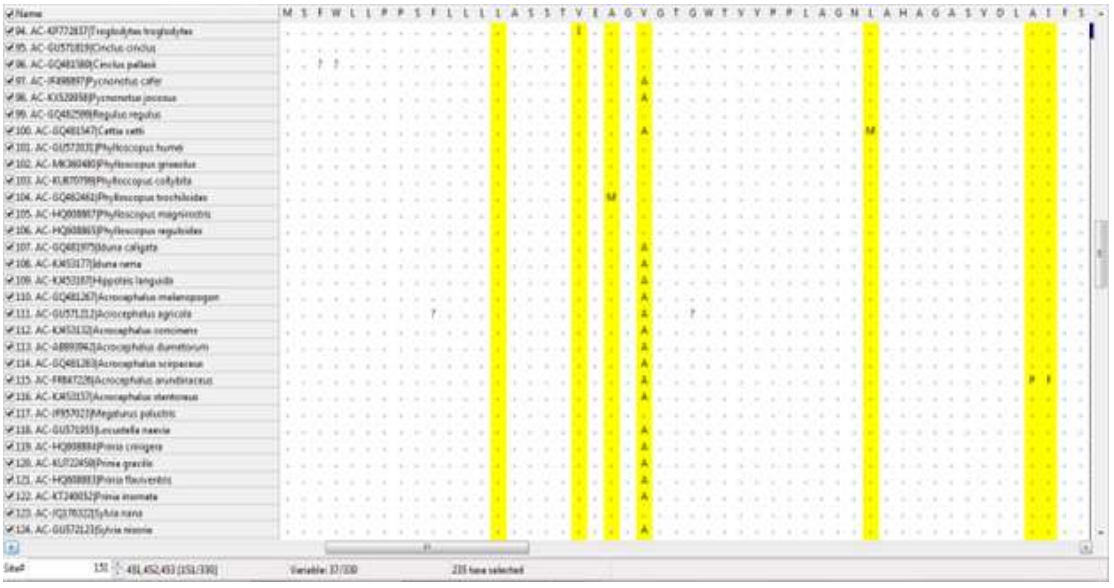


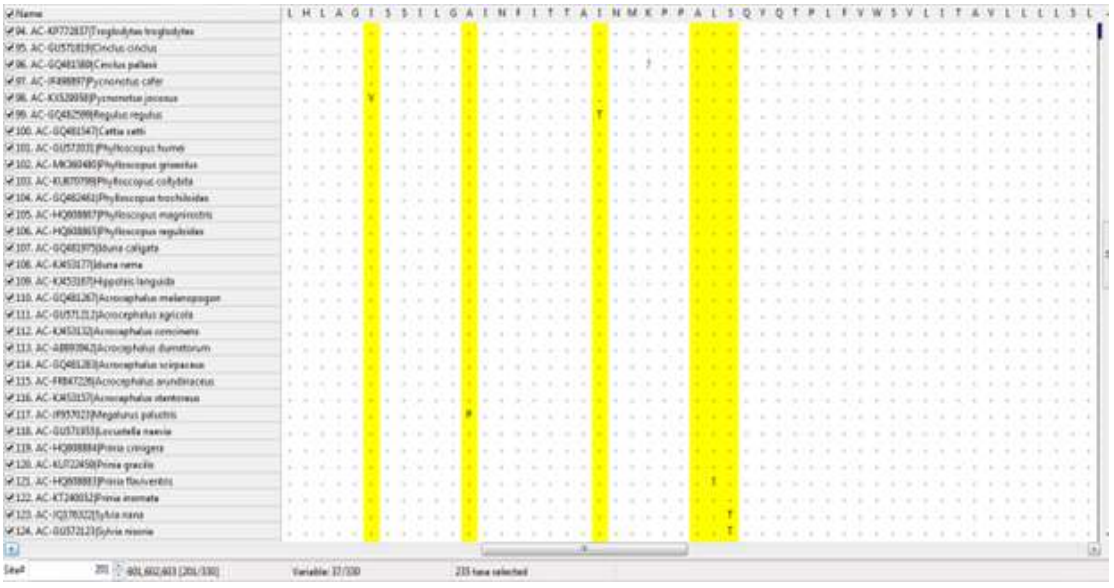


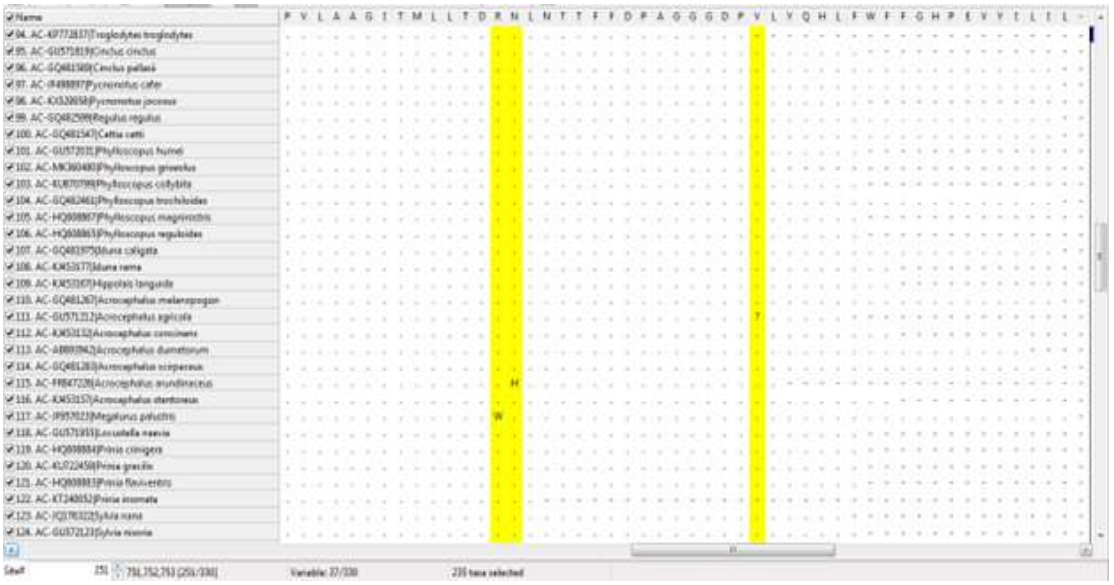


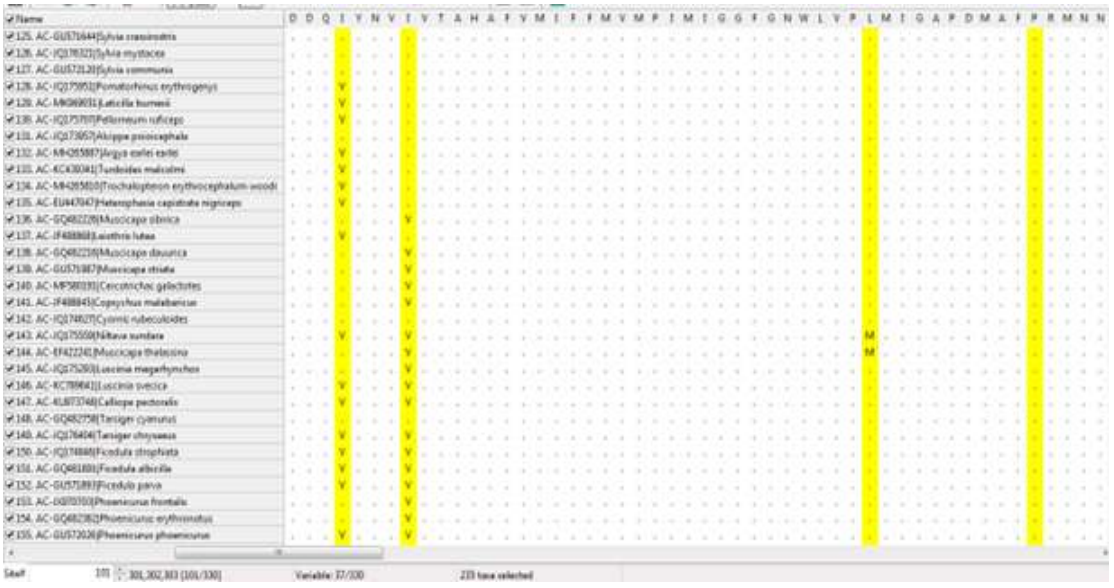


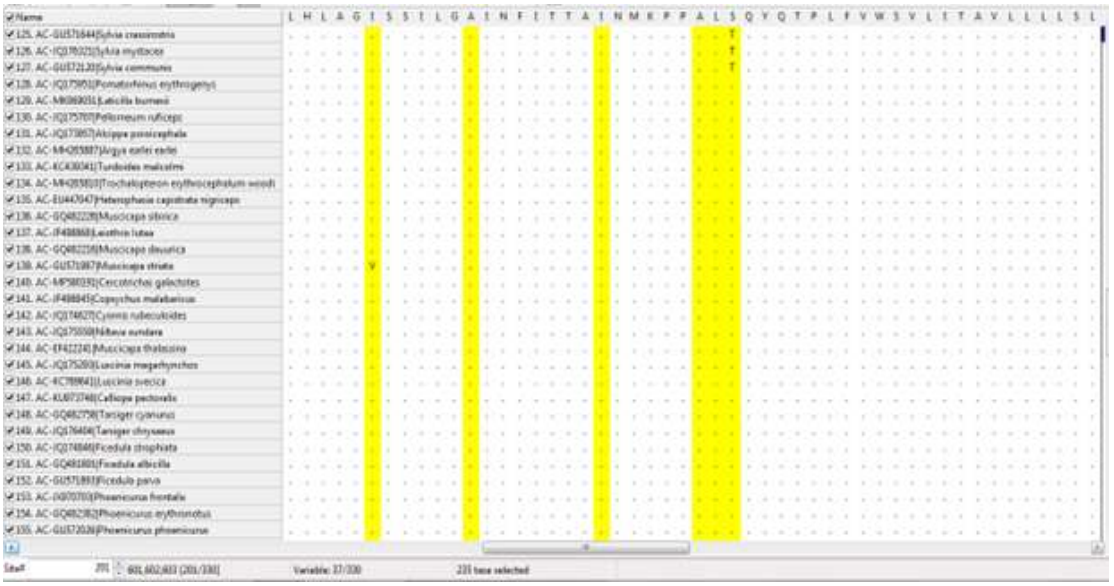


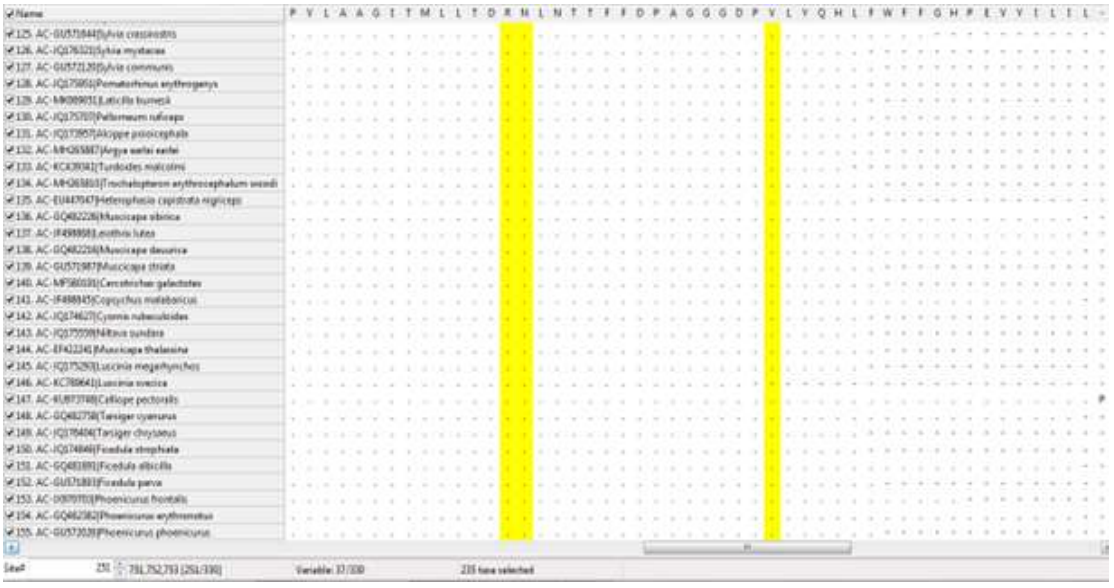


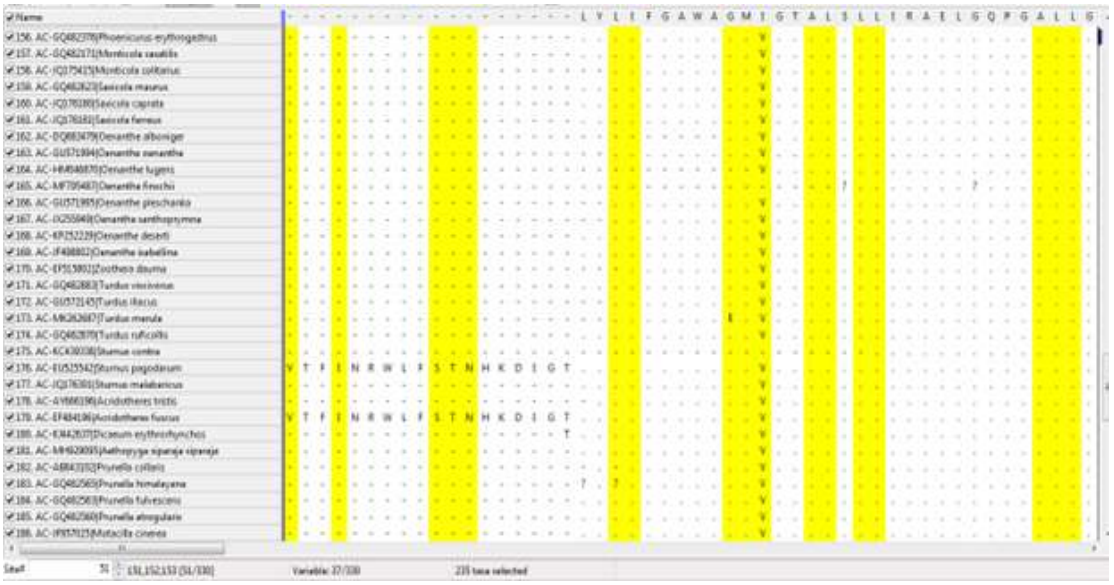


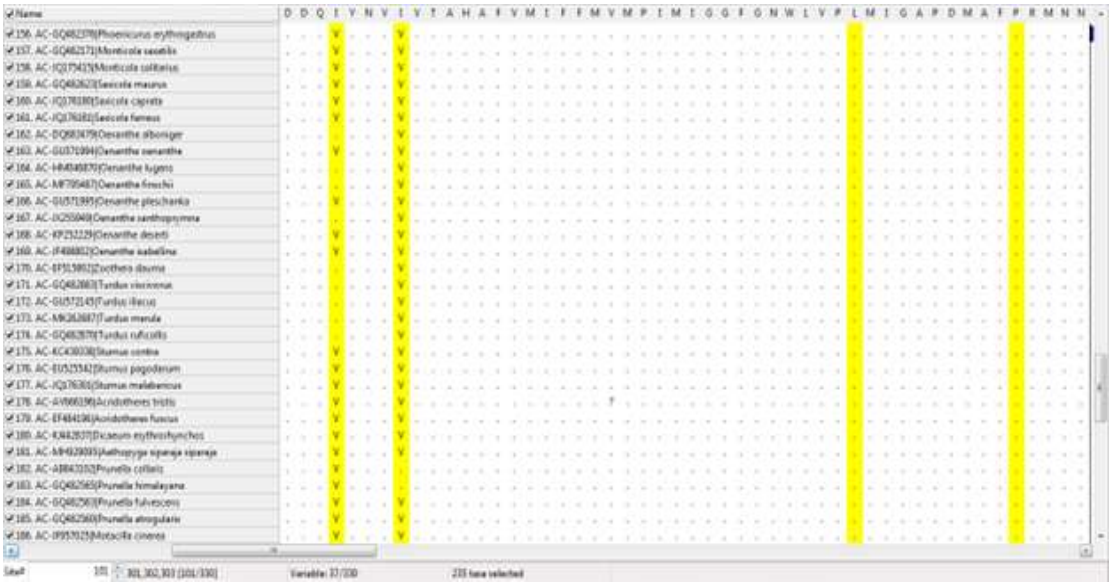


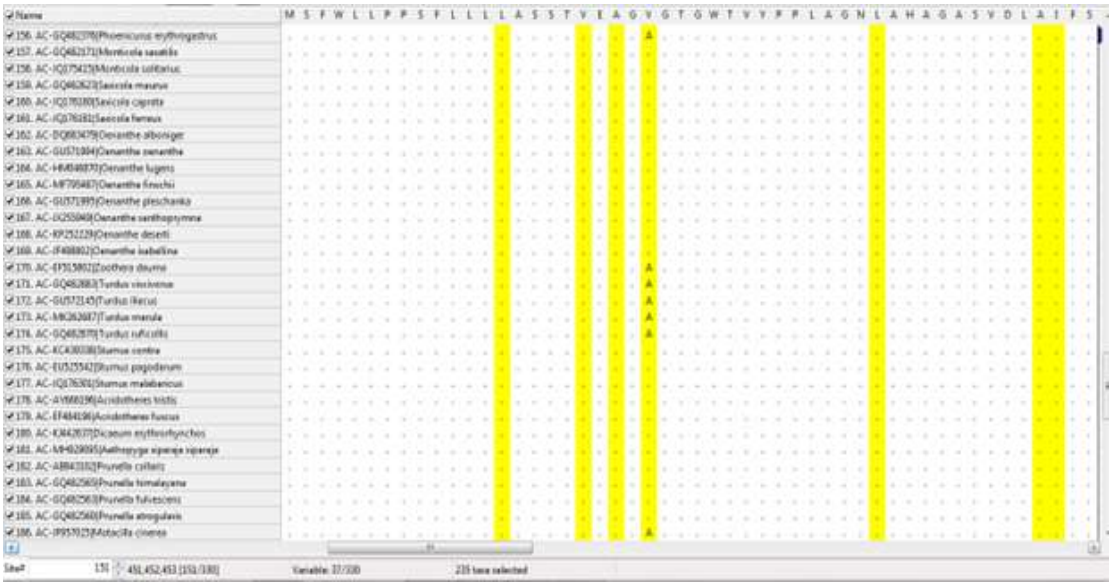


