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Study of Genetic Variations in Cytochrome B Gene of Grey Partridges from District Mianwali, Pakistan

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Abstract

Grey partridge is the native bird of Pakistan. It is present in Mianwali which is the least developed area. It is a game bird so it is a significant source of income for the area, but no data is available about its population in Mianwali. So, the purpose of this work is to study the cytochrome b gene of grey partridge to find out genetic variation in the Cytb gene of grey partridge and to gain information about the genetic diversity of this species. We collected blood samples of 8 individuals from 5 different sampling sites in the Mianwali. The length of DNA was 660 B.P. Neighbor joining, Minimum Evolution and UPGMA trees showed that there were a total of 1157 positions in the final dataset. The estimates of evolutionary divergence between Sequences results showed that a significant difference is present between birds of the same group and also among the groups. 99 % similarity Index through BLAST showed that the species that is present in this area is *Francolinus Pondicerianus*. Overall mean distance was found to be 2.76. Grey partridge shows more deviation. Average codons are found to be 302 in Codon usage bias.

Keywords: Partridges, Cytochrome b, Genetic variation, Mianwali, DNA, PCR, Pakistan.

INTRODUCTION

Birds, descendants of dinosaurs, have evolved into an incredibly diverse group, with approximately 9,000 species worldwide (Pathan et al., 2014). In Pakistan alone, 669 bird species are recorded (Grimmett et al., 2008). Among these, the Grey Partridge (*Francolinus pondicerianus*) is a notable species, originating from Asia and establishing itself in Europe and the Euro-Mediterranean region with agricultural advances (De Leo et al., 2004). The Grey

Partridge is an endemic bird of Pakistan, considered a significant game bird and a pest controller beneficial to agriculture (Mann and Chaudhry, 2000; Birdlife, 2007). Despite its classification as a species of "least concern" by the IUCN (2007), local observations suggest a decline in numbers due to hunting pressures (Roberts, 1991). Significant populations are protected within Lal Suhanra National Park (Khan, 2010).

Mitochondrial DNA (mtDNA) has proven invaluable for studying genetic variation due to its rapid mutation rate and maternal inheritance, providing insights into evolutionary processes and species relationships (Brown et al., 1982; Avise, 1994). The cytochrome b (cyt-b) gene, a critical component of the mitochondrial respiratory chain, is particularly useful for genetic studies due to its high mutation rate and well-characterized sequences (McClellan and McCracken, 2001; Nabholz et al., 2009).

In Pakistan, Grey partridges are found in the Makran and Lasbela mountains, Sindh, Punjab, the Thar Desert, and the Suleiman range (Roberts, 1992). They are omnivorous, consuming a variety of seeds and insects (Wijeyamohan et al., 2003). *F. p. interpositus* is North Indian Grey Francolin, found in northwest India and northern Pakistan. *F. p. mecranensis* is Baluchistan Grey Francolin, located in southeastern Iran and southern Pakistan. *F. p. pondicerianus* is Nominate subspecies found in southern India and Sri Lanka (Gmelin, 1789; Zarudny and Harms, 1913; Ali et al., 2021).

Imran Khaliq and his colleagues conducted a genetic study on grey partridges in the Soleiman range of Pakistan in 2011. They analyzed the genetic variation of mitochondrial DNA (mtDNA) from 29 grey partridges collected from four regions in the east and west of the Soleiman range. DNA was extracted and amplified to focus on a 511 bp segment of the mitochondrial control region. Genetic diversity and haplotype diversity were calculated using ARLEQUIN software, and AMOVA was used to analyze genetic variation within and between eastern and western populations. The study identified seven haplotypes across the samples, revealing five polymorphic sites and notable nuclear diversity. The mtDNA data indicated a high level of inherent variety within the grey partridge population, with the highest diversity indices found in the western population. Ghiyas and Rezaei's 2016 study focused on evaluating genetic diversity in grey partridges from Iran. Samples were collected from regions around Bandar Abbas in Hormozgan province and areas in Kerman province, including Jiroft and Kahnooj. After DNA extraction and sequencing a 439 bp fragment of the mitochondrial d-loop gene, a phylogenetic and haplotype tree was constructed to compare grey partridge populations in Iran and Pakistan. The study identified two distinct populations

with seven haplotypes across 29 specimens, showing high haplotype diversity but low nucleotide diversity, indicating closely related haplotypes. The phylogenetic analysis suggested a common ancestry between the black partridge and grey partridge. Parson et al. (2000) discussed the use of mitochondrial CYTB gene sequence analysis in forensic studies, highlighting its precision and consistency in species identification. The method relies on a single pair of primers and relative sequence analysis, making it a robust tool for forensic applications.

