

Sequencing Analysis of Its2 Gene of *Davaineidae* of Feral Pigeons

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ABSTRACT

The aims of this study to validate a specific primer to confirmatory identification of three *Raillietina* species in naturally infected feral pigeons (*Columba livia*). Initially intestinal examinations were conducted on 55 birds, revealing the presence of *Davaineidae* through morphological identification. The primers previously designed targeting ITS2 region. The results showed that 35 (63.6%) of the examined birds were infected with cestoda. Molecularly, DNA was extracted from 60 cestode samples. The primers successfully amplified specific species without cross-amplifying of other parasites or host DNA. The polymerase chain reaction (PCR) product were approximately 473, 352, and 397 bp, respectively. Subsequent Sanger sequencing revealed the molecular identification of *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*. The primers usefully can be used for detecting cysticercoids for all parasite stages in pigeons and other poultry for epidemiological surveys, as well as for the management and control of infection.

Key words:.. Iraq ,PCR *Raillietina* spp, specific primers.



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INTRODUCTION

The internal transcribed spacer 2 (ITS2) region, is an important molecular marker widely utilized in evolutionary studies to assess sequence variability at the species level. (Moorhouse-Gann et al., 2018 ; Al-saadi et al., 2016). In Iraq, pigeons are considered as a reservoir for many parasites including helminths. Birds can become infected and transmit infections by vectorized parasites through their ability to travel long distances (Alanad and Farhood, 2023; AL-lahaibi et al., 2021; Issa et al., 2021; Fadhel and Azawi, 2020). Also, the food nature of pigeons (seed, slugs, earthworm and insects) helps parasites to obtain infection. In *Davaineidae* family, three species of *Raillietina*, including *R. echinobothrida*, *R. tetragona*, and *Raillietina cesticillus* are globally distributed in poultry and have been documented in both chickens and ducks (Al-Moussawi et al., 2018; Mohammad and Al-Moussawi, 2013). *Raillietina* spp. are found in the small intestinal tract of the definitive host (Canids) and causes severe illness (impaired growth,

emaciation, weakness, and lowered production). The clinical manifestations and signs such as the epithelial cell inflammation may lead to digestive tract obstruction (Begum et al., 2019). While, cysticercoids (larval stage) reside in various intermediate invertebrate hosts, such as ants, beetles, small mini-wasps, or termites (Siddiqui et al 2023). DNA sequencing analysis in combination with morphological characterization are the most effective and recommended methods for the identification and speciation of parasitic helminths (Bogach, 2021). However, there are some variations of morphology between related *Raillietina* spp. It is difficult to discriminate between these parasites based on morphology only. Numerous DNA bases technologies are utilized for the classification, identification, and taxonomy of parasitic organisms (Nematoda, Cestoda, and Protozoa) with similar morphological characteristics. Polymerase chain reaction (Conventional PCR) is commonly used to amplify conserved rDNA fragments, such as the ITS1-ITS2

region, due to their high variation rates (Alhaboubi et al., 2021; Butboonchoo, 2016).

MATERIALS AND METHODS

The host and parasite collection: Fifty-five feral pigeons (*Columba livia*, Gmein, 1789), were obtained from the birds market from different regions in the south part of Baghdad, Iraq, during 2023. The pigeon's body weight averaged ~220 gm. The 50 birds were euthanized under the animal ethics rule and regulation of the scientific committee in the college of veterinary medicine/Baghdad, with no. P.G: 219 in 30/01/2025. The birds' digestive tracts were examined with the separation of intestine from the esophagus and vent. The intestine was then opened longitudinally to search for *Raillietina* spp. and other closely related species. Live tapeworms and their segments were collected with normal saline and transmitted to the parasitology laboratory, Department of Parasitology, College of Veterinary Medicine, University of Baghdad. The parasites undergo washing process several times with phosphate buffer saline (PBS) and preserved in 70% ethanol for further study.

Molecular study DNA extraction: A total of 60 tapeworm samples that collected were previously preserved in 70% ethanol and after primarily identification as cestodes worm, were subjected to genomic DNA extraction using EasyPure® Genomic DNA tissue Kit, manufacturing by Trans-Gen Biotech, China. The obtained genomic DNA was measured by

Nano-Drop. (Thermo-Scientific; California, USA) for determining concentration and purity.