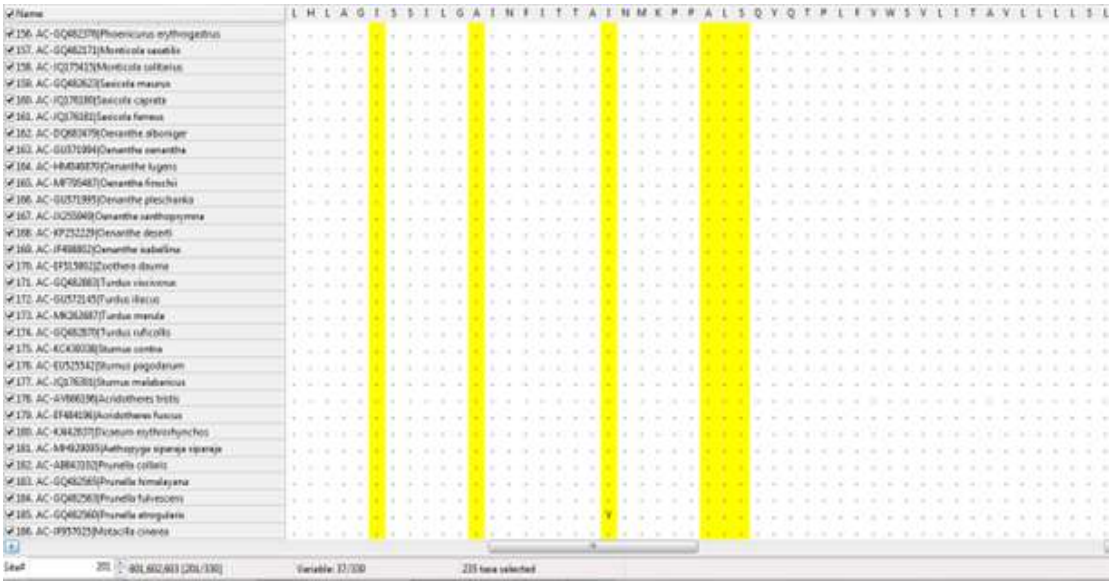


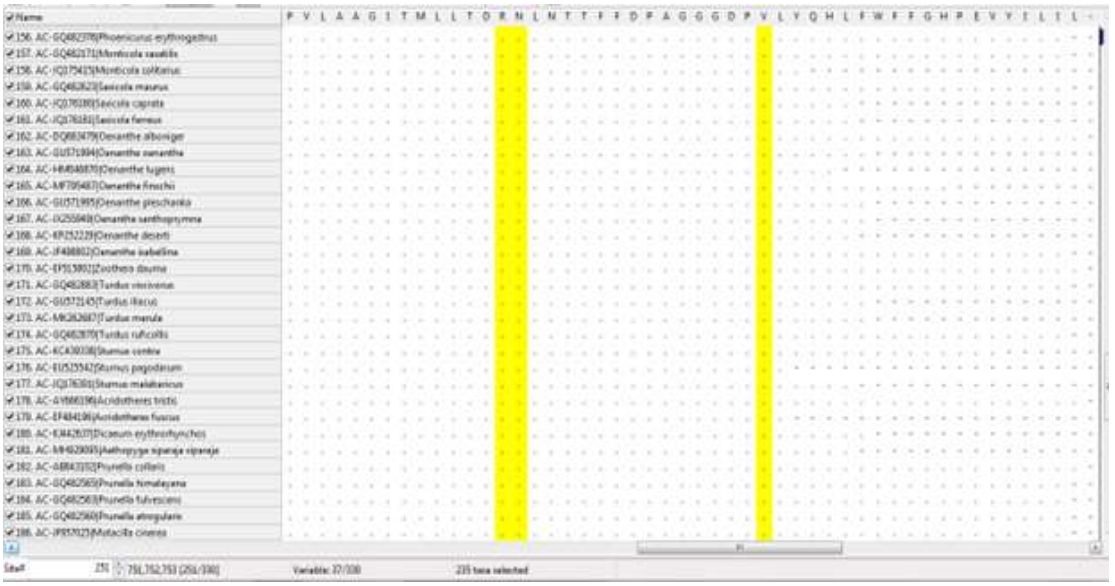


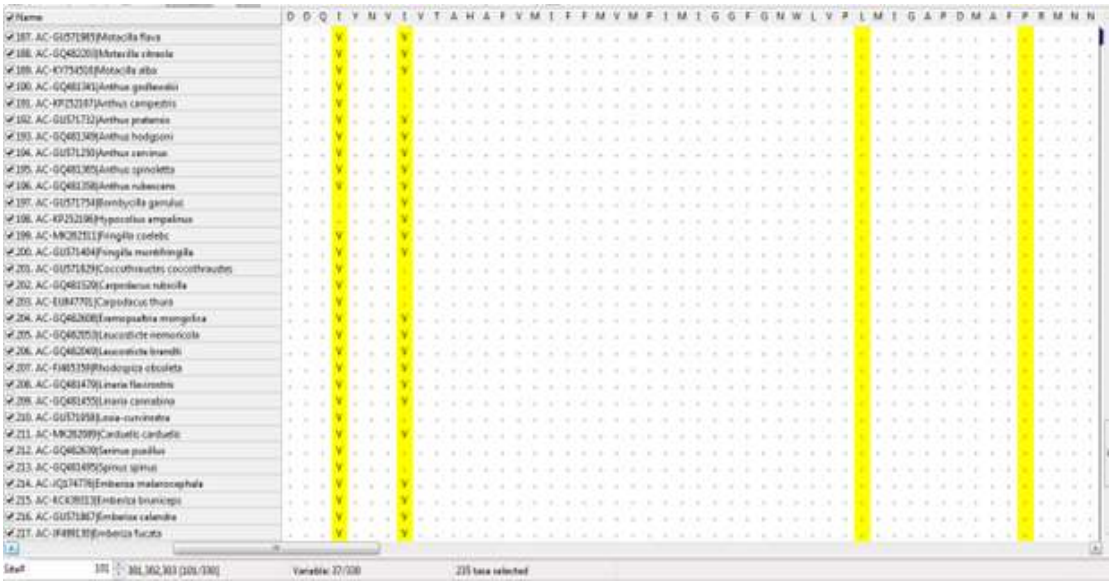


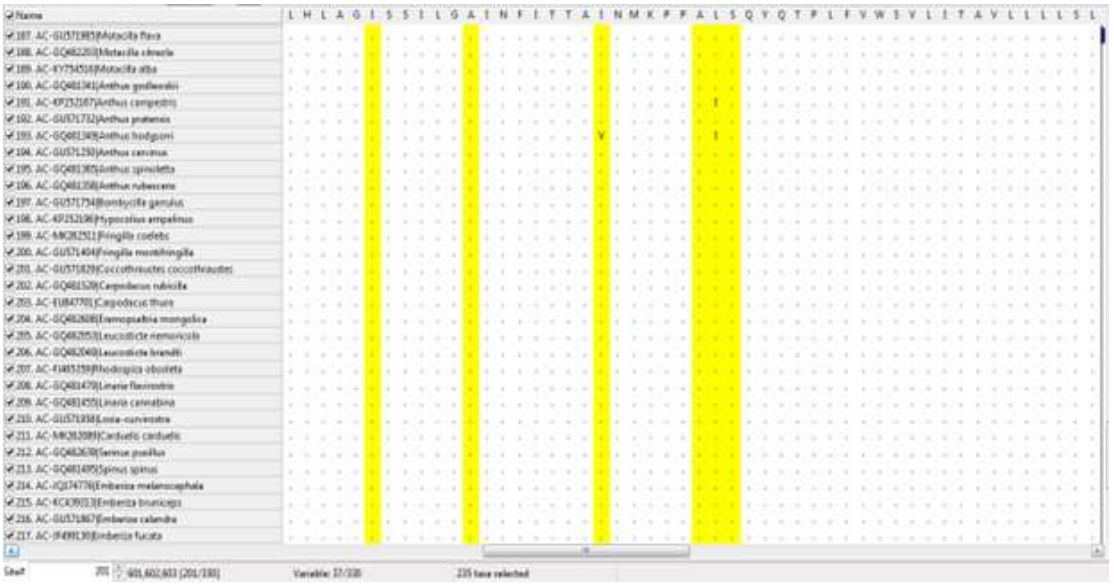


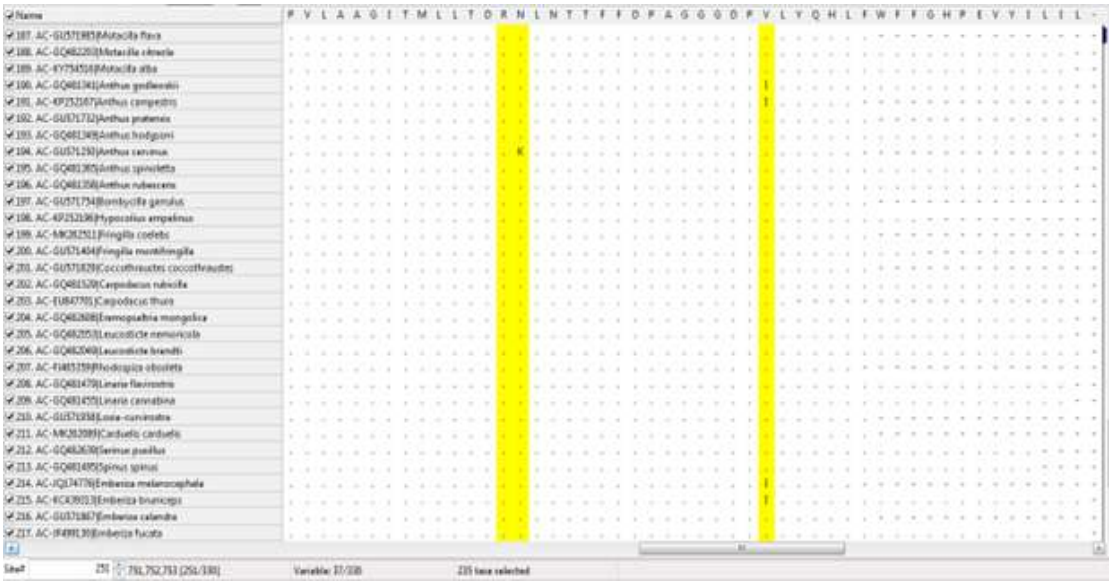


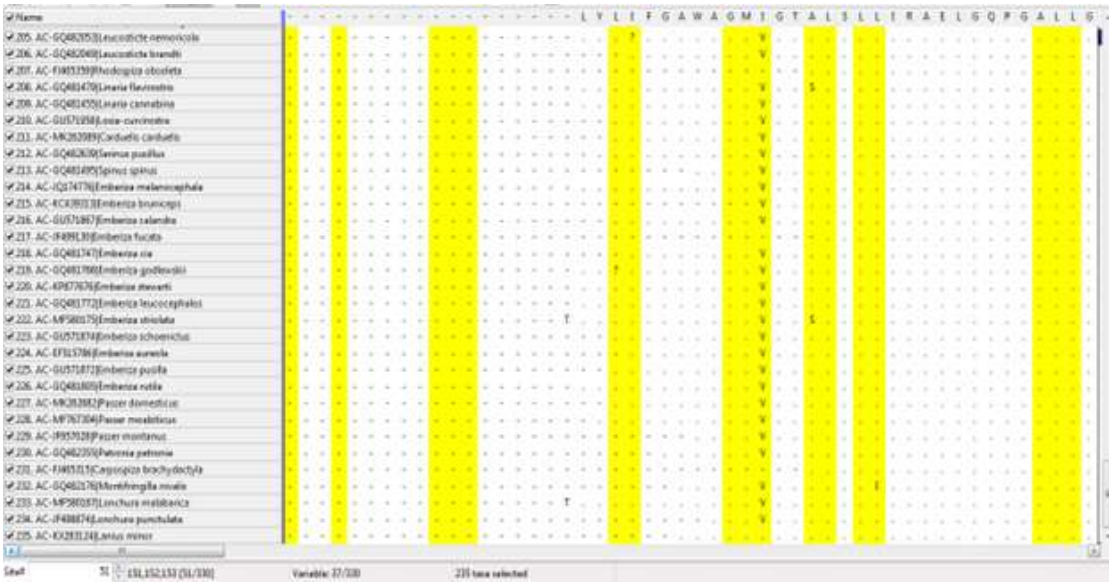


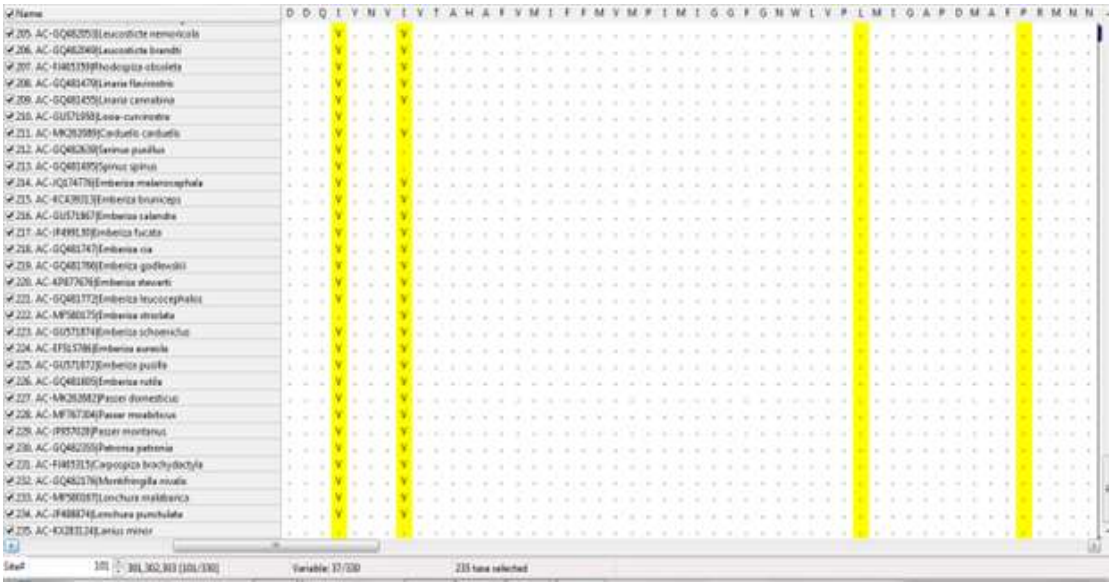


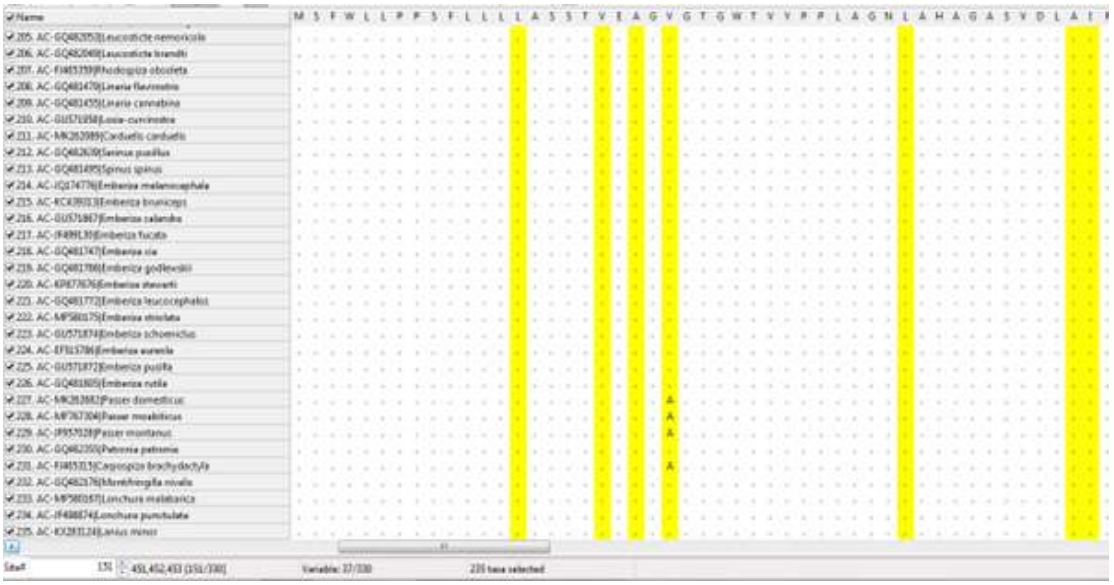


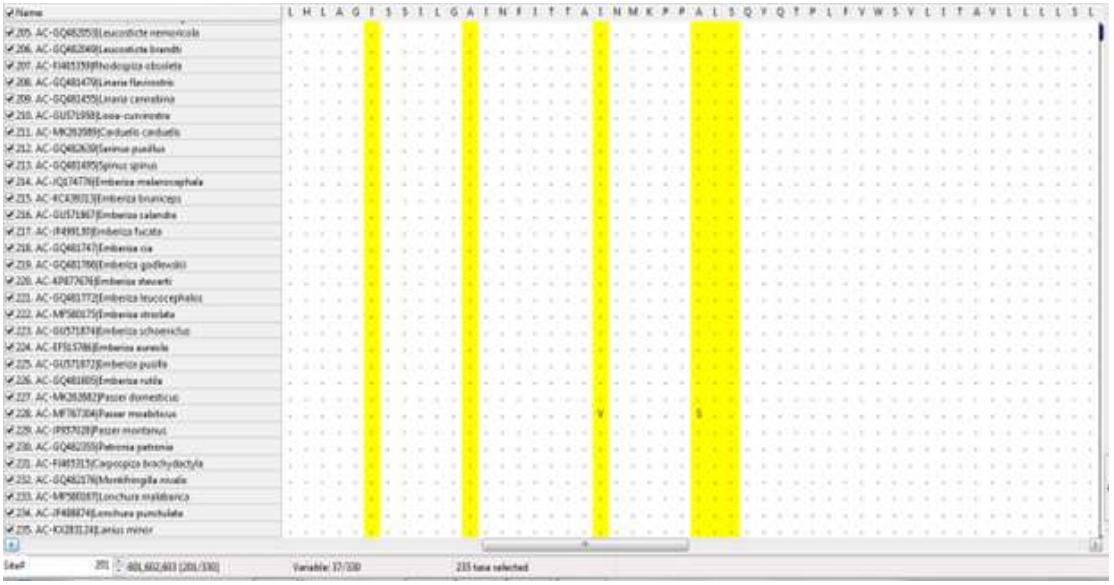