Dimshef et al. (2002) investigated the genetic variation in grey partridges by analyzing the cytochrome b gene sequence. The study supported DNA sequence alignments with inferred amino acid sequences. Kumar and Sharma (2016) studied the genetic diversity of grey and Asian black partridges in the Western Himalayas. Analyzing mtDNA control region fragments, they found 12 polymorphic sites across samples, indicating a recently emerged or slowly evolving group. The study provided initial insights into the genetic structure of partridge populations in the Himalayan region and suggested the control region's utility for comparative studies across species in the genus Francolinus.

Kang-Bao et al. (2010) examined the phylogenetic relationships among grey partridges and other Galliformes using mitochondrial cytochrome b and ND2 gene sequences. The study involved 44 species from the Phasianidae family and utilized maximum parsimony and Bayesian techniques. The phylogenetic analysis suggested that Tibetan Partridge was the ancestor of other species within the genus Perdix. Divergence among species was estimated to have occurred around 3.63 million years ago, highlighting the evolutionary history and genetic diversity within Galliformes.

MATERIALS AND METHODS

Study Area

This study was conducted in Mianwali District, located in the northwest of Punjab Province, Pakistan. Mianwali serves as a border district between Punjab and Khyber Pakhtunkhwa, covering an area of 5,840 square kilometers (2,250 sq mi). The Pothohar Plateau and Kohistan-e-Namak are situated to the north, while the southern side is part of the Thal Desert. The Indus River flows through the district, and the region's climate features long, hot summers and cold, dry winters. Mianwali has a diverse range of species, including the Grey Partridge *(Francolinus pondicerianus)*, which is found in areas such as Piplan, Harnoli,

Gulmeri, Chhidru, Beruli, Kamar Mushani, Kalabag, and Mochh. For this study, samples were collected from Harnoli, Piplan, Chhidru, Mianwali, and Mochh, where the Grey Partridge is present.

Settings

The research was conducted in the laboratory of the University of Lahore, Sargodha campus.

Study Duration

The study was carried out over seven months, with sample collection completed within the first month.

Equipment

The equipment used in the study included syringes, gloves, and EDTA tubes.

Sample Collection

Eight samples of Grey Partridges were collected from various areas of Mianwali. Two samples were obtained from Chhidru, two from Harnoli, one from Mianwali and one from Mochh. Additionally, two samples were purchased from hunters. The blood samples were collected from the jugular vein of the partridges' using syringes and stored in EDTA tubes, which were then kept at -20°C to preserve the samples for DNA extraction and further analysis.

Blood samples were collected and stored in EDTA tubes at -20°C to prevent degradation. For long-term storage, samples were kept at -80°C.

Ethical Considerations

All data was collected with integrity, ensuring no manipulation occurred. The Grey Partridges were treated humanely, with care taken to minimize harm during blood collection. After sampling, the birds were provided with food and water and subsequently released.

DNA Extraction Method

DNA extraction was carried out using the Phenol-Chloroform method. A 750 µl blood sample was mixed with an equal volume of RBC lysis solution (0.32 mM Sucrose, 10 mM Tris pH 7.5, 5 mM MgCl2, 1% Triton) and centrifuged at 13,000 rpm for 1 minute. The resulting pellet was resuspended in 500 µl of nuclear lysis solution and subjected to another round of centrifugation. The lysis process was repeated with an additional 500 µl of lysis solution containing 10 mM Tris, 400 mM NaCl, and 2 mM EDTA. Subsequently, Proteinase K and 20% SDS were added to the samples, followed by overnight incubation at 55°C. The following day, 500 µl of a phenol, chloroform, and isoamyl alcohol (PCI) mixture was added, and the samples were centrifuged at 13,000 rpm for 10 minutes. The aqueous phase was carefully transferred to a new tube and treated with chloroform and isoamyl alcohol, followed by a second centrifugation. The final aqueous layer was moved to a 1.5 ml centrifuge tube, and 55 µl of sodium acetate along with 500 µl of chilled isopropanol were added. The samples were incubated at -20°C for 45 minutes and then centrifuged. The supernatant was discarded, and the DNA pellet was washed with 500 µl of 70% ethanol, followed by centrifugation at 7,500 rpm for 5 minutes. After air-drying, the DNA pellet was resuspended in TE buffer (Tris EDTA) and stored at 4°C.