DNA Amplification: A conventional PCR was applied for DNA amplification targeting (ITS2) region of *Raillietina*. Firstly, all extracted DNA (60 samples) underwent the 1st amplification reaction using a specific set of primers for *R. echinobothrida*. three species of the local *Raillietina* genus through applying a specific primer of the ITS2 region. Table 1. The 2nd reaction included the negative samples from the 1st reaction using a set of primers specific for *R. tetragona*. Finally, the negative DNA samples from the previous reactions were subjected to the 3rd amplification using a primer set for *R. cesticills* (Panich and Chontanarith 2021). The PCR reaction components included the use of GoTaq® Green Master Mix 2x (Promega/USA) and the other components. A typical PCR reaction is prepared (25µl) as total volume. This includes (12.5µl) of Master Mix, (1.5µl) of DNA template, 1 µl each of ITS2 forward Primer (at 10 pmol/µL) and ITS2 Reverse Primer (at 10 pmol/µl), and finally, 9 µl of PCR water to bring the reaction to the desired final volume. The PCR protocol starts by an initial denaturation phase at (94°C) for 3 minutes, for 1 cycle. Followed by 35 cycles of three steps: denaturation at (94 °C) for 45 seconds, annealing at (58°C) for 30 seconds, and final extension (72°C) for one minute.

Table 1. Three sets of primers used for *Raillietina* spp

Species	primer's sequence	Amplicon size
<i>R. echinobothrida</i>	EchiF: 5'-TTCTTCACCCTCTTAGCCCTTCTA-3'	~ 473(bp)
	EchinR: 5'-TCCCACCAAACCTCCTGCTG-3'	
<i>R. tetragona</i>	TetrF: 5'-CGT TGT GTG TTG TGT GTG TA-3'	~352(bp)
	TetrR: 5'-ACCCACAGT ACC CAC AG-3'	
<i>R. cesticills</i>	CesF: 5'-GGATTAAGTGTCTATGGCTGG TT-3	~397(bp)
	'CesR: 5'-CTC ACG CAC ACA CAT CG-3'	

The protocol concludes with a final extension at (72°C) 1 cycle. Gel-electrophoresis run with (1.5%) agarose concentration for, yielding a fragment approximately length with a reaction profile (70 volts and 65 amps for 1 hour). The results were visualized under UV light. (Thermo Fisher Scientific, Global biotechnology, USA).

Sequencing analysis: Positive PCR products were sent for performing Sanger sequencing to

parasites speciation confirmation. The obtained DNA sequence analysis was performed by the Basic Local Alignment Search Tool (BLAST). The phylogenetic tree was constructed by the software program MEGA. 11. (Tamura et al., 2021).

Statistical analysis: SAS analysis was used to affect differences in the research. Chi-square test was used to significantly compare throw percentages.

RESULTS AND DISCUSSION

Sixty adult tapeworms were acquired from the 35 infected birds with (63.6%) infection rate which all subjected to DNA extraction and conventional PCR. Three different species groups were targeted using three amplification reactions. The PCR analysis revealed the

following prevalence rates: *R. echinobothrida* 8.33% (5/60), with a DNA fragment of approximately ~473 bp; *R. tetragona* 10.90% (8/55) with a length of approximately ~352 bp; Finally, *R. cesticillus* 4.25% (2/47) of DNA fragment of ~397 bp. **Table 2.**

Table 2. The PCR results of three amplification reactions for different species

No. of sample	<i>R. echinobothrida</i>	<i>R. tetragona</i>	<i>R. cesticillus</i>
60 adults	(5/60) 8.33%	(8/55)10.90%	(2/47) 4.25%
			P-Value: 0.368

Gelelectrophoresis profiles for these are presented in Figures 1, 2, and 3. The chi-square test indicates no statistically differences in the prevalence rates ($p \geq 0.05$). The sequences obtained from all three PCR

products were registered within the GenBank database. The accession numbers assigned were *R.echinobothrida* (OQ947167.1); *R.tetragona* (OQ947168.1); and *R.esticillus* (OQ947169.1).