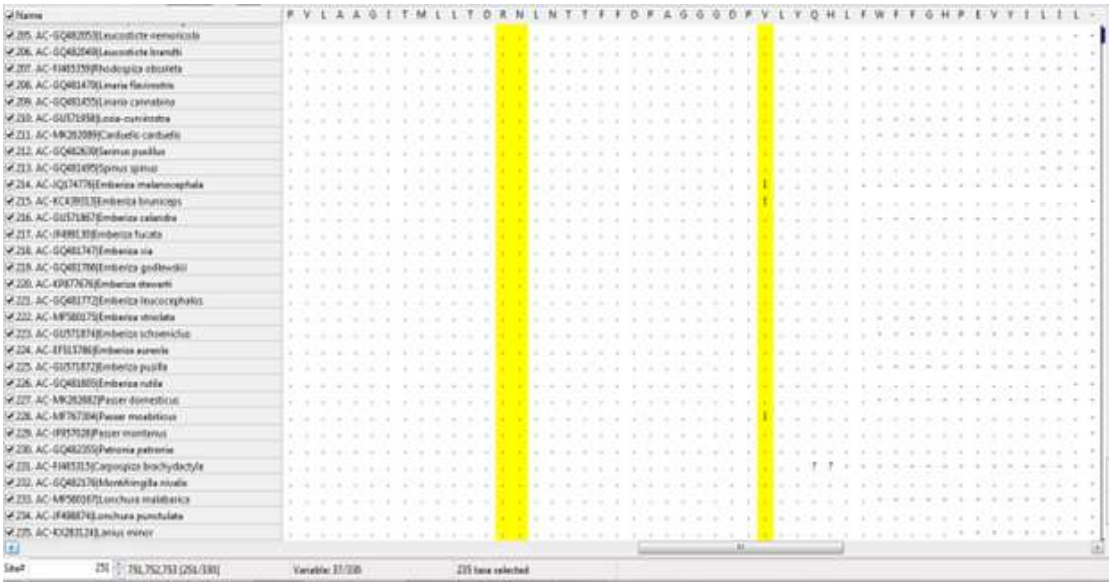




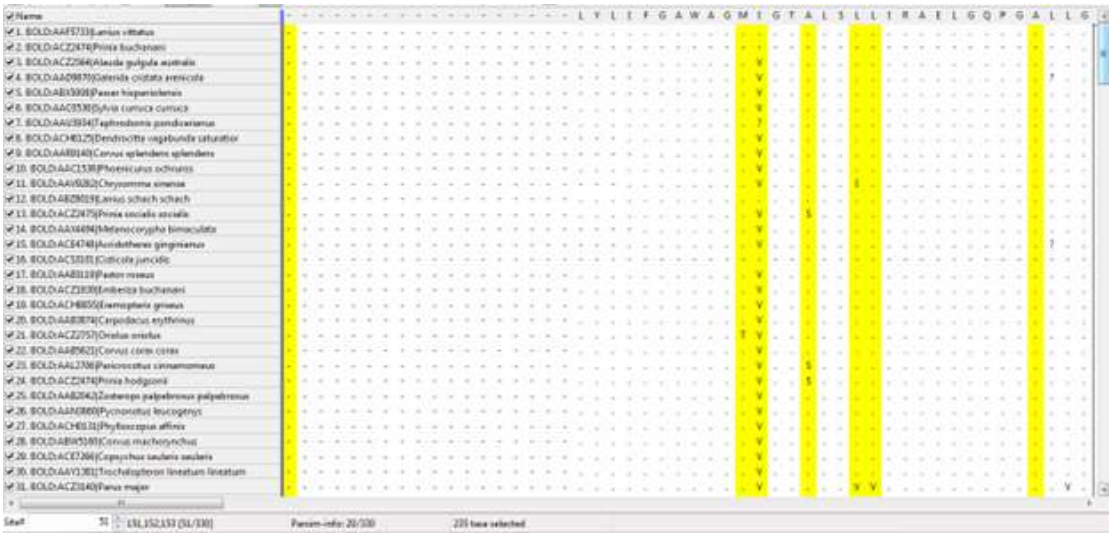


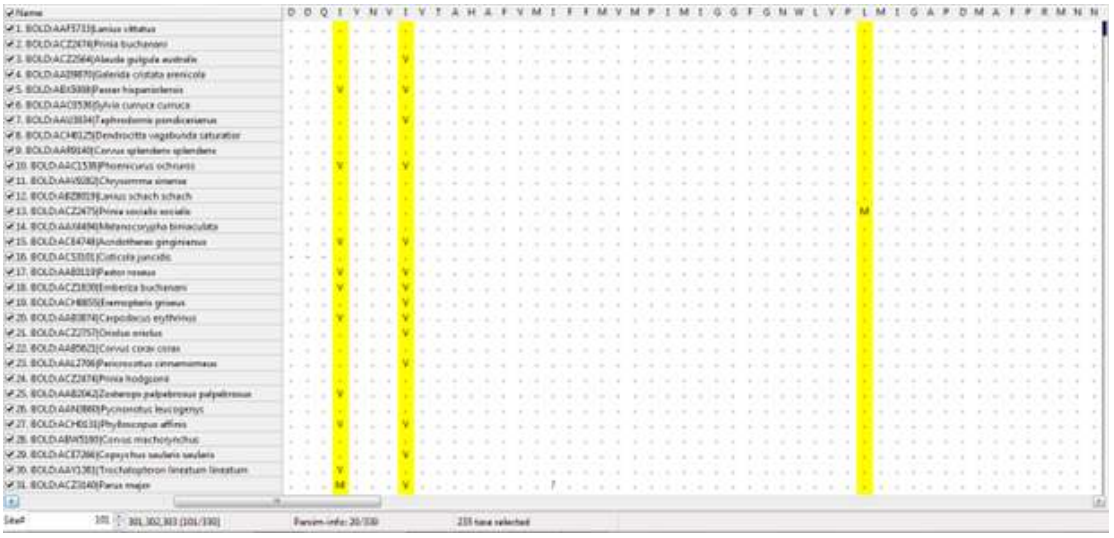


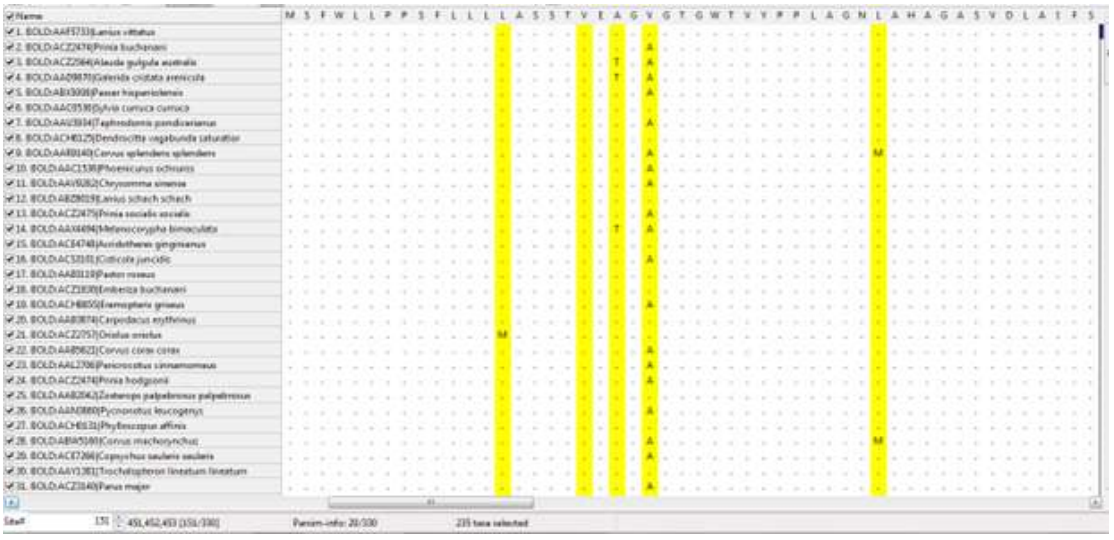


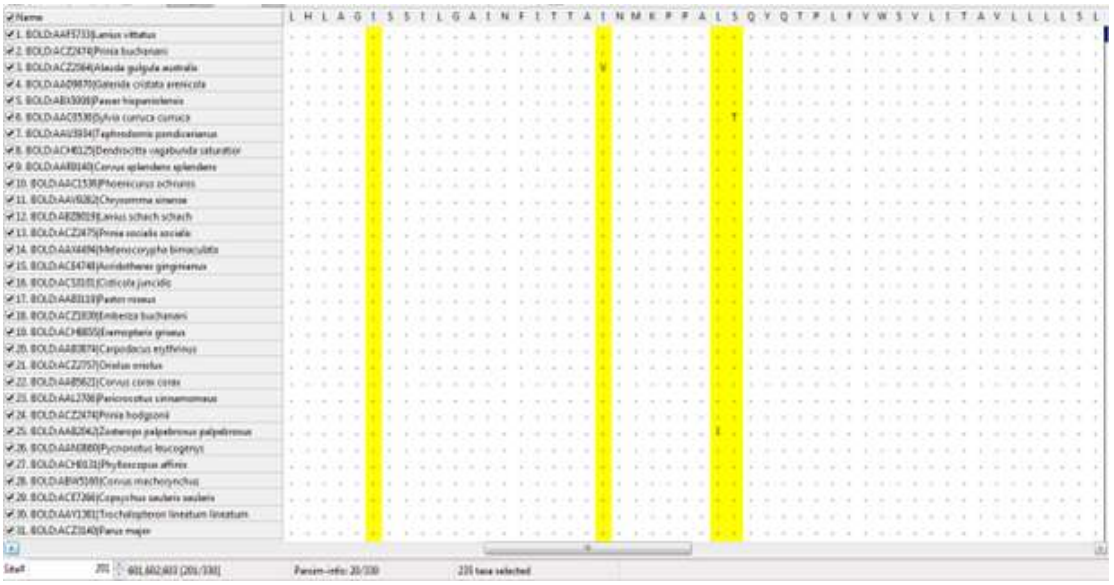


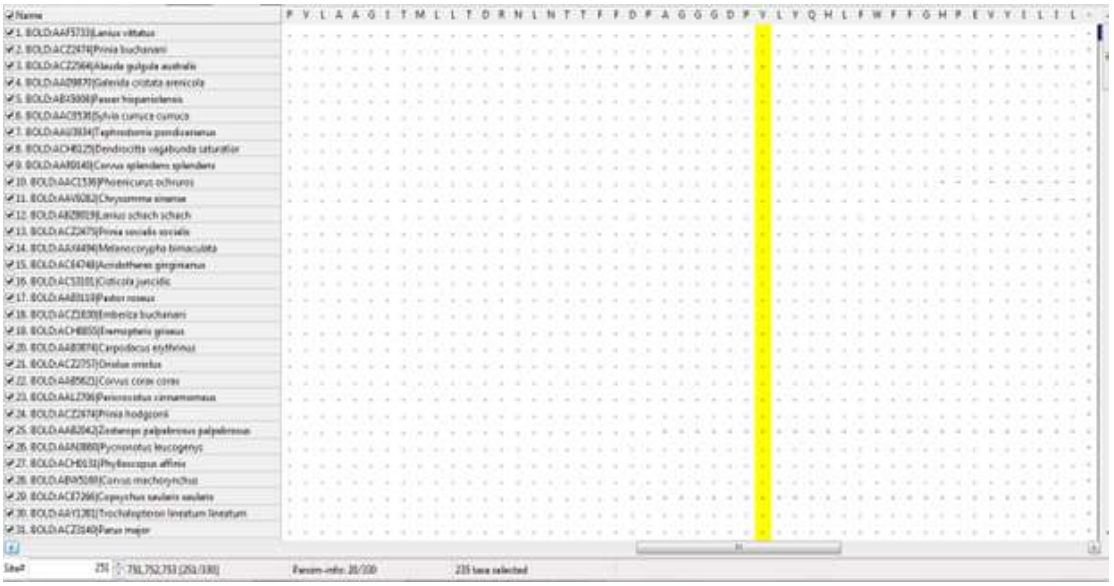
(iii): Variation at Amino Acid Level (Parsimony Informative Sites) in Sequences of Passeriformes Computed by MEGAX