Agarose Gel Electrophoresis

Gel electrophoresis was performed using 1% agarose gel. One gram of agarose was dissolved in 100 ml of 1X TAE buffer and heated to clarify. After adding 7 µl of Ethidium Bromide, the gel was poured into a casting tray with combs to create wells. DNA samples mixed with loading dye were loaded into wells, and the gel was run at 500 mA and 70 volts for 60 minutes. The gel was visualized under a UV transilluminator to assess the DNA quality, showing intact DNA bands compared to a 1KB ladder. Primers F1484B and R1485B were used to amplify the Cyt b gene in blood samples. The primers were designed to bind specifically to the target DNA sequences, enabling the amplification of the desired gene region.

RESULTS

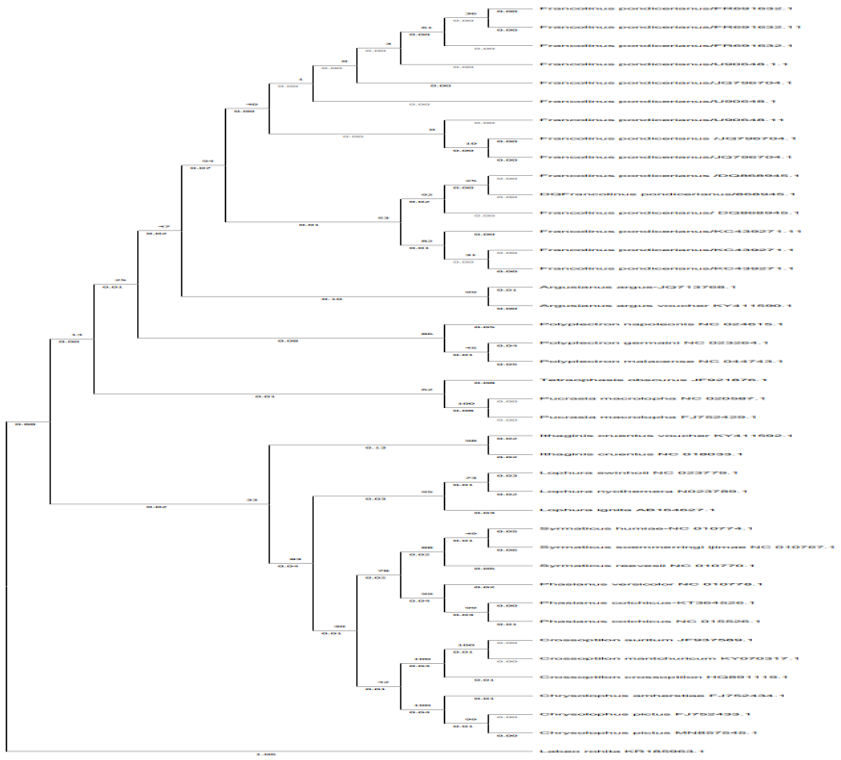
DNA extraction and quantification

DNA extraction from all 8 bird samples was completed and later confirmed on the agarose gel having a concentration 1 %. The DNA was extracted by using the Russell and Sambrook technique. After DNA extraction completion the quality of DNA samples was checked on gel documentation system and pictures were saved in the computer system for further use.

Polymerase Chain Reaction Results (PCR)