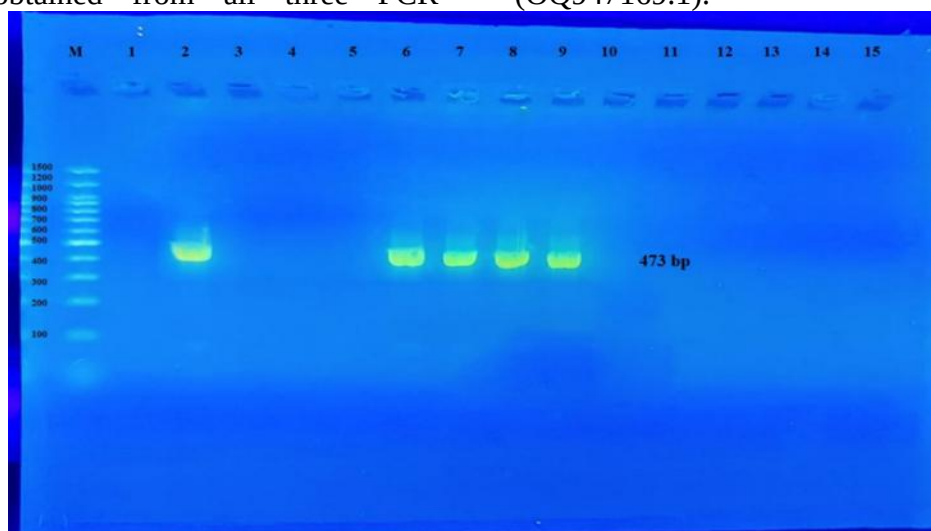


Figure 1. Gel electrophoresis (1.5%) was performed for *R. echinobothrida* esticillus, yielding a fragment approximately ~ 473 bp in length. M: DNA marker 100 bp. The reaction at 70 volts for 1 hour

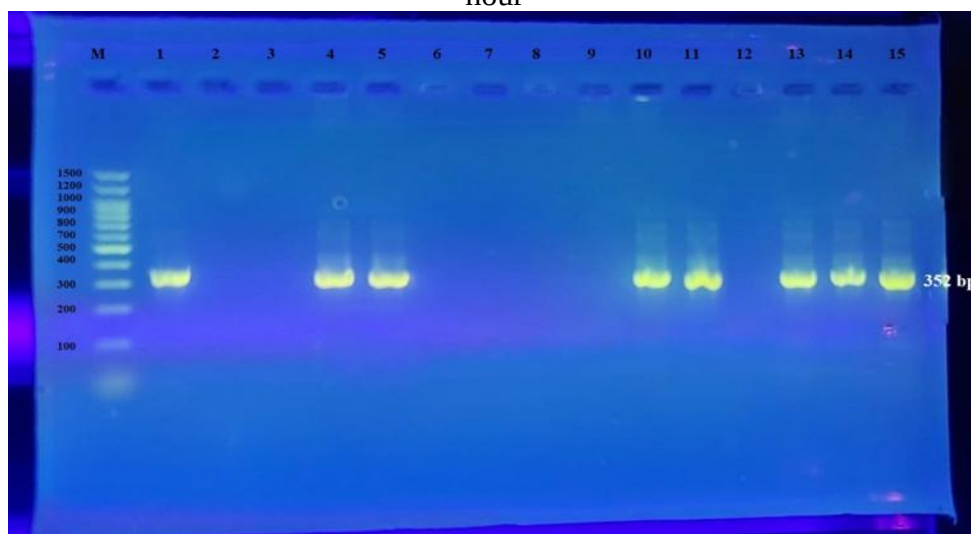


Figure 2. Gel electrophoresis (1.5%) was performed for *R. tetragona*, yielding a fragment approximately ~352 bp in length. M: DNA marker 100 bp. The reaction at 70 volts for 1 hour