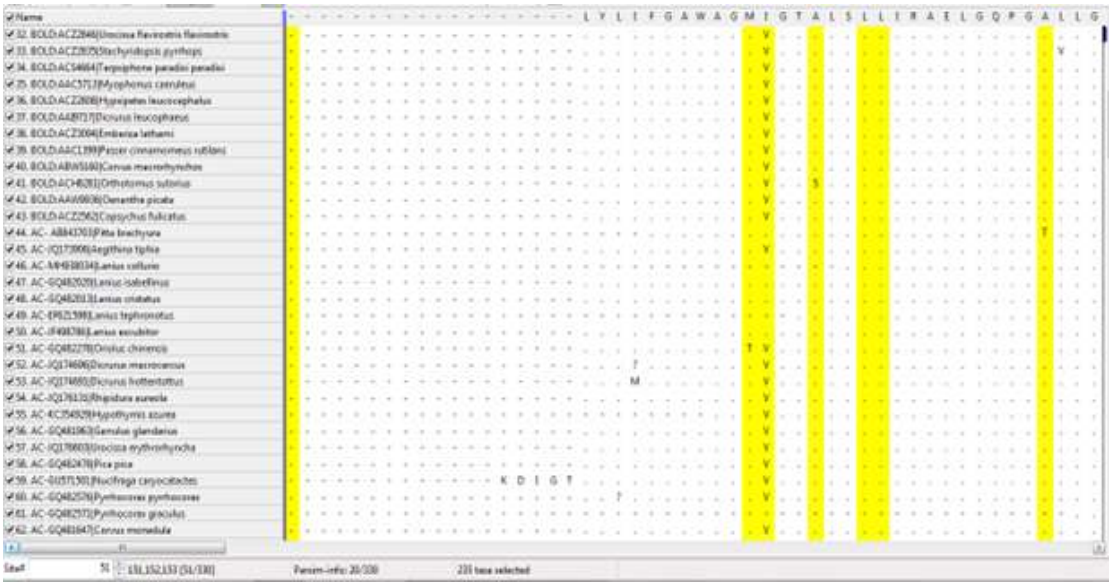


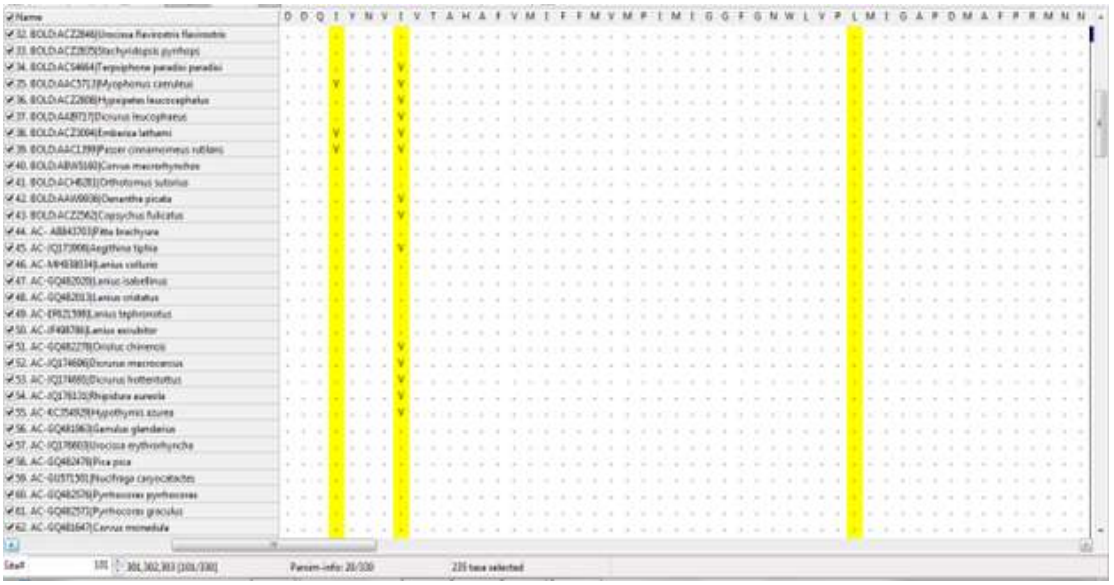


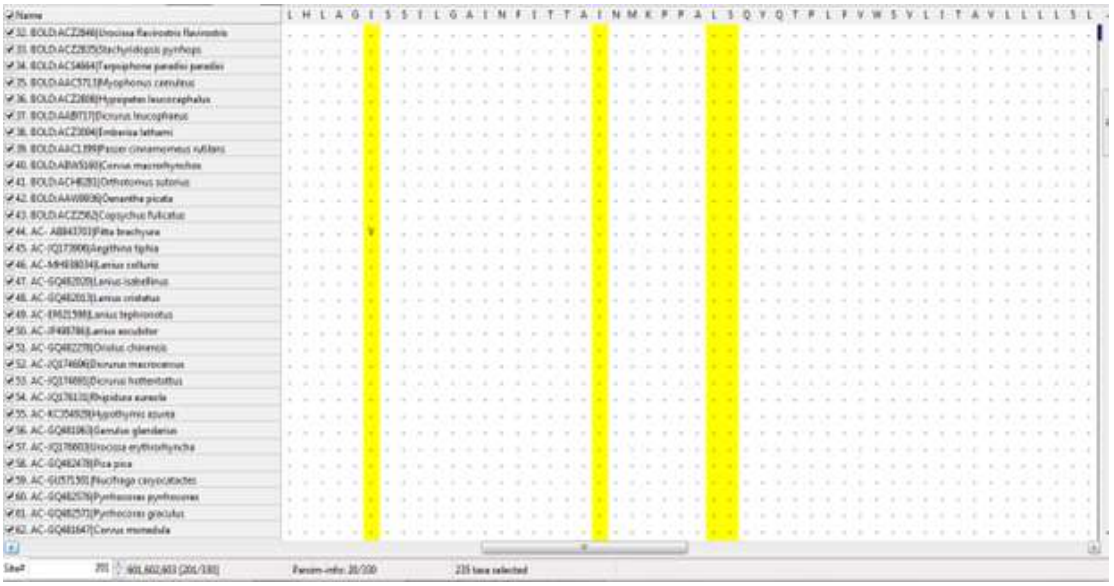


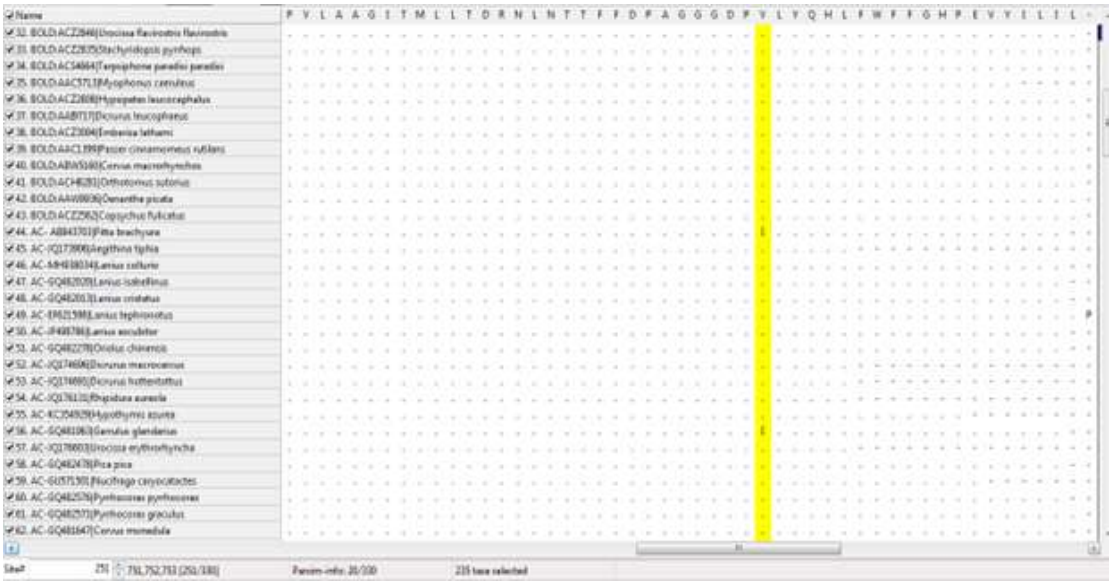


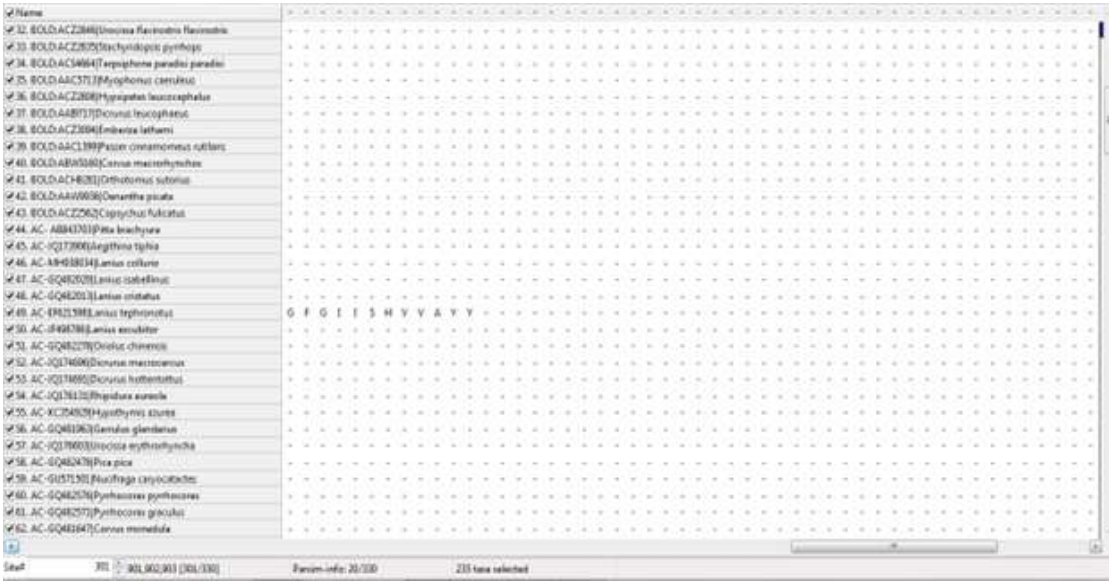


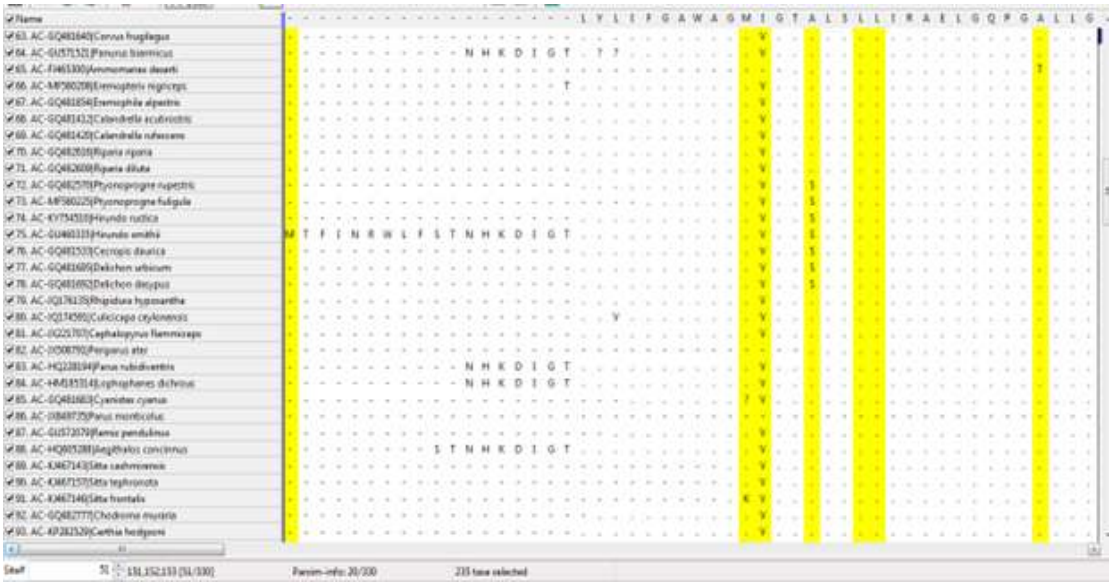


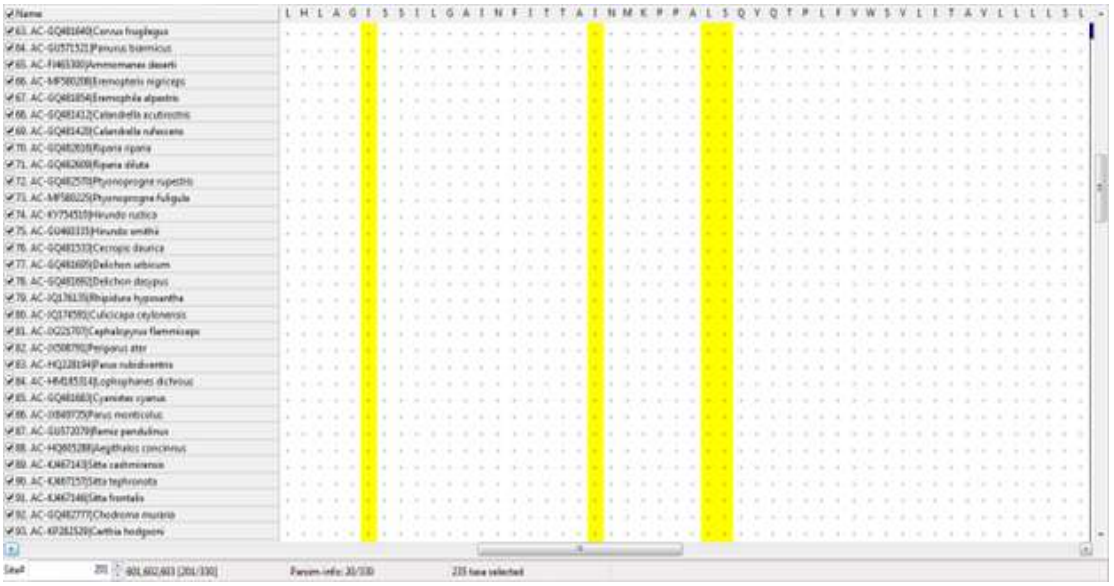