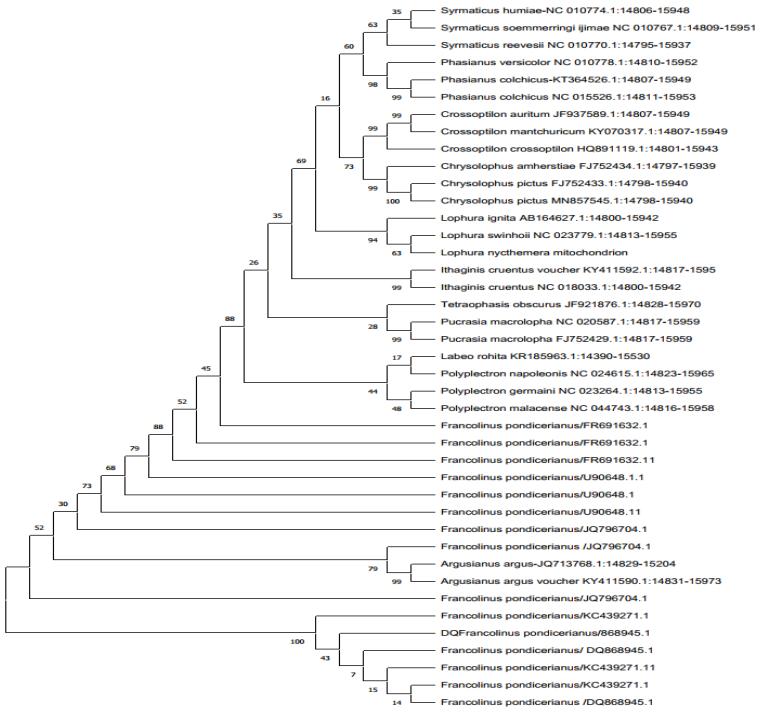
In order to amplify the desired region of Cyt B gene the polymerase chain reaction of extracted genomic DNA of grey partridge had carried out.

Maximum Likelihood method



Evolutionary relationships of taxa by Maximum Likelihood method

Using the Maximum Likelihood method and General Time Reversible mode evolutionary olden times was hypothesised (Nei M. and Kumar S, 2000). The tree with the maximum log probability (-7658.86) has been shown. The percentage of trees grouped together by the related taxa is seen next to the branches. Initial tree(s) for the heuristic search has been obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. To model evolutionary rate variations between sites (5 groups (+ G, parameter = 0.02371)), a discrete Gamma distribution was used. The tree is scale-drawn, with the length of the branch determined by the number of substitutions per site. 41 nucleotide sequences were included in this study. In the final dataset, there were a total of 1145 locations. In MEGA X, evolutionary studies were performed (Kumar S et al., 2018).



Evolutionary relationship of taxa by Neighbour joining method

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree, with a branch length of 17.7344, was constructed, and bootstrap values (1000 replicates) indicate the percentage of times associated taxa clustered together (Felsenstein, 1985). The tree, scaled to branch lengths representing evolutionary distances, was drawn using the Maximum Composite Likelihood method (Tamura et al., 2004), which measured base substitutions per site. A gamma distribution (shape parameter = 1) modeled rate variation among sites. The study included 41 nucleotide sequences, with codon positions 1st+2nd+3rd considered, and unclear positions deleted via pairwise deletion. The final dataset contained 1157 positions. The evolutionary analysis was conducted using MEGA X (Kumar et al., 2018).

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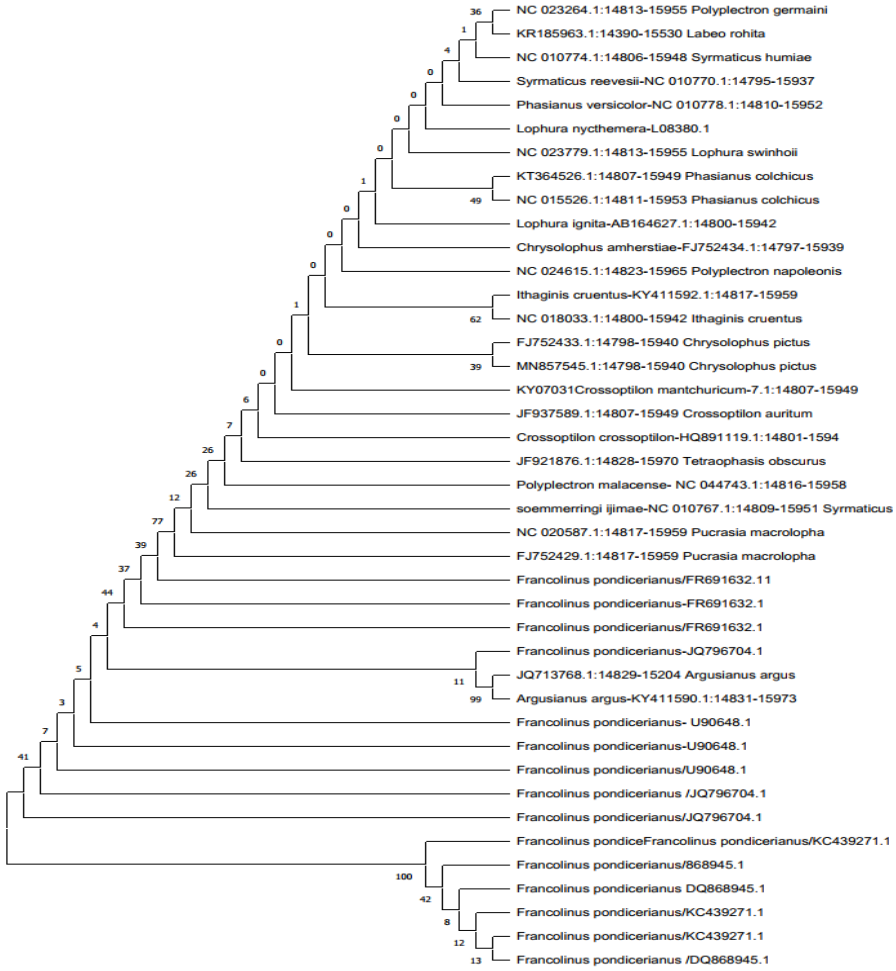

