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Molecular detection of cestoda in black francolin (*Francolinus francolinus*) of Babylon Province, Iraq

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Abstract

Cestodes or tapeworms (Class Cestoda, phylum Platyhelminthes) are widespread parasites of birds, as they cause various damages, which may lead to the death of their hosts, especially when the infection is severe. The Black Francolin belongs to the Phasianidae family of pheasants and, in the order Galliformes, the gallinaceous birds, and it is called *Francolinus francolinus*. Considering it as a local bird in Asia, it was referred to as the black partridge. The current study was conducted from January to October 2019 to determine the cestoda infections within *F. francolinus*. Thirty-five birds from *F. francolinus* were examined for the presence of cestoda, of which 17 birds (48.57%) were infected with cestoda parasites. Molecular diagnosis based on sequences of cytochrome c oxidase 1 (COI) gene regions was performed to confirm the diagnosis of this cestoda. This revealed a close identification of up to 93.44-100% for COI gene regions with other cestoda species obtained from GenBank. Phylogenetic analysis supported the placement of this species within three families, Davaineidae, Taeniidae, and Hymenolepididae, with close relationships to the previously described species of *Raillietina* sp., *R. mahnerti*, *R. hymenolepidoides*, *Ophryocotyloides dasi*, *Rodentolepis nana* and *Hydatigera parva* based on the COI gene regions. This is the first study in Iraq to diagnose Cestoda in birds by molecular methods, where five species of cestoda parasitic on *F. francolinus* were recorded for the first time in Iraq.

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Introduction

Birds are hosts to a variety of parasitic worms that have complex life cycles, and most of the time, they act as the definitive host (1). Cestoda is the name given to a class of parasitic flatworms of the phylum Platyhelminthes (2). More than 1400 species of tapeworms have been described in domestic and wild birds; many are harmless or have mild pathogenicity (3,4). Parasitic worms disrupt the digestion and absorption of foods (5). Tapeworms infect more than 1700 of the approximately 4000 nominal species of birds (6). Adult cestodes may cause damage to the gizzard lining (Gastrotaenia), intestinal blockage, localized damage to the intestinal wall at the site of attachment, or irritation of the intestinal lining. Inflammation is the most common host reaction to cestode infection, and it appears to be most severe when the host and parasite are in contact for an extended period (7,8). Tapeworms may lead to the death of their hosts, especially when the infection is severe (9). The Black Francolin, *Francolinus francolinus* (Linnaeus 1766), known as the black partridge, is a medium-sized game bird that belongs to the Phasianidae family of the order Galliformes and has a global distribution. All of this family's birds are wild and are called the same name as the male and female as well as they rely on the grains as food (Graniivorous) (10-13). They may be found worldwide, from the high

Himalayan peaks to the deep rain forests of Southeast Asia. There are 41 species in the genus *Francolinus*, five of which are limited to Asia and the remainder to Africa (14,15). Male Black Francolins are particularly conspicuous for their black underparts, often boldly spotted or streaked with white, prominent white cheeks, and chestnut neck collar. In addition to their outward variations from females, they have a distinctive high, loud voice, especially during the breeding season, while females maintain an inaudible sound (16,17). *F. francolinus* is known as "a farmer's buddy" since it feeds on insects. It commonly eats caterpillars, seeds, shoots, beetles, bugs, aphids, and ants, among other things (18,19). The population of this *francolin* in Asia is on the decrease. Illegal hunting, excessive gun uses for hunting, excessive predation, loss of food, overgrazing, habitat damage, drought, and agricultural chemicals have all been suggested as contributors to the decline (20). The morphological examination is generally suitable for identifying some parasites, except for some organisms that are morphologically difficult to distinguish, especially regarding species level (21). Intestinal tapeworms are frequently identified based on morphological features, transmission patterns, or pathological effects on their hosts (22). However, these criteria are often insufficient to establish a specific identity (23). Compared with other diagnostic techniques, molecular methods have higher sensitivity and specificity in diagnosing tapeworms than other methods (24,25). Molecular diagnosis and sequencing are the only reliable tool for identifying tapeworms at the species level (26). Recently, RNA and mitochondrial genes have been widely used to identify and study the evolutionary relationships between species due to their rapid rates of evolution at the species and genus levels (27). The results of studies using COI showed that it has the ability and good efficacy to determine a strain and genetic variation (28).