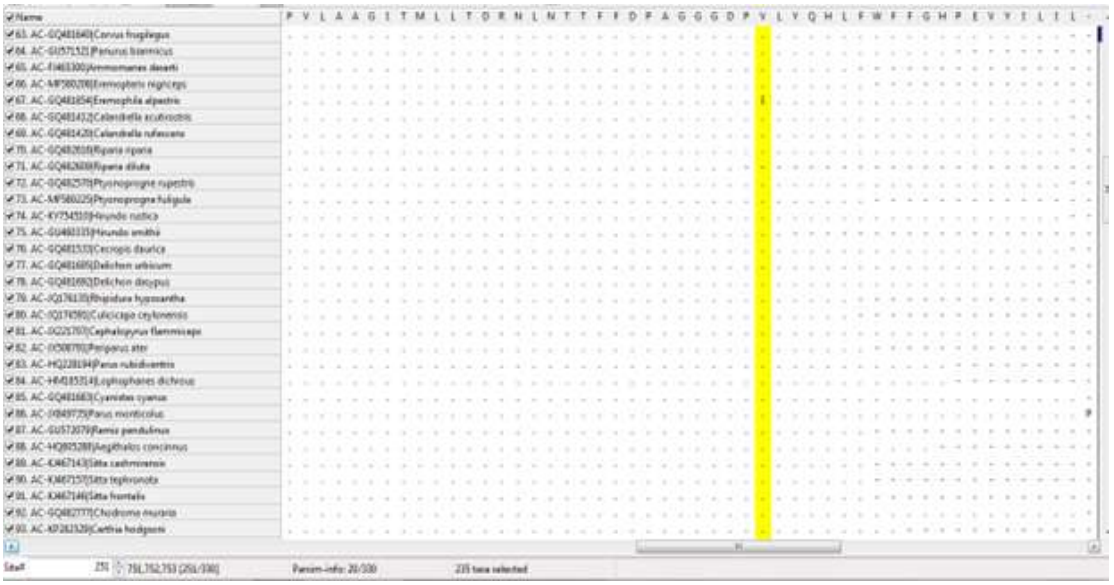


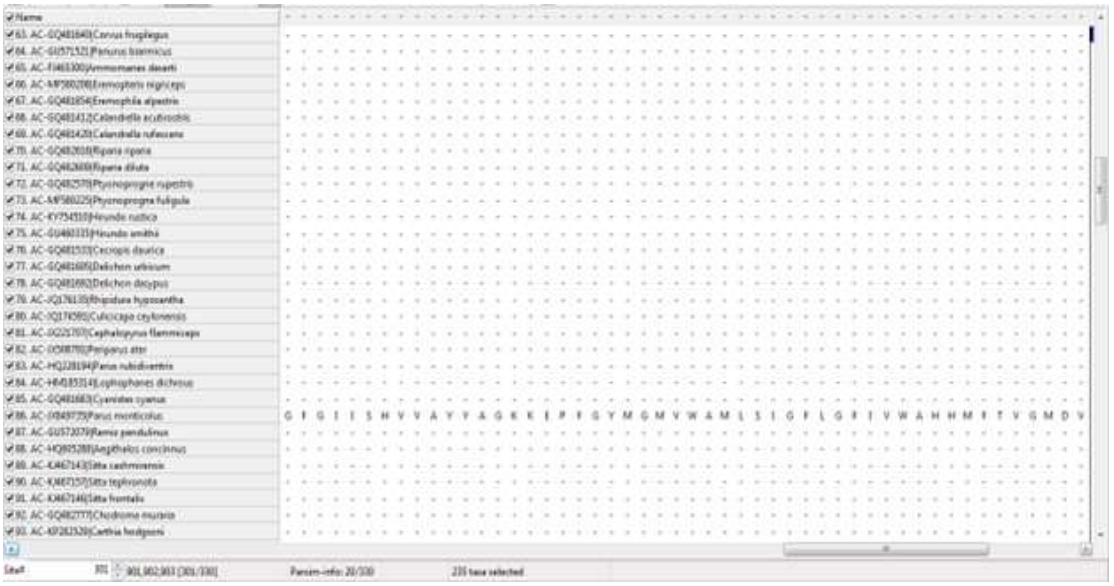


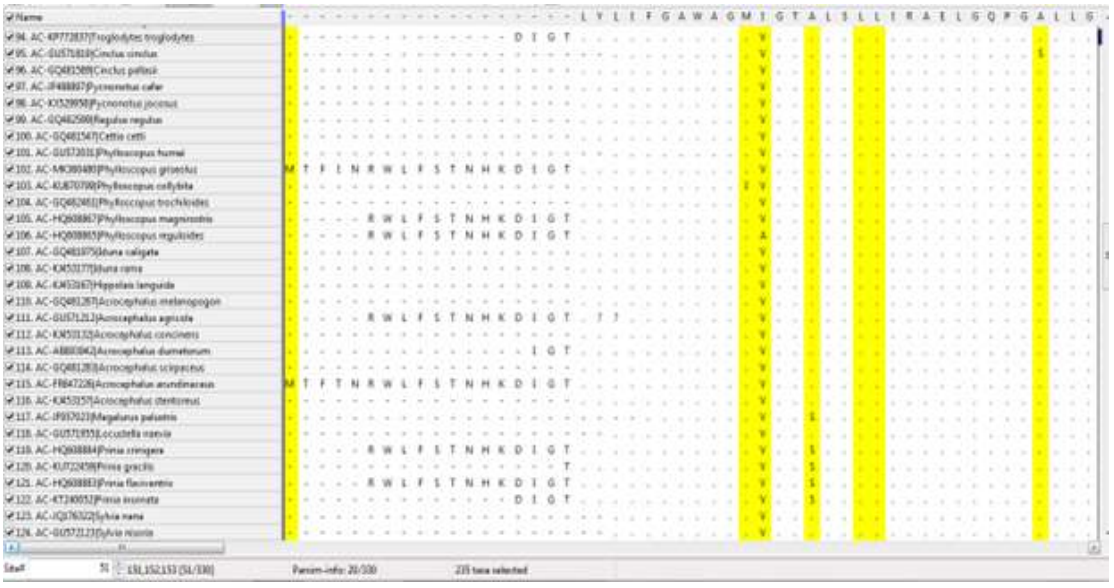


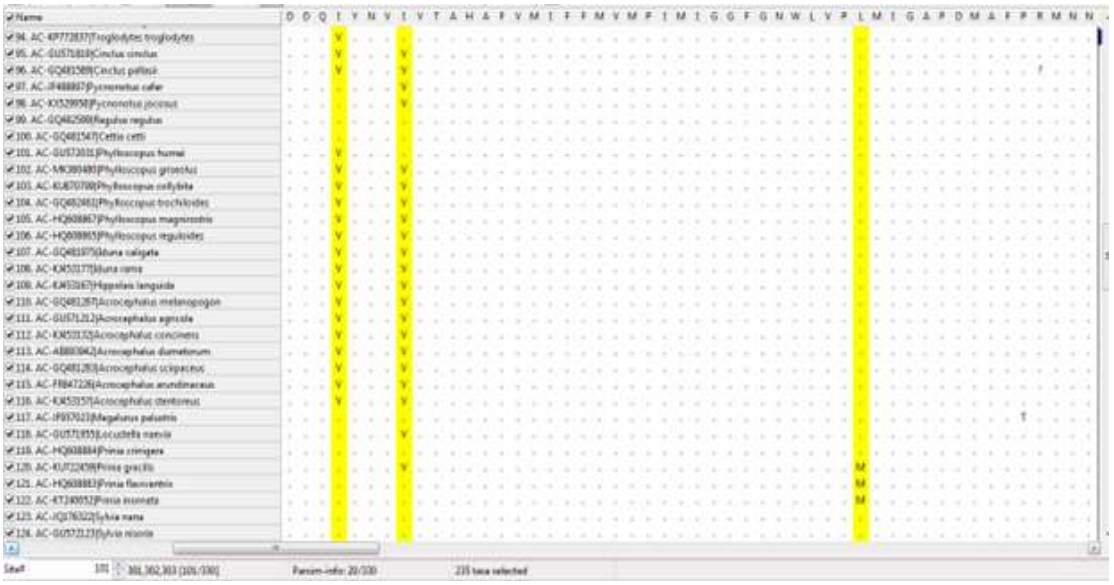


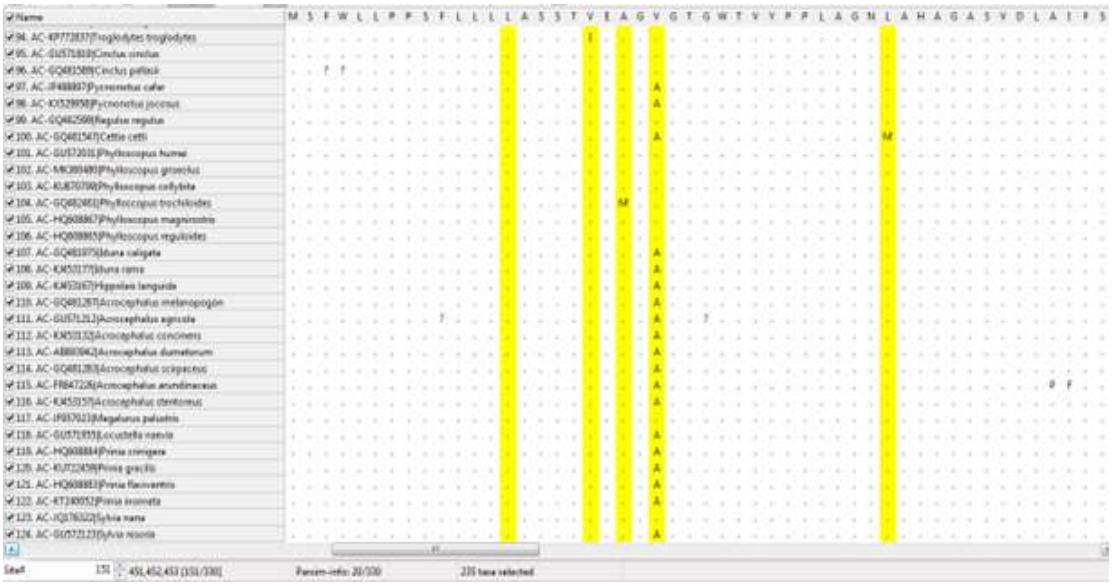


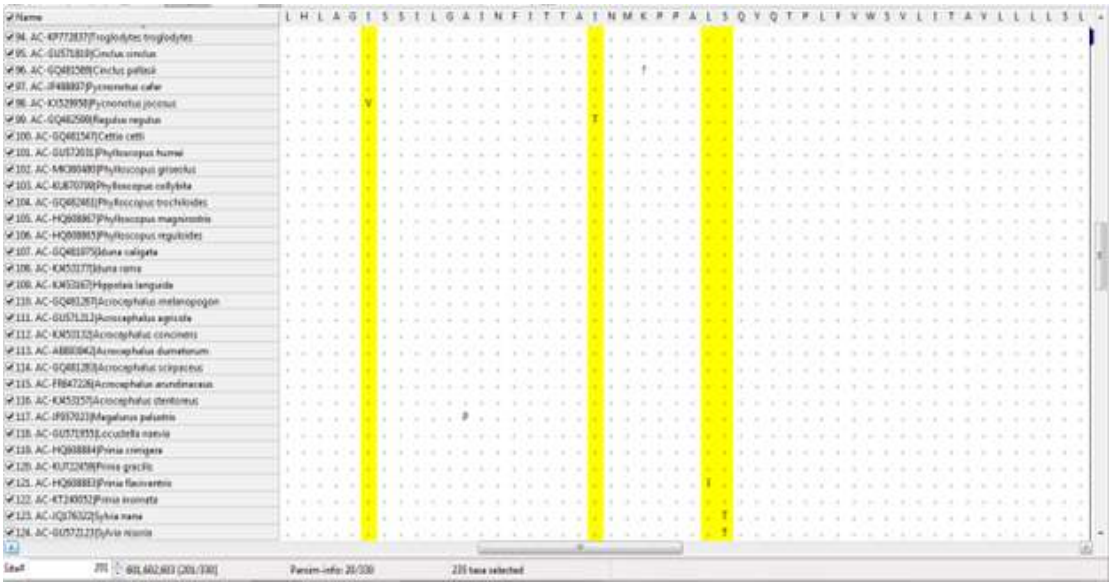


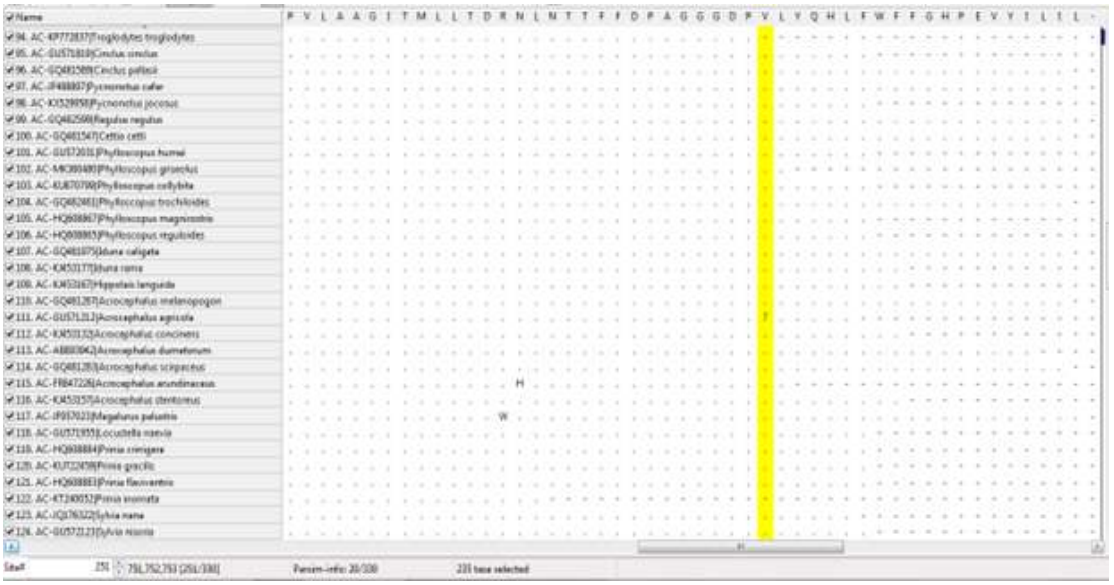


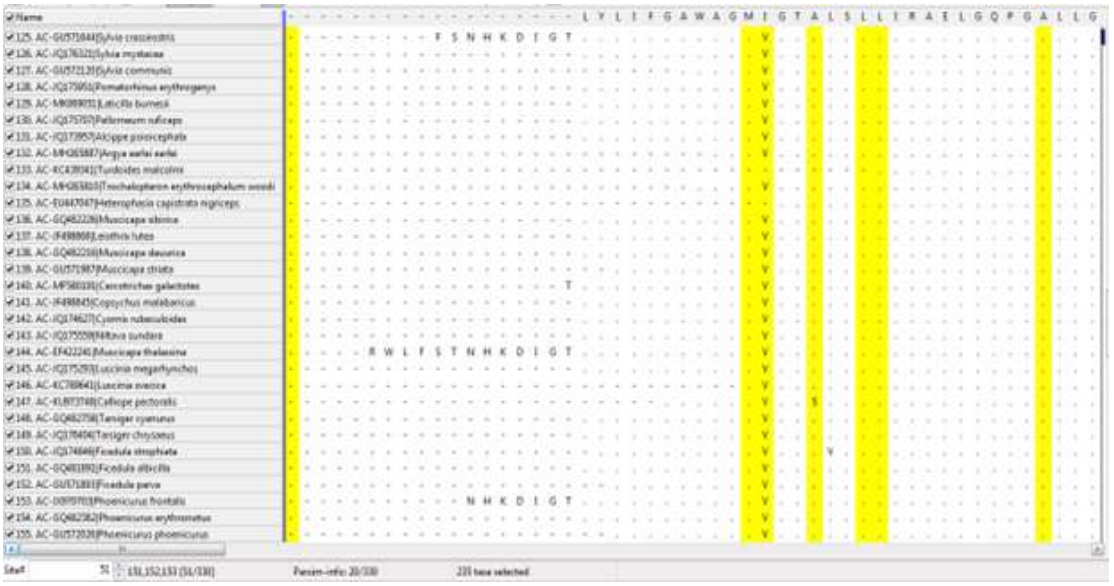


