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Molecular phylogeny of *Francolinus francolinus* in Iraq based on mitochondrial cytochrome c oxidase I gene diversity

S.A. Abid

Department of Biology, College of Science for Women, University of Baghdad, Baghdad, Iraq

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Correspondence:
S.A. Abid
shaimaaa_bio@csw.uobaghdad.edu.iq

Abstract

The Iraqi avifauna, such as the black francolin, faces habitat loss and illegal hunting, necessitating their protection to conserve genetic diversity. The cytochrome c oxidase I gene is a precise and widely used genetic marker for identifying bird diversity and protecting native species. This study used the cytochrome c oxidase I (COI) gene as a barcode, isolating genomic DNA from the skin and feathers of 14 samples. In addition, a specific primer set was designed for amplification of the cytochrome c oxidase I gene by polymerase chain reaction (PCR), and the resulting cytochrome c oxidase I sequences were aligned with sequences in the NCBI GenBank. The black francolin COI sequence divergence varies significantly among individuals from Iraq and different regions. The evolutionary tree was constructed within *Francolinus francolinus* to determine closely related species and diversification. The Iraqi black francolin falls within the first and second clades in the tree, with 62%-100% affinity to the original species. The outcome emphasizes that the cytochrome c oxidase I gene selected offers a reliable indicator for the classification of F. francolinus and for determining this species' viability in the Iraqi environment. The newly sequenced F. francolinus sequences are recorded in GenBank under accession numbers PV199482.1, PV199483.1, PV199484.1, PV199485.1, PV199486.1, PV199487.1, and PV199488.1. Finally, the present study may contribute to the development of a reference DNA barcoding database of Iraqi birds and confirmed the important role of genetic diversity in assessing the black francolin's ability to survive.

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Introduction

Humans have historically transported wildlife, such as the black francolin, from the Eastern Mediterranean and Middle East to the Indian subcontinent, impacting current biodiversity (1,2). The black francolin (*Francolinus francolinus*, Linnaeus, 1766), a terrestrial gamebird, which belongs to the Galliformes order of the family Phasianidae, is classified as Least Concern (3). Morphologically, the male is recognized by a black face and a dark rust-brown neck collar. At the same time, the brownish female has thick black-and-white patterns throughout the body. This bird inhabits a variety of habitats worldwide and eats grains, seeds, insects, and worms (4). This bird's threat in Asia, especially in Iraq, is due to increased predation, illegal hunting, trapping, habitat deterioration and fragmentation, continuous use of handguns for hunting, and chemical residues in agriculture. Additionally, human activities, including the expansion of urban areas, affect Iraq's biological diversity (5-7). Asian francolins are diverse in form, the environment, behavior, and their geographic distribution patterns (8). The bird challenge of morphological analysis (primarily of feather features) and of recognizing diverse developmental phases. Recently, the researchers began using DNA tools to conserve biological diversity and species taxonomy (9,10). Short fragments of DNA from a standard portion of the genome serve as a DNA barcode, the mitochondrial cytochrome c oxidase I (mtCOI)

gene, to identify individuals at the species level, elucidate evolutionary relationships among closely related species, and recognize genetic diversity (11-13). DNA barcoding has gained wide acceptance in taxonomic research and in ecology and evolution for its ability to quickly and accurately identify species (14-16). Forcina *et al.* (17) conducted an evolutionary sequence analysis of the entire mitochondrial DNA control region in Asian francolin species. Furthermore, there are few studies on the mt *COI* gene in Iraqi birds. Balog *et al.* (18) assessed the genetic diversity of Iraqi Red and Raabi pigeons using the COI area.

The current study aimed to use a valuable mitochondrial DNA marker (the partial mtCOI gene) to identify native F. francolinus in Iraq and to determine genetic variation at the species level. Mitochondrial DNA is an effective marker, and the COI gene is used as a DNA barcode.

Materials and methods

Ethical approval

The experiment was approved by the local ethical committee under approval number 862 (8/10/2024) at the University of Baghdad, College of Science for Women, Department of Biology.

Sampling and DNA extraction

Fourteen *F. francolinus* (19) samples were gathered from various locations in Baghdad during the period from August to October 2024 (Figure 1). Genomic DNA was extracted from skin and feathers using the DNeasy Blood and Tissue Kit (Qiagen, Germany), as modified from (20,21). The DNA concentrations were measured using NanoDrop to verify the DNA quality of all samples at two wavelengths (260 and 280 nm).