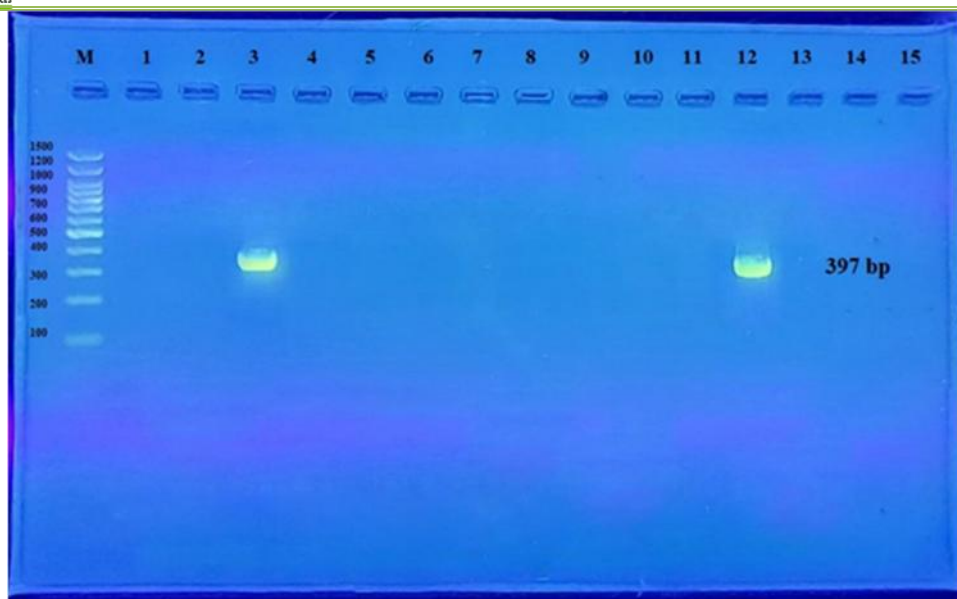


Figure 3. Gelelectrophoresis with (1.5%) was performed for *R. cesticillus*, yielding a fragment approximately 397 bp in length. M: DNA marker 100 bp. The reaction at 70 volts for 1 hour

Fadhel and Azawi, 2020, in earlier Iraqi study successfully amplified the 18S rDNA gene of *Rallietina* for the molecular conformation without sequence analysis, depending only on the expected band size seen on the gel electrophoresis profile.

Phylogenetic tree: In this study, we unfortunately only obtained partial ITS region sequences for three isolates: *Rallietina echinobothrida*, *Raillietina tetragona*, and *Raillietina cesticillus*. The remaining sequences were unsuitable for inclusion in the analysis, likely due to PCR artifacts or

sequencing defects. The phylogenetic analysis, by using the unweighted pair group method with arithmetic mean method (UPGMA) produced three phylogenetic trees exhibiting highly similar topologies. Figures 4,5,6. The homology with other GenBank isolates showed varying levels of identity, ranging from low homology (70%) with an isolate from India to high (99%) with an isolate from Thailand. The final dataset for the inquiry species comprised 194, 298, and 319 positions, respectively, as detailed in Table 3.

Table 3. The ITS2 region sequences data found in NCBI/GenBank correspondent to *Raillietina* spp

No	Access. no	Country	Source	Identity
1.	ID: MN902341.1	Thailand	<i>Raillietina echinobothrida</i>	99%
2.	ID: MN902343.1	Thailand	<i>Raillietina echinobothrida</i>	99%
3.	ID: MF426021.1	Egypt	<i>Raillietina echinobothrida</i>	71%
4.	ID: JN797628.1	India	<i>Raillietina echinobothrida</i>	70%
5.	ID: MN121084.1	India	<i>Raillietina echinobothrida</i>	70%
6.	ID: MN902346.1	Thailand	<i>Raillietina tetragona</i>	99%
7.	ID: MN902345.1	Thailand	<i>Raillietina tetragona</i>	99%
8.	ID: MN902344.1	Thailand	<i>Raillietina tetragona</i>	99%
9.	ID: MH421996.1	China	<i>Raillietina tetragona</i>	99%
10.	ID: MH421995.1	China	<i>Raillietina tetragona</i>	99%
11.	ID: LC706474.1	Bangladesh	<i>Raillietina tetragona</i>	99%
12.	ID: MW291975.1	China	<i>Raillietina tetragona</i>	99%
13.	ID: OM101136.1	India	<i>Raillietina tetragona</i>	99%
14.	ID: KP893422.1	Germany	<i>Raillietina cesticillus</i>	99%
15.	ID: MW291981.1	China	<i>Raillietina cesticillus</i>	99%
16.	ID: MW291960.1	China	<i>Raillietina cesticillus</i>	98%
17.	ID: MW291982.1	China	<i>Raillietina cesticillus</i>	98%
18.	ID: MN902347.1	Thailand	<i>Raillietina cesticillus</i>	93%
19.	ID: MZ427929.1	Thailand	<i>Raillietina cesticillus</i>	86%
20.	ID: OL825001.1	India	<i>Raillietina cesticillus</i>	86%
21.	ID: OM112301.1	India	<i>Raillietina cesticillus</i>	85%