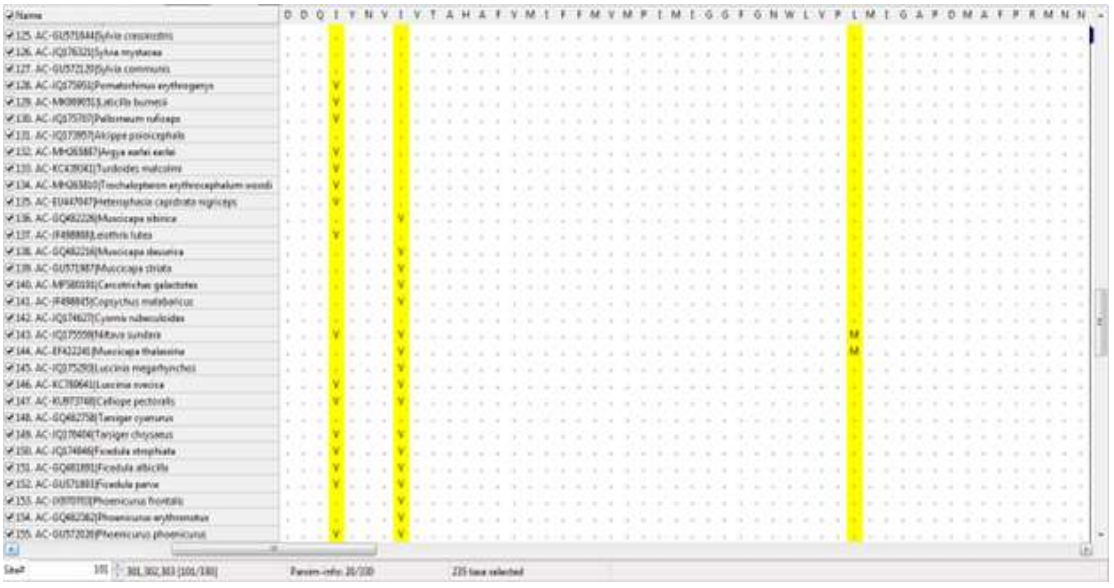


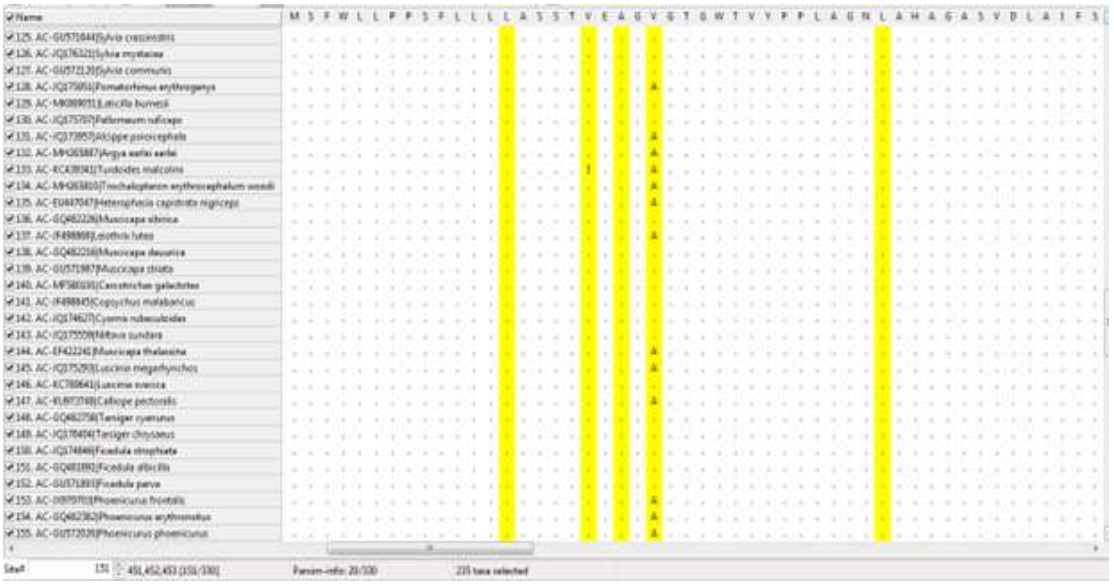


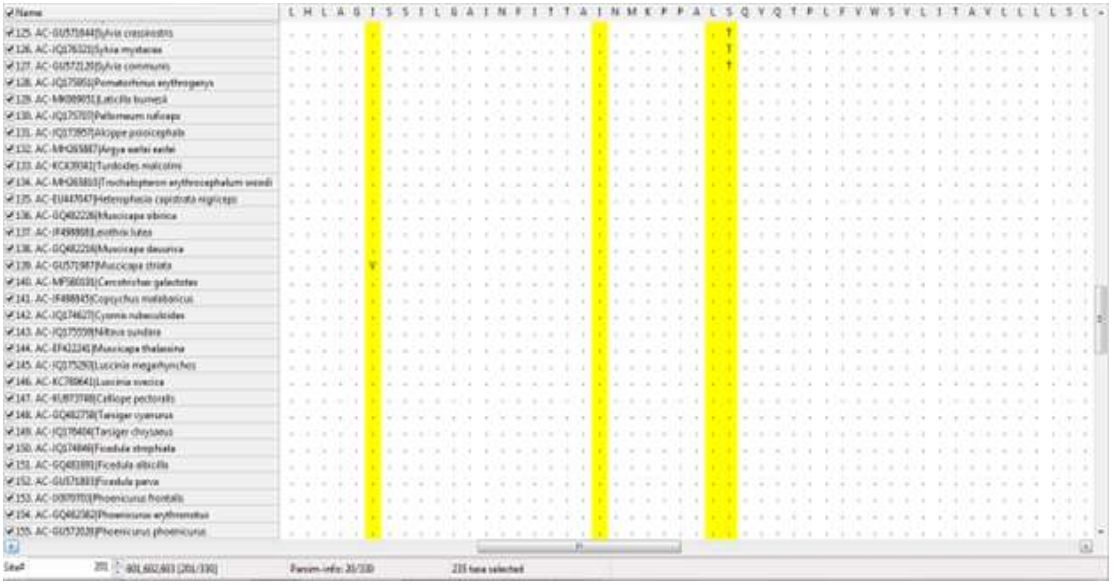


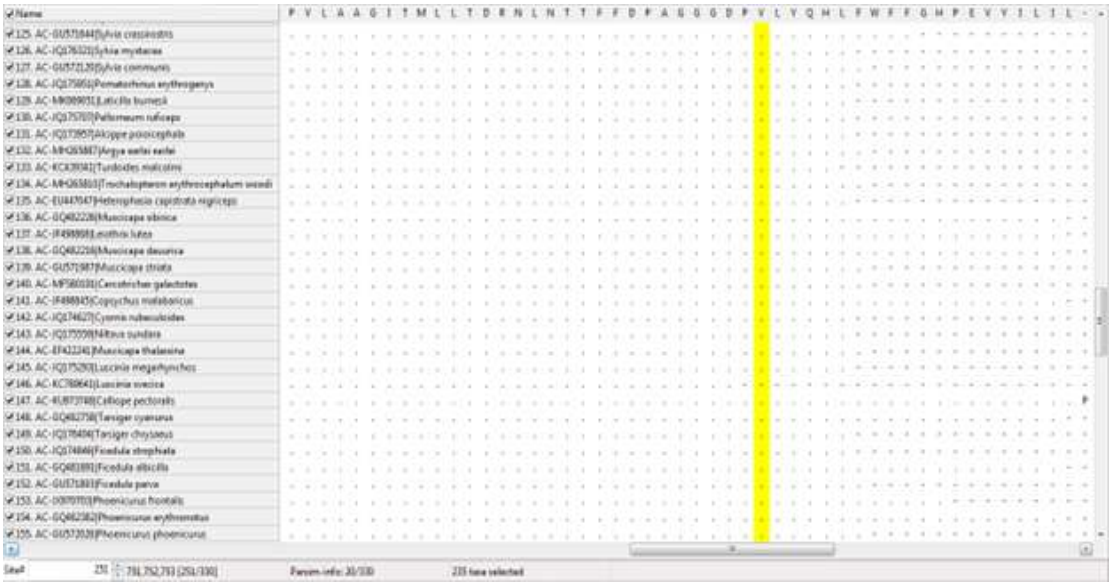


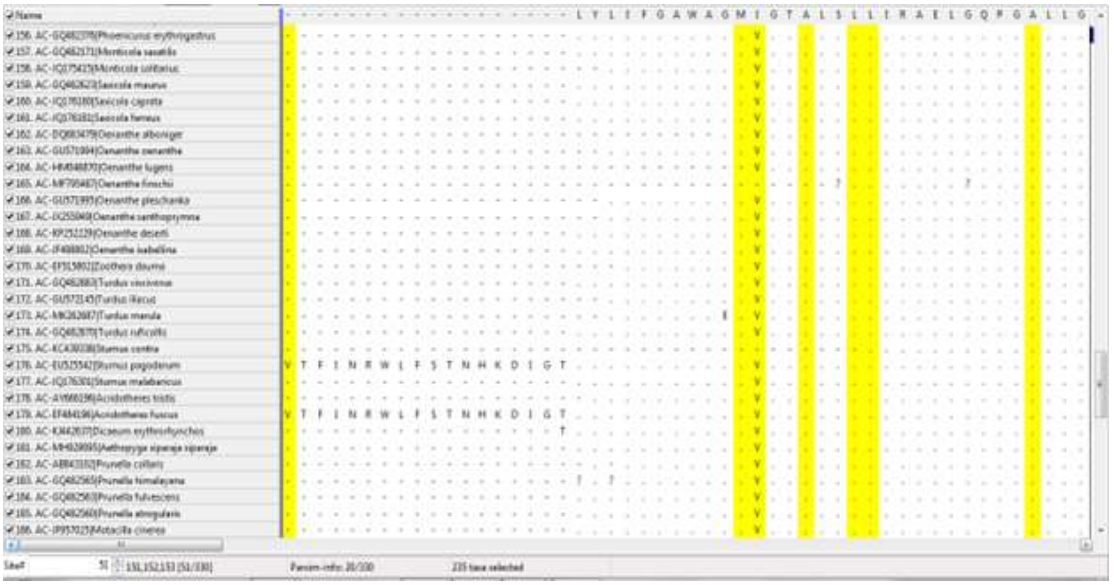


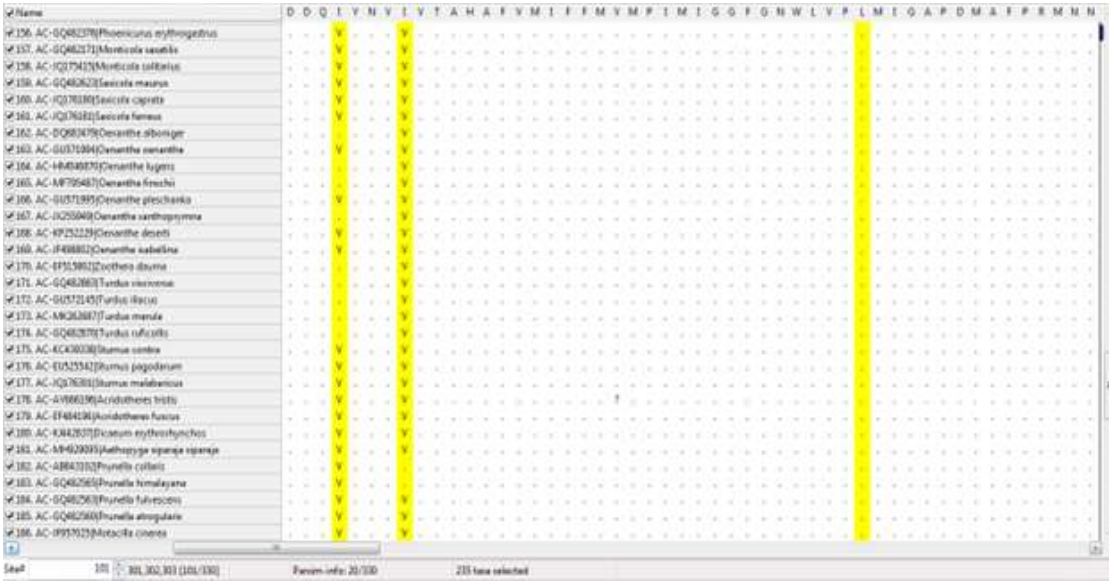


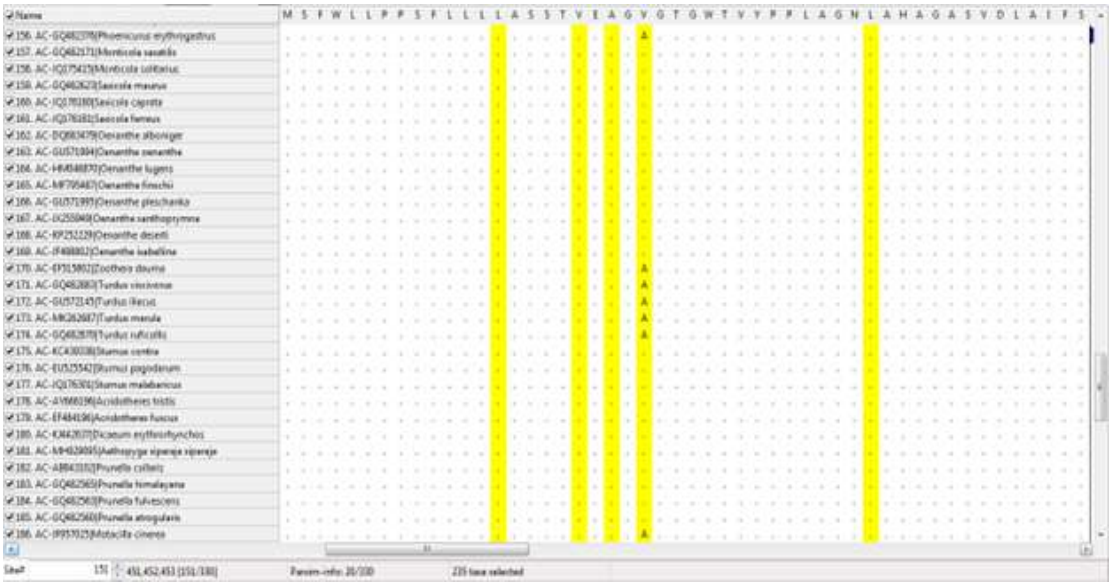