Black partridge in Iraq does not have many studies to search for tapeworms, except for three studies by Sawada and Mohammad (29), who recorded the species *Raillietina francolini* sp. nov. for the first time in the pheasant in Baghdad. Moreover, Mohammad (30) recorded the infection of black partridge in Baghdad province with two species of tapeworms, *Rallietina tetragona*l and *Cotugnia digonopora*, and Al-Aredhi (31) recorded two species of tapeworms *Raillietina tetragona*l. *Cotugina* sp. is parasitic on partridge in Al-Diwaniyah province. The current study aimed to conduct a molecular diagnosis of the tapeworms in *F. francolinus* based on the nested polymerase chain reaction technique in the province of Babylon.

Materials and methods

Ethical approve

The approval was given to conduct this scientific work by the University of Basra/ College of Education for Pure Sciences in their book No. 3/7/3061 on 14/12/2020.

Collection of bird specimens

A total of 35 birds of *F. francolinus* (18 males and 17 females) (Figure 1) were hunted from different locations in the province of Babylon during the period from January to October 2019 and identified by Allouse (32).

Collection of tapeworms

Dissected the birds from the chest till the end of their abdomen, then isolated their digestive tract and divided them into parts. The isolated tapeworms were placed in normal cold saline until their parts were relaxed and then fixed with a solution of alcohol, formalin, and acetic acid (AFA) 10 ml of formaldehyde, 50 ml of alcohol (95%), 5 ml of glacial acetic acid, and 45 ml of distilled water, heated to 60-63°C. Tapeworms were washed with ethanol 70%, then kept in single plastic containers with 70% ethanol, adding a few drops of glycerin (33).

Molecular analysis

Using an AddPrep tissue kit (Addbio, Korea), genomic DNA was extracted from ethanol-preserved worm's tissues according to the manufacturer's instructions. For the polymerase chain reaction, 20 ng of genomic DNA was employed (PCR). Primers for the cytochrome c oxidase 1 (COI) gene region (Table 1) were developed for PCR amplification (34). Cycling conditions (Initial denaturation 95ºC, 5 min (One cycle), denaturation 95ºC, 30 sec, annealing 60ºC, 35 sec, extension 72ºC 35 sec (39 cycle), final extension 72ºC, 5 min (One cycle). On a 1.5 percent agarose gel, the PCR products were validated. A BLAST search was performed to find relevant sequences in the NCBI database. Sequences were aligned directly with cestoda parasite sequences obtained from GenBank using SnapGene software.

Table 1: The single nucleotide sequence of the nitrogenous bases of the primers and the size of the output Nested-PCR reaction

Primer name	The first round of PCR sequencing 5-------3	Amplicon size
PBI-Cox1F-PCR	CATTTTGCTGCCGGTCARCAYATGTTYTGRTTTTTTGG	562 bp
cox1R-PCR	CCTTTGTCGATACTGCCAAARTAATGCATDGGRAA	
PBI-cox1F-seq	CATTTTGCTGCCGGTCA	
PBI-cox1R-seq	TAATGCATDGGRAAAAAAC	

Results

Nested-PCR

Seventeen out of 35 birds of both sexes from *F. francolinus* were found naturally infected with cestoda parasites (genus: *Ophryocotyloides*, *Rodentolepis*, *Hydatigera*, *Raillietina*), resulting in a prevalence of 48.57%. The incidence rate in females was higher than in males at 58.82% (10 of 17) and 41.17% (7 of 18), respectively. The Nested-PCR technique for detecting the COI gene shows a 562bp molecular weight (Figure 1).