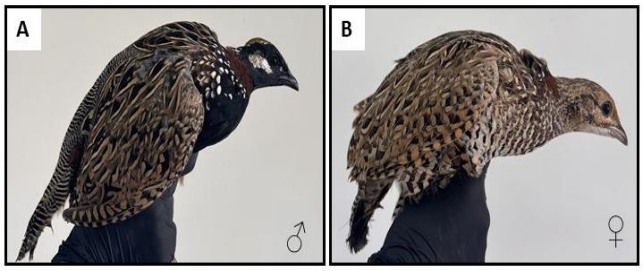

Figure 1: Lateral view of the black francolin (*Francolinus francolinus*); A: Male, B: Female.

Amplification and sequencing:

The PCR amplification of the *COI* gene was done using specific primer pairs FrCO-FWD: 5′-ACAGCACTCAGCCTACTAAT-3′ and FrCO-REV: 5′-GGTGTCCGAAGAATCAGAAT-3′. Each PCR reaction had a final volume of 35 µl containing 3 µl of genomic DNA, 1.5 µl of each forward and reverse primer, and 17.5 µl of GoTaq® G2 Hot Start GreenMaster Mix 2X, and was completed by adding 11.5 µl of free nuclease-distilled water. The optimum conditions of COI amplification in the thermocycler were set as follows: one cycle of 95°C initial denaturation for 5 min, followed by thirty-three cycles of 95°C denaturation for 45 sec, annealing at 55°C/45 sec, 1 min for extension at 72°C, and one cycle for final extension at 72°C for 7 min. The reaction was held at 4°C for 15 min. The PCR products were electrophoresed on a 1.5% agarose gel, and the bands were visualized using a gel imaging system. The PCR outcomes of COI were sequenced using an automated DNA sequencer. The COI sequences were analyzed and aligned with reference sequences available at the National Center for Biotechnology Information (NCBI, USA) (https://blast.ncbi.nlm.nih.gov/Blast.).

Data analysis

The taxonomic rank of *F. francolinus* was assessed to align it with the most closely related species in NCBI. The Mega 11 program was used to estimate pairwise sequence distances for divergence analysis (22) and to construct a phylogenetic tree for this species. Some of the nucleotide sequences were unsuccessful. The phylogenetic tree was built using the Maximum Likelihood (ML) method and validated with 1000 bootstrap replicates (23) to determine the evolutionary history of *F. francolinus*.

Results

PCR amplification of the partial mtCOI gene was performed to identify *F. francolinus*. The PCR outcomes indicate that the 631 bp segment of the mt *COI* gene for the black francolin birds (Figure 2) was subsequently sent for sequencing. The sequence analysis confirms that the mt *COI* gene belongs to *F. francolinus*. The partial COI sequence of *F. francolinus* was registered in NCBI GenBank under accession numbers PV199482.1, PV199483.1, PV199484.1, PV199485.1, PV199486.1, PV199487.1, and PV199488.1, respectively. The intraspecific genetic distances ranged from 0.00% to 5.6%, which explains evolutionary divergences in the mtCOI sequence within *F. francolinus* (Table 1). The phylogenetic analysis revealed clear clustering patterns among *F. francolinus* individuals from different geographic regions. Iraqi samples formed a well-supported clade, exhibiting moderate bootstrap values, indicating a close genetic relationship and possible shared ancestry among these individuals. One Turkish sample (NLAVE321-11) was nested within this Iraqi clade, suggesting historical gene flow or a common ancestral lineage between populations in Iraq and adjacent regions of Turkey. The Turkish samples overall were placed in separate positions, reflecting genetic divergence within the population. Conversely, individuals from the USA and Cyprus formed clades, indicating significant genetic differentiation likely due to geographic isolation (Figure 3).

Table 1: Pairwise distances for estimates of evolutionary divergence between COI sequences of *F. francolinus*

Species	1	2	3	4	5	6	7	8	9	10	11	12
PV199482.1:Iraq	-											
PV199483.1: Iraq	0.019											
PV199484.1: Iraq	0.019	0.000										
PV199485.1: Iraq	0.027	0.039	0.039									
PV199486.1: Iraq	0.019	0.000	0.000	0.040								
PV199487.1: Iraq	0.022	0.002	0.005	0.038	0.002							
PV199488.1: Iraq	0.024	0.005	0.005	0.040	0.005	0.002						
KT803620.1: Cyprus	0.010	0.009	0.009	0.038	0.011	0.014	0.016					
JF498851.1: USA	0.027	0.026	0.026	0.051	0.030	0.030	0.030	0.018				
JF498852.1: USA	0.032	0.031	0.031	0.056	0.035	0.035	0.035	0.019	0.005			
NLAVE173-11: Turkey	0.014	0.014	0.014	0.038	0.016	0.018	0.011	0.003	0.017	0.019		
NLAVE174-11: Turkey	0.014	0.014	0.014	0.038	0.016	0.018	0.011	0.003	0.017	0.019	0.000	
NLAVE321-11: Turkey	0.014	0.014	0.014	0.038	0.016	0.018	0.011	0.003	0.017	0.019	0.000	0.000