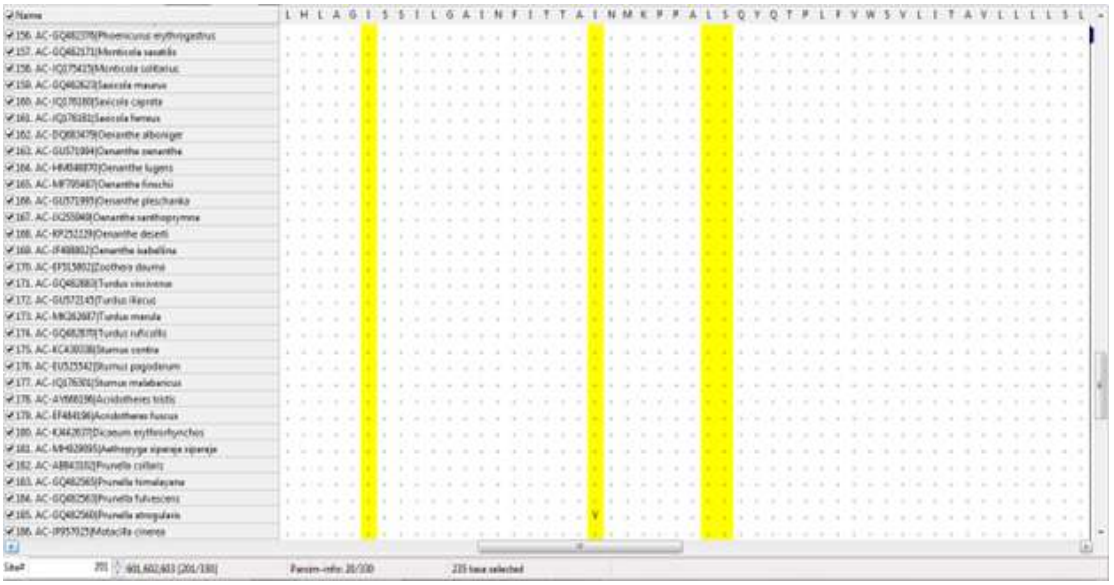


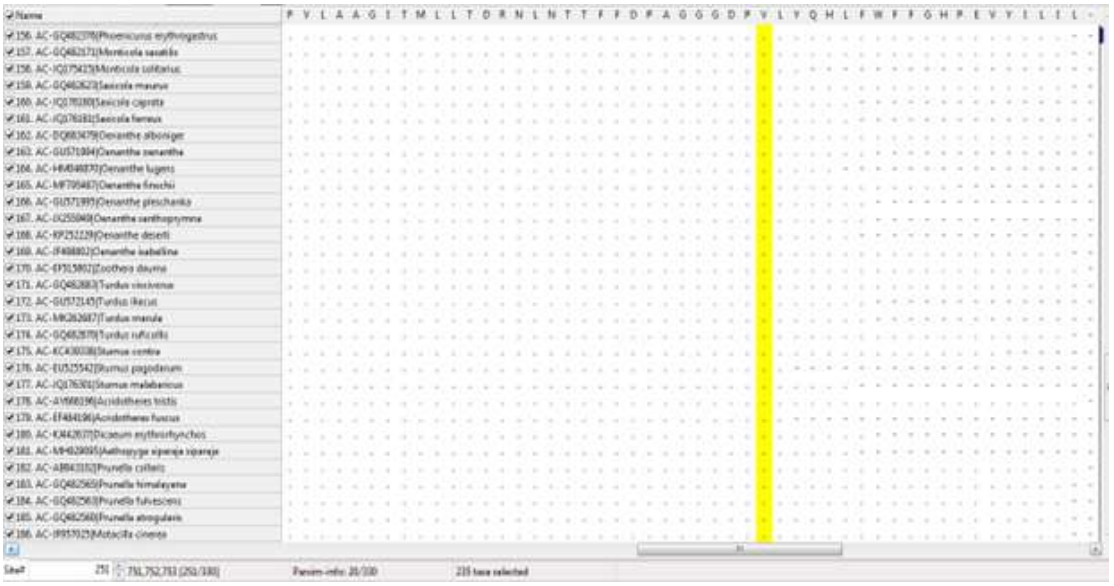


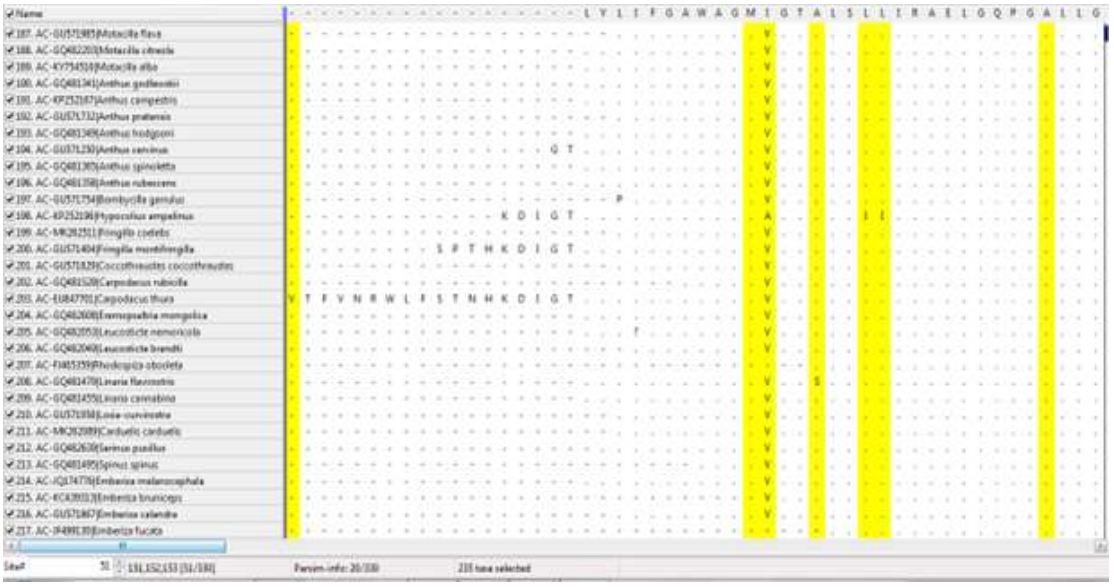


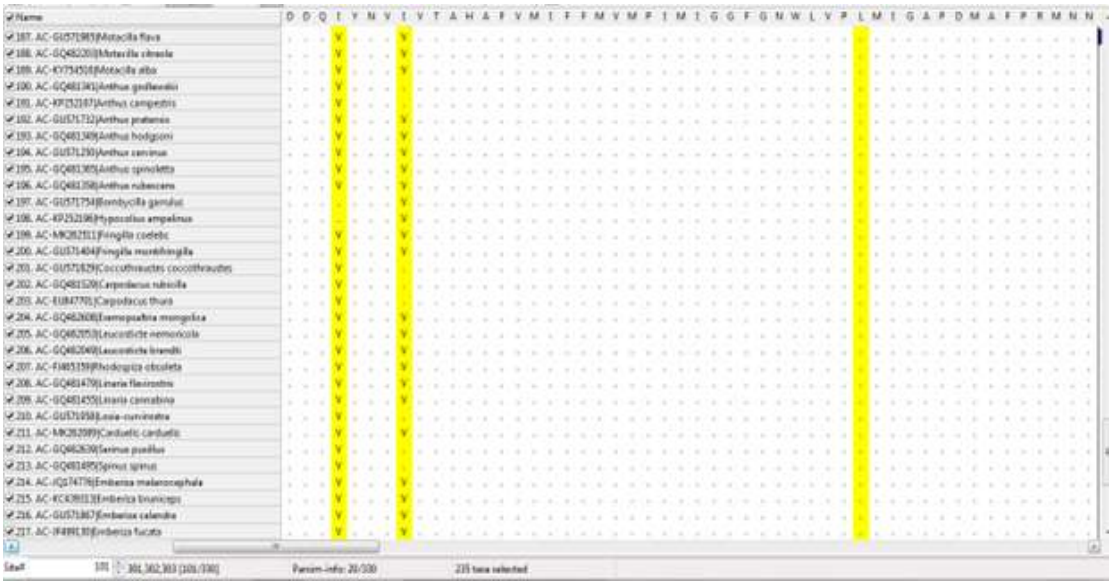


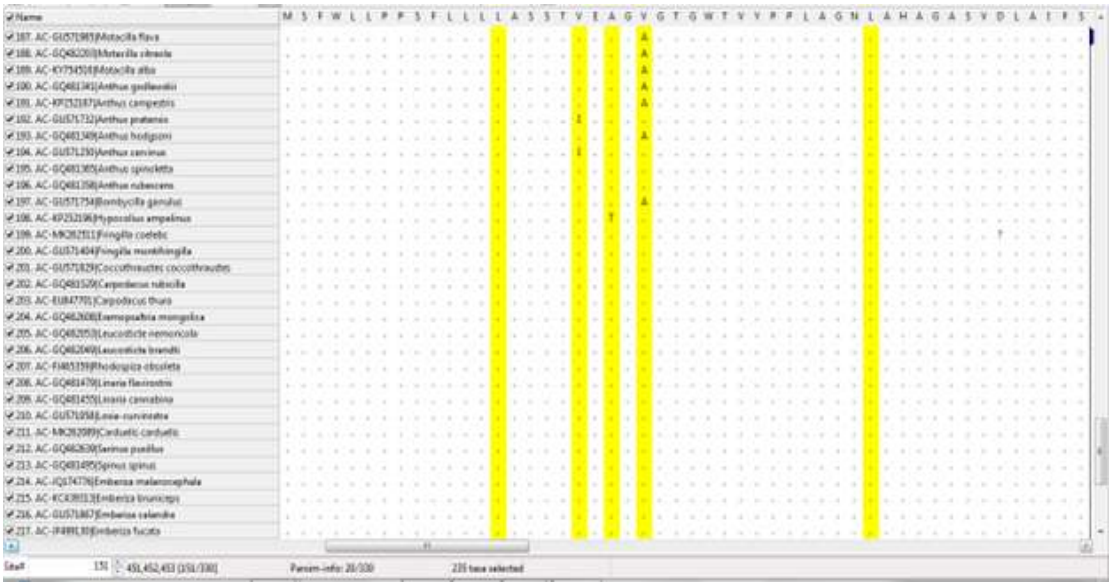


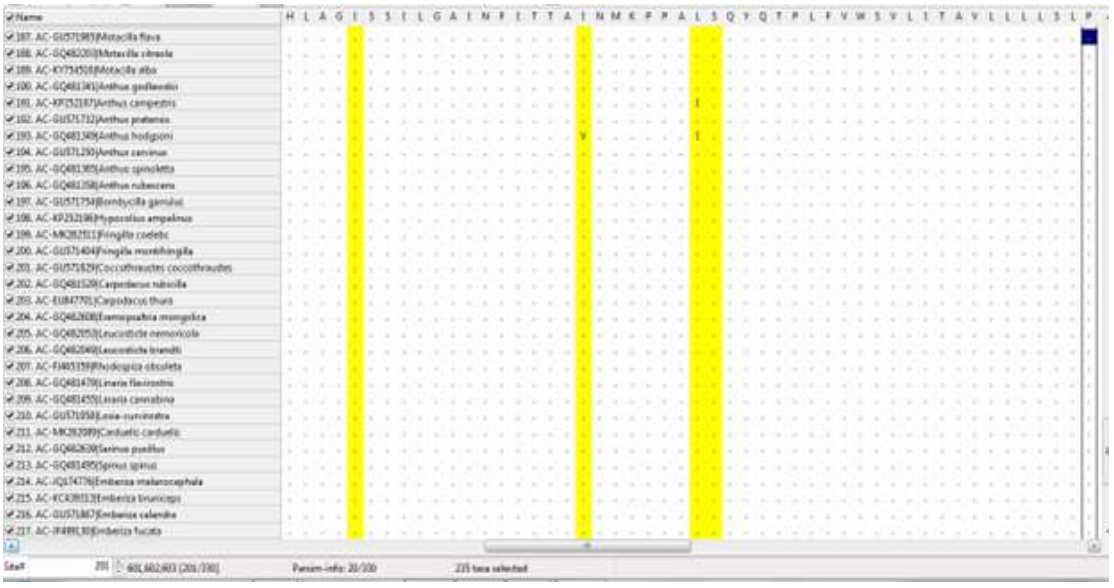


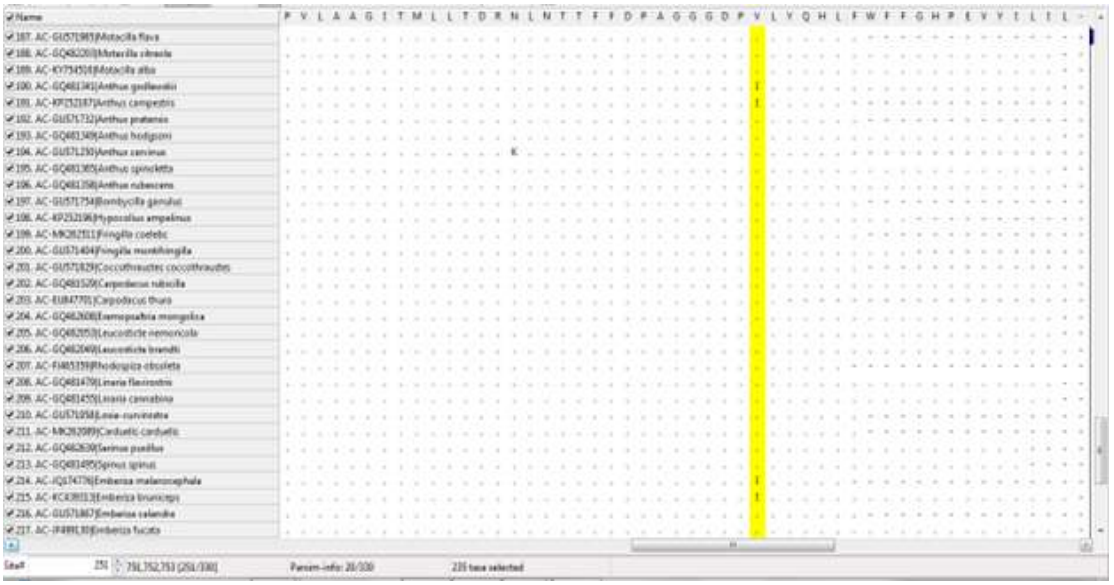