The evolutionary history using the siting of the UPGMA (unweighted pair group method with arithmetic mean method). The phylogenetic tree drawn to scale including the branch length =559.62126850 in units, presenting the evolutionary distances. Six nucleotide sequences were involved in the analysis. The three-codon position were included plus the non-coding without gaps setting result in total

of 194 location in the sequence. *Rallietina echinobothrida* phylogenetic tree in Figure 4, shows two main clades, and the first one contained the study isolate (Iraq) with isolates from Thailand. The second clade includes isolates from Egypt and India. The evolutionary analyses show a different range of similarity with Thailand, Egypt and India. Table 4.

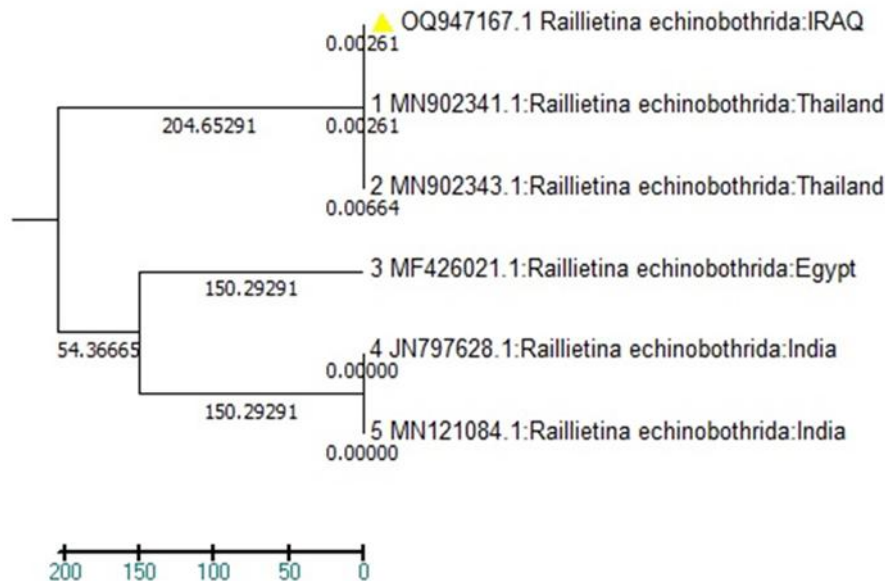


Figure 4. Phylogenies based on the partial sequences of the ITS2 region for one *Rallietina echinobothrida*. Accession numbers with yellow triangle based on ~473 bp with the GenBank corresponding available sequences

Table 4. Evolutionary relationships of taxa

	1	2	3	4	5
1. OQ947167.1 <i>Rallietina echinobothrida</i> :IRAQ					
2. 1 MN902341.1: <i>Rallietina echinobothrida</i> :Thailand	0.005				
3. 2 MN902343.1: <i>Rallietina echinobothrida</i> :Thailand	0.016	0.011			
4. 3 MF426021.1: <i>Rallietina echinobothrida</i> :Egypt	424.243	424.243	424.243		
5. 4 JN797628.1: <i>Rallietina echinobothrida</i> :India	401.857	401.857	401.857	300.586	
6. 5 MN121084.1: <i>Rallietina echinobothrida</i> :India	401.857	401.857	401.857	300.586	0.000

The evolutionary history using the setting of the UPGMA. The phylogenetic tree drawn to scale including the branch length = 0.01778525 in units, presenting the evolutionary distances which computed using the setting of Maximum composite likelihood method. Nine of the nucleotide sequences were included in the analysis. A result of total

of 298 positions. The *Rallietina tetragona* phylogenetic tree in Figure 5, shows one main clade branched 98.66% with the Iraqi isolate, and the clade contained isolates from China, Thailand and Bangladesh. The evolutionary analyses show the range of similarity with Thailand, China, Bangladesh and India. Table 5.