Figure 4.5 Evolutionary relationship of taxa by Minimum evolution method

The evolutionary history was inferred using the Minimum Evolution method (Rzhetsky et al., 1992), with an optimal tree having a branch length sum of 18.1236. Bootstrap values (1000 replicates) indicate the percentage of times associated taxa clustered together (Felsenstein, 1985). The tree, scaled to branch lengths representing evolutionary distances, was constructed using the Maximum Composite Likelihood method (Tamura et al., 2004), which measured base substitutions per site. Rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The Minimum Evolution tree was refined using the Close-Neighbor-Interchange (CNI) algorithm (Nei and Kumar, 2000) with a search level of 1, starting from an initial Neighbor-Joining tree (Saitou et al., 1987). The study included 41 nucleotide sequences, with vague positions removed via pairwise deletion, leaving 1157 sites

in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

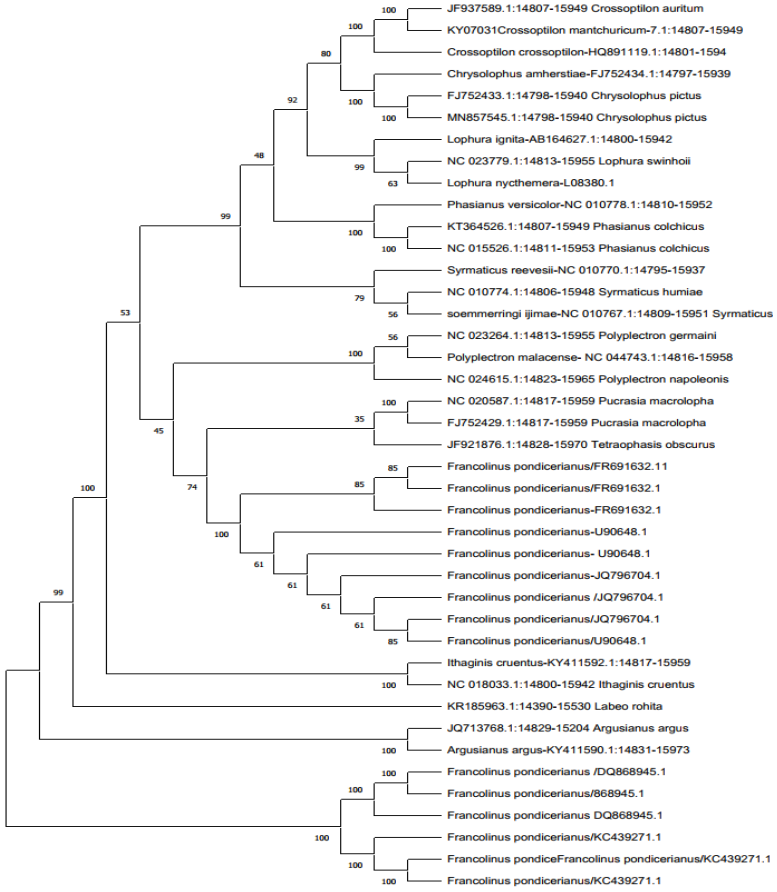

Figure 4.6 Evolutionary relationship of taxa by UPGMA method

The evolutionary history is inferred by using the UPGMA method (Sneath P and Sokal R,1973). The optimal tree is shown which has the sum of branch length (12.56941292). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) has been shown next to the branches (Felsenstein J, 1985). By using the Maximum Composite Likelihood method, the evolutionary distances are computed (Tamura K et al., 2004) and are in the units of the number of base substitutions per site. The rate variation among sites is modeled with a gamma distribution (shape parameter = 1).41 nucleotide sequences are involved in this analysis. All ambiguous positions were removed for each sequence pair (pairwise deletion option). Total of 1157 positions in the final dataset are present. Evolutionary analyses are conducted in MEGA X (Kumar S et al., 2018; Bilal and Ullah, 2021).