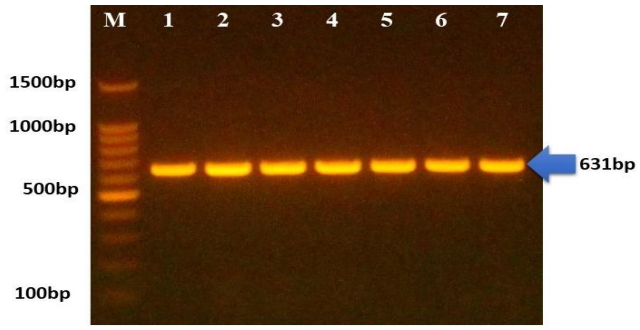

Figure 2: The PCR amplicons of the *COI* gene were electrophoresed on a 1.5% agarose gel at 70 V/60 min. The 631 bp product is indicated by a blue arrow. Lane DNA ladder (M), lanes (1-7) represent samples of PCR products.

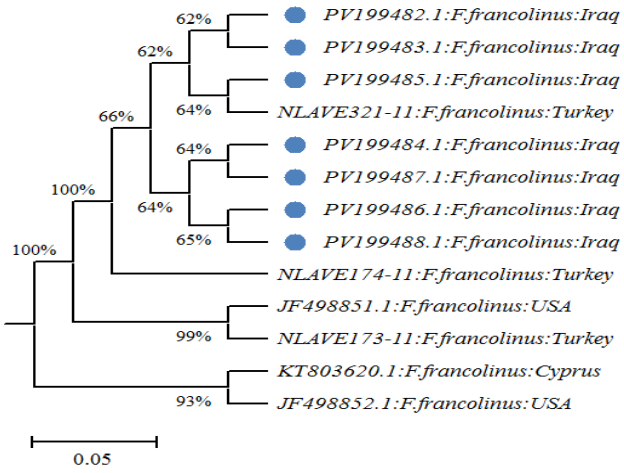

Figure 3: Phylogenetic tree analysis of the mtCOI sequence of *F. francolinus* by using Mega 11 (ML). This tree showed the geographical distribution in Iraq and in different countries (the sample from Iraq was marked with a blue circle).

Discussion

The global population growth, urbanization, deforestation, climate change, and illegal hunting have severely reduced wildlife habitats. Furthermore, species may adapt to their environment to survive or migrate, or their numbers may decline over time, which impacts bird diversity (6,24,25). Therefore, mitochondrial DNA is a powerful genomic tool for better understanding species and assessing the diversification of ecologically distinct species, thereby promoting biodiversity conservation (26,27). According to Khaliq *et al.* (28), the overhunt would threaten the survival of *Francolinus pondicerianus* in Pakistan. As a result, use mitochondrial DNA to better understand the genetic diversity of this bird and its conservation. Genetic diversity within the black francolin population was demonstrated through partial mitochondrial gene sequencing of individuals from various regions. Additionally, deforestation, flooding, and habitat destruction might result in further separation and smaller populations that may have decreased genetic diversity (29,30).

The black francolin is generally considered a non-migratory gamebird, typically remaining within the same area where it hatched. Nevertheless, it may undertake short-distance movements in response to environmental fluctuations, such as habitat degradation or seasonal changes in food availability, often in search of suitable breeding grounds or foraging habitats (31,32). Alternatively, the chicks or eggs of the black francolin in exposed nests may be susceptible to predation by raptors such as *Elanus caeruleus* (33). Due to its sensitivity to habitat disturbances, targeted conservation efforts and practical habitat management (e.g., maintaining forest sustainability) are vital to maintaining stable populations and preserving its ecological role in native ecosystems (32,34). The current study highlighted that the target COI genes were selected because of their widespread use in species taxonomy. Despite their partiality, these genes provide sufficient

sequence divergence to meet the requirements of this study. Here, the *COI* genes were PCR-amplified using specifically designed primer pairs, which were demonstrated to be a stringent complement to the conserved region for species identification. These results are generally in agreement with other studies, but used universal primers to amplify conserved regions of the COI gene (15,35). DNA barcodes provide high-quality genetic information for species identification (36). Therefore, these findings will assist in developing the GenBank database and can be used for additional phylogenetic analysis on other avian species. The high intraspecific divergence (0.00%-5.6%) of the *F. francolinus* COI sequence among individuals from Iraq and different regions (e.g., USA, Cyprus, Turkey). This elevated divergence may reflect historical geographic isolation among populations, resulting in limited gene flow and the accumulation of substantial genetic differences over time. The phylogenetic analysis of the COI sequence revealed clear genetic structuring among F. francolinus populations across regions.