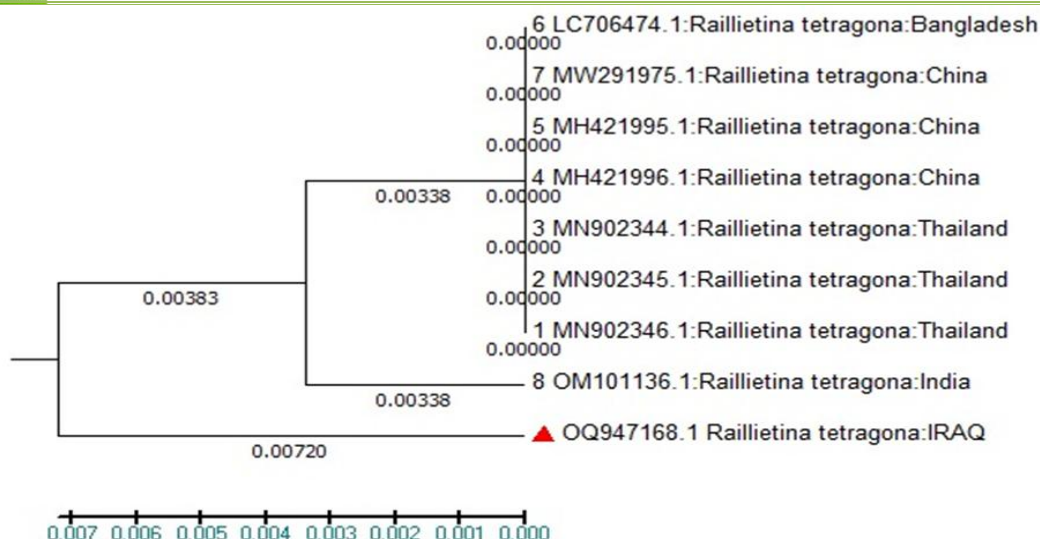


Figure 5. Phylogenies based on the partial sequences of the ITS2 region for one *Raillietina tetragona* . Accession numbers with red triangle based on ~352bp with the GenBank available corresponding sequences

Table 5. *Raillietina tetragona* evolutionary relationships of taxa

	1	2	3	4	5	6	7	8
1. OQ947168.1 Raillietina tetragona:IRAQ								
2. 1 MN902346.1:Raillietina tetragona:Thailand	0.014							
3. 2 MN902345.1:Raillietina tetragona:Thailand	0.014	0.000						
4. 3 MN902344.1:Raillietina tetragona:Thailand	0.014	0.000	0.000					
5. 4 MH421996.1:Raillietina tetragona:China	0.014	0.000	0.000	0.000				
6. 5 MH421995.1:Raillietina tetragona:China	0.014	0.000	0.000	0.000	0.000			
7. 6 LC706474.1:Raillietina tetragona:Bangladesh	0.014	0.000	0.000	0.000	0.000	0.000		
8. 7 MW291975.1:Raillietina tetragona:China	0.014	0.000	0.000	0.000	0.000	0.000	0.000	
9. 8 OM101136.1:Raillietina tetragona:India	0.020	0.007	0.007	0.007	0.007	0.007	0.007	0.007

The phylogenetic tree of *R. cesticillus* in Figure 6 consists of three main clades. The first clade includes two isolates from China and one from India. The second clade comprises the Iraqi isolate, grouped with an

isolate from Germany, showing 99.7% similarity. The third clade contains isolates from China and Thailand. Evolutionary analyses reveal a high similarity among isolates from Germany, China, India. Table 6.