Sequences result

From matching the sequences of the tapeworms recorded in the current study with the sequences of tapeworms registered in the NCBI Gene bank for the same cytochrome c oxidase 1 (COI) gene, it was a high degree of similarity of 93.44-100% between the tapeworms samples isolated in the current study (*Ophryocotyloides dasi*, *Rodentolepis nana*, *Hydatigera parva*, *R. mahnerti*, *R. hymenolepidoides*, *Raillietina* sp.) with the species registered in the National Center for Biotechnology Information (NCBI) (Table 2).

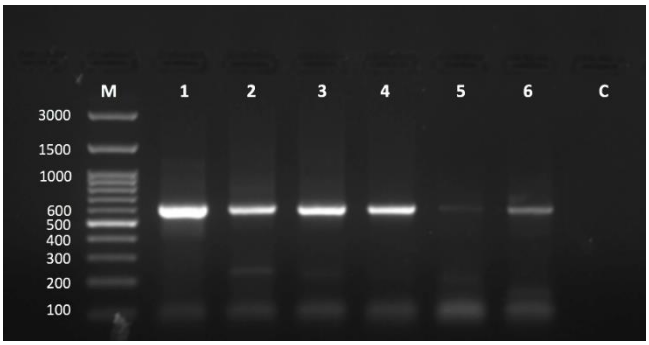

Figure 1: Gel electrophoresis image (agarose= 1.5%) shows the positive amplicons of the detected cestodes (1-6); C controls negative in which similar reaction components of PCR were used except H2O was added instead of DNA, M is a molecular marker from Genedirex.

Phylogenetic tree

After reading the sequences of the cytochrome c oxidase 1 (COI) gene of the tapeworms isolated in the current study, a phylogenetic tree analysis was drawn using MegaX software (Figure 2). This shows the evolution history using the neighbor-joining method. When comparing the diagnosed species, a percentage of affinity of 66-87 was found for the original *Rodentolepis nana*. In the two species, *Ophryocotyloides dasi* and *Raillietina* sp., the percentage of affinity is 66%, while the difference is 34% from the original. For the species *R. hymenolepidoides*, the percentage of affinity is 81%, while the difference is 19% from the original. For the species *R. mahnerti*, the percentage of affinity is 82%, while the difference is 18% from the original. For the species *Hydatigera parva*, the percentage of affinity is 87%, while the difference is 13% from the original.

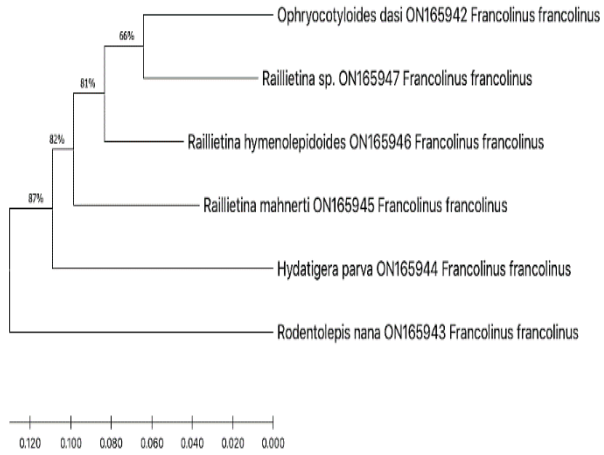

Figure 2: Phylogenetic tree analysis of the identified cestode sequences followed by the obtained accession numbers and the identified hosts. This shows the evolution history using the neighbor-joining method carried out by MegaX software.

Table 2: Comparison of the identified cestodes in *F. francolinus* with global deposited sequences at NCBI

Accession number	Similar to	Percentage identity	Relevant global accession number	Country
ON165942	*Ophryocotyloides dasi*	100	MN590292	UK
ON165943	*Rodentolepis nana*	98.19	MT093855	Egypt
ON165944	*Hydatigera parva*	100	MH036506	UK
ON165945	*R. mahnerti*	94.30	MN590291	UK
ON165946	*R. hymenolepidoides*	94.30	MN590289	UK
ON165947	*Raillietina* sp.	93.44	LC535261	Japan