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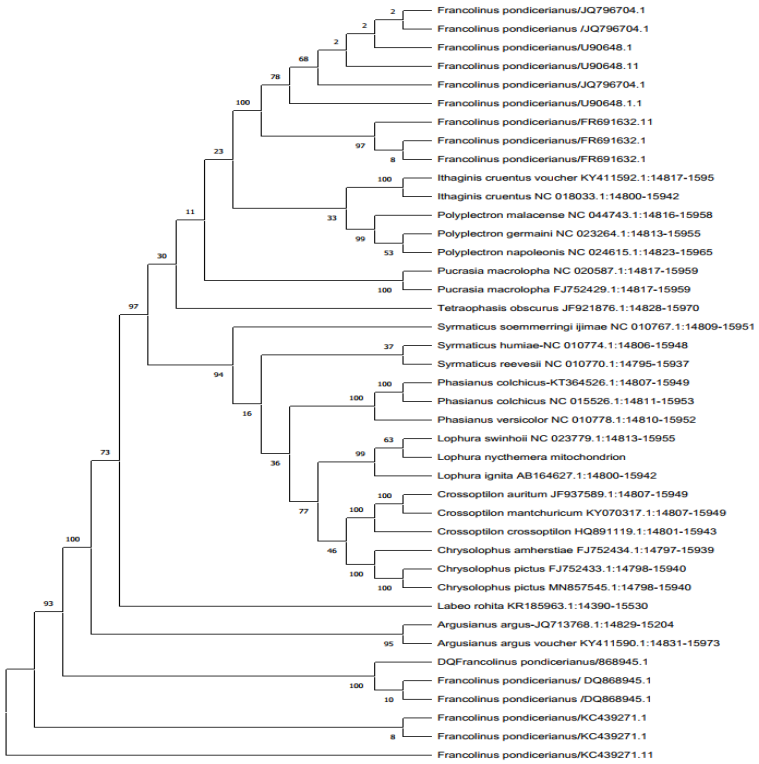
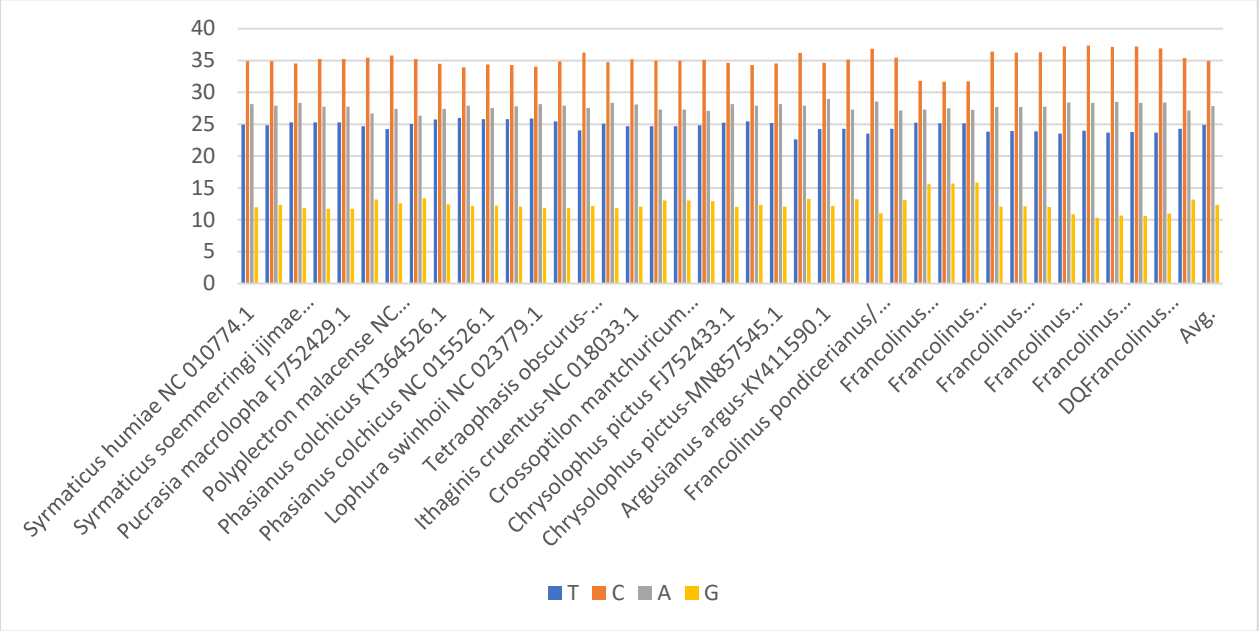