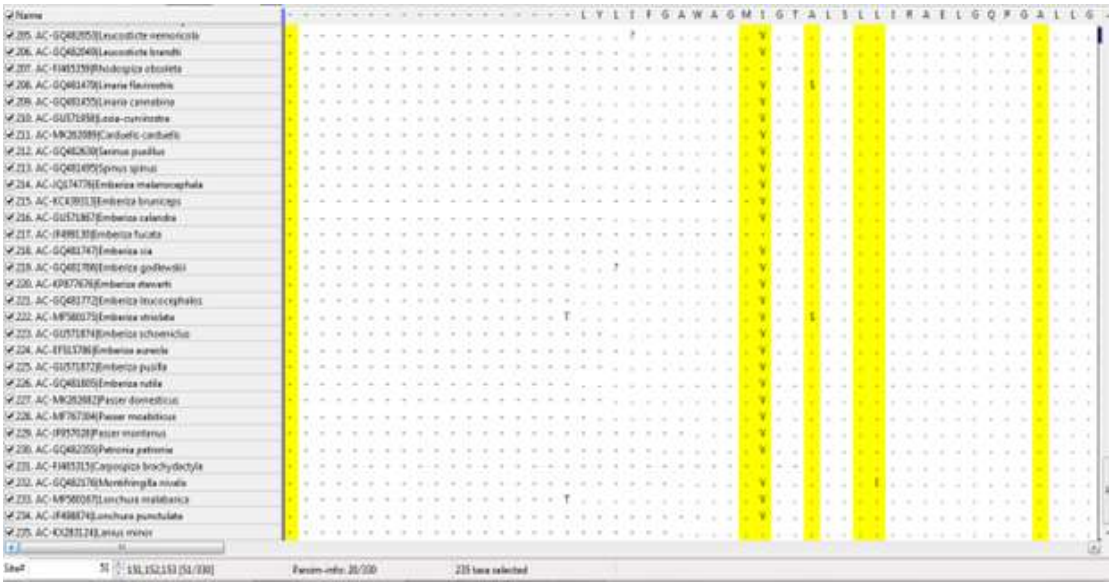


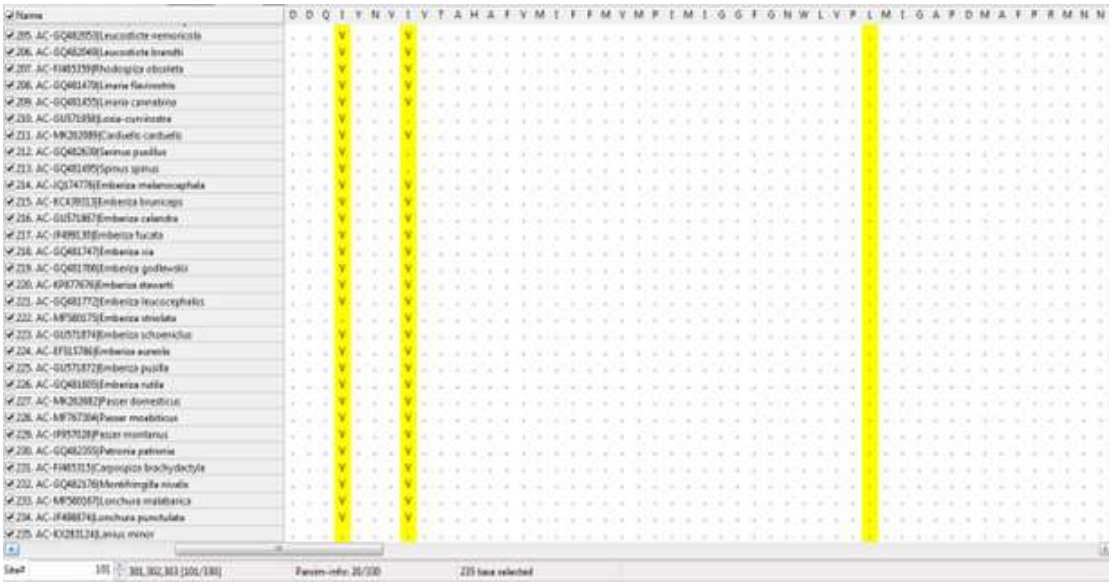


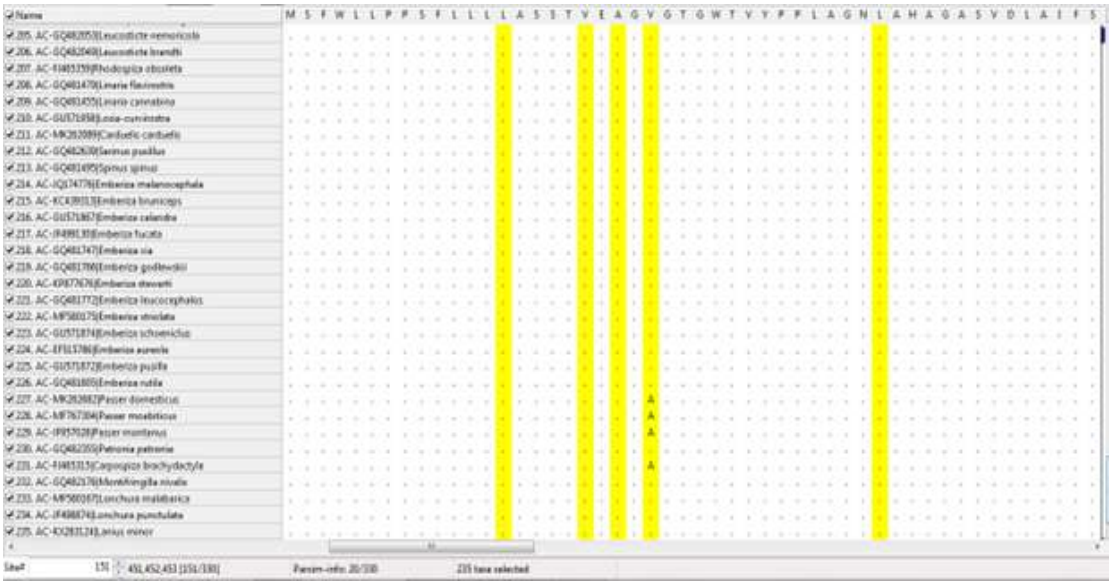


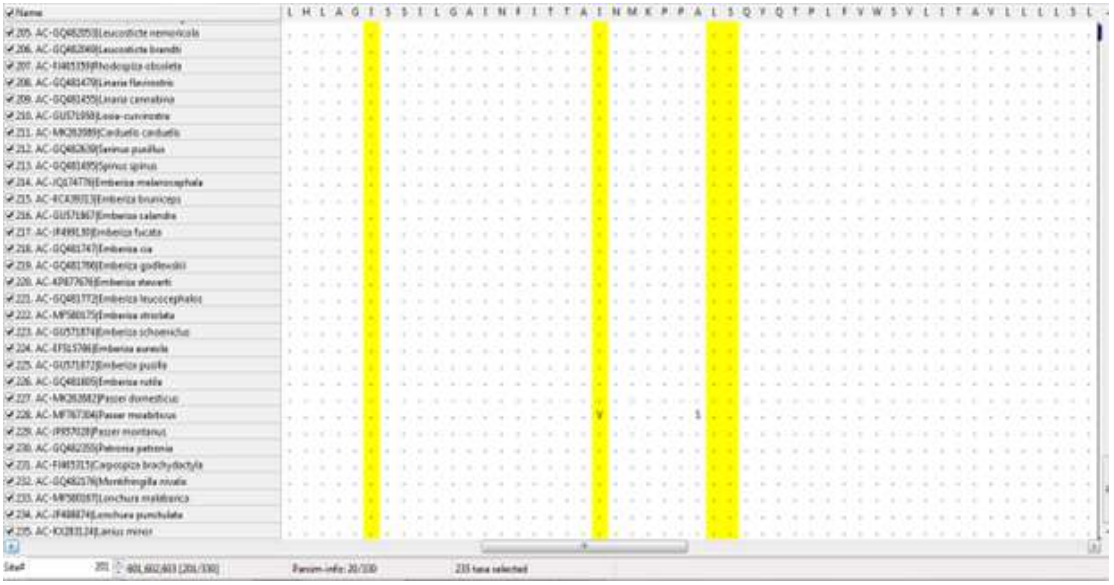


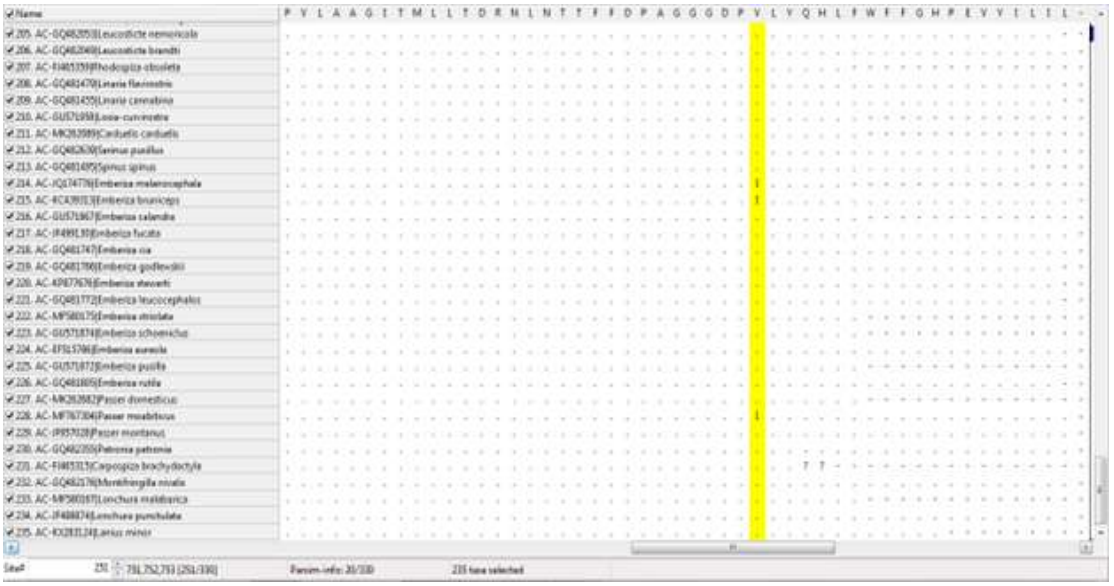












(iv): Variation at Amino Acid Level (Singleton Sites) in Sequences of Passeriformes Computed by MEGAX