Discussion

The current study's infection rate of tapeworms is lower than that of Mohammad (30) in Baghdad Province and Al-Aredhi (31) in Al-Diwaniyah Province, which are 61.9% and 76.13%, respectively. Also, the infection rate was lower than the percentage recorded in Pakistan by Naz *et al.* (35), which was 85%. In this study, *Raillietina sp.* was found to be

parasitic on *F. francolinus* in Iraq, and Mohammad and Al-Moussawi (36) had earlier discovered it in *P. domesticus* in Baghdad. In comparison, Abbas (37) found that *S. senegalensis* in Babylon province was infected with tapeworms *Raillietina* spp. *Raillietina hymenolepidoides* was first isolated from *F. francolinus* in Iraq, and Mariaux and Georgiev (38) were the first to record this type of worms, *R. hymenolepidoides* sp. nov., in the Malaysian on dove *Chalcophaps indica*. In this study, *Ophryocotyloides dasi* was isolated for the first time from *F. francolinus* in Iraq, and Mariaux and Georgiev (38) isolated it from *Psilopogon henricii* in Malaysia. The species *Rodentolepis nana* (syn. *Hymenolepis nana*) was isolated for the first time in Iraq from *F. francolinus*, and in Bulgariait was isolated by Iliev *et al*. (39) from the black rat, *Rattus rattus*, while Zonta *et al*. (40) managed to record this type of worms in *Leopardus geoffroyi* in Argentina, and isolated by Grano-Maldonado (41) from *Aspiculuris tetraptera* and *Syphacia obvelata* in Mexico. For the first time, *Raillietina mahnerti* was isolated from *F. francolinus* in Iraq. Mariaux and Georgiev (38) described it as a new species, *R. mahnerti* sp. nov., infecting the dove *Chalcophaps indica* in Malaysia. The species *Hydatigera parva* was isolated for the first time in Iraq from *F. francolinus*, and in Senegal was isolated by Catalano *et al*. (42) from *Mastomys huberti*, while Julius *et al*. (43) managed to record this type of worms in *Mastomys coucha* in South Africa.

Conclusions

From the present work results, it can be concluded that *F. francolinus* is infected with six types of tapeworms, five of which were recorded for the first time in Iraq as parasitic on *F. francolinus*.

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Conflict of interest

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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الكشف الجزيئي عن الديدان الشريطية في الدراج الأسود في محافظة بابل، العراق

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الخلاصة

الديدان الشريطية (صنف الشريطيات، شعبة الديدان المسطحة) هي من الطفيليات الشائعة جدا في الطيور، حيث تسبب أضرارا مختلفة، والتي قد تؤدي إلى هلاك مضيفيها، خاصة عندما تكون الإصابة شديدة. ينتمي الدراج الأسود إلى عائلة التدرجيات، في رتبة الدجاجيات، والطيور الدجاجية تسمى الدراج الأسود. نظرا لكونه طائرا محليا في آسيا، فقد تمت الإشارة إليه باسم الحجل الأسود. أجريت الدراسة الحالية لتحديد الإصابة بالديدان الشريطية في الدراج الأسود. تم فحص ٣٥ طير من الدراج الأسود للبحث عن الديدان الشريطية، منها ١٧ طير (٤٨,٥٧٪) مصابة بطفيليات الديدان الشريطية. تم إجراء التشخيص الجزيئي بناء على تسلسل مناطق جين السيتوكروم سي أوكسيديز الأول لتأكيد تشخيص هذه الديدان. كشفت الدراسة عن هوية قريبة تصل إلى ٩٣,٤٤ - ١٠٠٪ لمناطق جين السيتوكروم سي أوكسيديز الأول مع أنواع الديدان الشريطية الأخرى التي تم الحصول عليها من بنك الجينات. دعم التحليل الوراثي وضع هذه الأنواع ضمن ثلاث عائلات مع علاقات وثيقة مع الأنواع الموصوفة سابقا وبناء على مناطق جينات السيتوكروم سي أوكسيديز الأول. هذه الدراسة هي الأولى في العراق لتشخيص الديدان الشريطية في الطيور بالتقنية الجزيئية، حيث تم تسجيل خمسة أنواع من طفيليات الديدان الشريطية متطفلة على الدراج الأسود لأول مرة في العراق.