Figure 4.7 Evolutionary relationship of taxa by Maximum parsimony method

The evolutionary history was inferred using the Full Parsimony method, revealing Tree #1 out of the three most parsimonious trees (length = 2045). The consistency index is 0.5252, the retention index is 0.7670, and the composite index is 0.4733 (0.4028 for parsimony-informative sites). Bootstrap values (1000 replicates) indicate the percentage of times associated taxa clustered together. The most parsimonious tree (MP tree) was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar, 2000) with a search level of 1 and initial trees generated by random sequence addition (10 replicates). The study included 41 nucleotide sequences with codon positions 1st+2nd+3rd considered. The final dataset contained 1157 positions. Evolutionary analyses were conducted using MEGA X (Kumar et al., 2018; Bilal et al., 2024).



Nucleotide composition based on CYT B gene

DISCUSSION AND CONCLUSION

The Grey Partridge (*Francolinus pondicerianus*), originally native to Asia, expanded into Europe and the Euro-Mediterranean region with the advancement of agriculture (De Leo et al., 2004; Rizwan et al., 2024). In Pakistan, this species is endemic and particularly noted in Mianwali, a less developed area. Despite its presence in this region, genetic data on the Grey Partridge is sparse. Research by Khalil et al. (2015) highlights the bird's habitation in open, arid parts of Asia, including Pakistan, southeastern Iran, India, Bangladesh, and northern Sri Lanka, where it finds suitable conditions in grassy and cultivated areas (De Leo et al., 2004; Iftikhar et al., 2024; Rasheed et al., 2024). The genetic structure of the Grey Partridge in the Salt Range, such as Kalabag, remains largely unexplored. Previous studies by Imran Khaliq and colleagues focused on mitochondrial DNA (mtDNA) in the Soleiman Range of Pakistan, revealing high genomic diversity and low nucleotide variability, with significant haplotype diversity in the western populations. mtDNA, known for its rapid evolution and limited repair mechanisms, is a valuable marker for phylogenetic studies due to its uniform gene content and evolutionary rate (Cann & Wilson, 1983; Allan et al., 1985; Shahin et al., 2024).

In this study, we aimed to analyze the cytochrome b (cyt b) gene of the Grey Partridge to assess genetic variation and diversity. Blood samples from eight individuals across five sites in Mianwali were collected, DNA was extracted, and the cyt b gene was amplified using PCR. Sequencing of the 660 bp amplified DNA revealed significant genetic variation. Phylogenetic

analysis using Neighbor Joining, Minimum Evolution, and UPGMA methods showed a total of 1157 positions in the final dataset, with notable differences between individual birds and among groups. The 99% similarity index from BLAST confirmed the species as *Francolinus pondicerianus*. The cyt b gene showed higher deviation and a codon usage bias with an average of 302 codons. The high rate of mtDNA evolution, driven by mutation pressure and relaxed constraints on translation components, supports its utility in studying genetic variations (Cann et al., 1984).

The cyt b gene is well-characterized and widely used for phylogenetic studies due to its high substitution rate, universal primer sequences, and extensive data availability (Richman & Price, 1992; Bridge et al., 2005). It provides insights into species' genetic divergence and helps establish phylogenetic relationships, as evidenced by studies showing a close relationship between birds and crocodilians (Hedges, 1994). In conclusion, this study has provided valuable genetic data on the Grey Partridge from Mianwali, revealing significant variation in the cyt b gene. The results highlight the importance of mtDNA as a marker for genetic and evolutionary studies. However, conservation efforts are crucial as illegal hunting, habitat destruction, and other pressures threaten the Grey Partridge population. Effective conservation strategies and further genetic studies are needed to ensure the survival of this species and maintain its genetic diversity.

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