Iraqi samples form a monophyletic clade, suggesting localized evolution or limited gene flow with other populations. In contrast, the USA samples cluster separately with high bootstrap support, indicating significant genetic divergence from the Iraqi group. Turkish samples appeared in two separate branches within the tree. One Turkish sample was closely related to the Iraqi clade. In contrast, the others were more closely related to the USA and Cyprus samples. These findings may suggest population isolation due to geographic barriers in the Middle East and other regions. The phylogenetic structure observed supports the hypothesis of deep genetic divergence within species, potentially shaped by historical biogeographic events or habitat fragmentation (37). DNA sequence data in public repositories enable phylogenetic inference of avian species taxonomy (38). Therefore, a terrestrial gamebird is essential to combine ecological monitoring with molecular tools, such as genome-wide association studies, to further evaluate genetic diversity, population connectivity, and ecological adaptability of black francolins, thereby contributing to the development of conservation strategies (39,40).

Conclusion

This investigation demonstrates that genetic diversity is significant for assessing the viability of *F. francolinus* in Iraq. Seven novel COI haplotypes from Iraqi *F. francolinus* were deposited in GenBank (accessions PV199482.1-PV199488.1), representing the first such records from Mesopotamia. These genetic data suggest that Iraqi black francolins represent an evolutionarily significant unit meriting: (1) habitat corridors between remaining wetlands, and (2) stricter enforcement of hunting regulations.

Acknowledgment

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Conflict of interest

There is no conflict of interest.

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التطور الجزيئي *Francolinus francolinus* في العراق اعتماداً على تنوع جين المايتوكوندريا السيتوكروم سي اوكسيديز الأول

شيماء عواد عبد

قسم علوم الحياة، كلية العلوم للبنات، جامعة بغداد، بغداد، العراق

الخلاصة

تواجه الطيور في العراق مثل طائر الدراج الأسود فقدان موائلها والصيد غير المشروع، مما يستلزم حمايتها من أجل الحفاظ على التنوع الجيني. تعتبر العلامات الجينية (جين السيتوكروم سي أوكسيديز الأول) أداةً مهمةً ودقيقةً لتحديد تنوع الطيور وحماية الأنواع المحلية. استخدم في هذه الدراسة جين السيتوكروم سي أوكسيديز الأول كباركود، وتم تحقيق ذلك بعزل الحمض النووي من الجلد والريش لأربع عشرة عينة. بالإضافة إلى ذلك، تم تصميم مجموعة برايمرات خاصة لتضخيم جين السيتوكروم سي أوكسيديز الأول بواسطة تقنية تفاعل البوليميراز المتسلسل، وثم مطابقة تسلسلات السيتوكروم سي أوكسيديز الأول مع التسلسلات في بنك جينات للمركز الوطني لمعلومات التكنولوجيا الحيوية. أن تباعد تسلسلات COI للدراج الأسود يختلف بشكل كبير بين الأفراد من العراق والمناطق المختلفة. إن الشجرة التطورية تم إنشاؤها ضمن نوع طائر الدراج الأسود لتحديد الأنواع وثيقة الصلة وتنوعها. يقع الدراج الأسود العراقي ضمن الفرعين الأول والثاني في الشجرة بنسبة تقارب تتراوح بين ٦٢% إلى ١٠٠% من الأنواع الأصلية. تؤكد النتائج أن جين سيتوكروم أكسيداز المايتوكوندريا الأول (COI) الذي تم اختياره يقدم مؤشراً موثوقًا لتصنيف طائر الدراج الأسود وتحديد مدى قابلية هذا النوع للبقاء في البيئة العراقية. سُجلت التسلسلات الجديدة لطائر الدراج الأسود في بنك الجينات تحت أرقام الوصول PV199482.1، PV199483.1، PV199484.1، PV199485.1، PV199486.1، PV199487.1، و PV199488.1. وأخيراً، قد تساهم الدراسة الحالية في تطوير قاعدة مصادر البيانات التشفير الشريطي للحمض النووي للطيور العراقية وتؤكد الدور المهم للتنوع الجيني في تقييم قدرة طائر الدراج الأسود على البقاء.