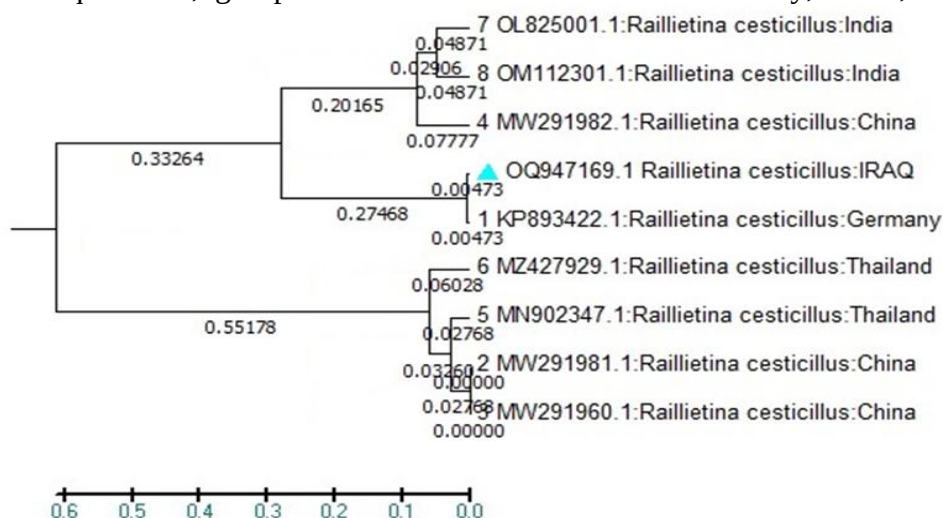


Figure 1. Phylogenies based on the partial sequences of the ITS2 region for one *Raillietina cesticillus*. Accession numbers with blue triangle based on ~ 397bp with the GenBank available corresponding sequences

Table 6. *Raillietina cesticillus* evolutionary relationships of taxa

	1	2	3	4	5	6	7	8
1. OQ947169.1 <i>Raillietina cesticillus</i> :IRAQ								
2. 1 KP893422.1: <i>Raillietina cesticillus</i> :Germany	0.009							
3. 2 MW291981.1: <i>Raillietina cesticillus</i> :China	1.318	1.320						
4. 3 MW291960.1: <i>Raillietina cesticillus</i> :China	1.318	1.320	0.000					
5. 4 MW291982.1: <i>Raillietina cesticillus</i> :China	0.376	0.366	1.521	1.521				
6. 5 MN902347.1: <i>Raillietina cesticillus</i> :Thailand	1.102	1.104	0.055	0.055	1.415			
7. 6 MZ427929.1: <i>Raillietina cesticillus</i> :Thailand	0.843	0.845	0.127	0.127	1.218	0.108		
8. 7 OL825001.1: <i>Raillietina cesticillus</i> :India	0.653	0.638	1.190	1.190	0.150	1.158	1.027	
9. 8 OM112301.1: <i>Raillietina cesticillus</i> :India	0.660	0.661	1.336	1.336	0.161	1.275	1.127	0.097

Panich and Chontanarath, (2021) has successfully developed species -specific primers for the identification of the *Raillietina echinobothrida*, *R. tetragona*, and *R. cesticillus* in the poultry. These primers offer a high-performance diagnostic tool, with the potential to detect the gene in parasite's various stages, including adult forms, eggs in the environment, and cysticercoids in intermediate hosts. In Iraq, the molecular investigation of veterinary studies shortening time required for diagnosis, improving accuracy over morphological methods (Abid and Karawan, 2026; Saleh et al., 2025). While studies have investigated endoparasite including *Raillietina* spp., in both domestic and wild birds, these investigations have primarily relied on morphological characteristics and prevalence assessment (Chaudhury, 2022; Chontanarath, 2022; Butboonchoo et al., 2016). However, depending upon morphology resulting in an inaccurate identification due to shared close morphological characterization of cestode like SEM (Al-Sudani and Al-Amery, 2024). Resent molecular tool, has been used as an alternative or confirmative to the traditional diagnostic methods, and has started to be more reliable, inexpensive, accurate, and sensitive than those of morphology-based methods (Ashfiyah et al., 2022; Al quraishy, et al., 2019). Cestoda in general identified by several molecular procedures, including the RT-PCR. (Chen and Li., 2014), random amplified polymorphic DNA polymerase chain reaction (RAPD-PCR), phylogenetic analysis and different annealing temperatures and nonamplified polymorphic. (Elsayed et al., 2025; Safi-Eldin et al., 2019). A new analytical method called duplex loop-mediated isothermal amplification (d LAMP) complemented with a lateral flow

dipstick (LFD) was used. This method showed a low analytical sensitivity of up to 5ng/μL for real time tracing. In another study (Lamprou et al., 2025; Panich et al., 2024), this method was noted that the simultaneous detection of *Raillietina* spp. and *Ascaridia galli* parasites in chicken feces.

CONCLUSION

Sanger sequencing confirmed the molecular identification of *R. echinobothrida*, *R. tetragona*, and *R. cesticillus*. The primers developed in the current can be effectively used for the detection of cysticercoids and other developmental stages of these parasites in pigeons and other poultry. This molecular approach may be useful for epidemiological surveys and for improving the management and control of these infections.

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CONFLICT OF INTEREST

The authors declared on conflict

AUTHOR/S DECLARATION

We confirm that all Figures and Tables in the manuscript are original to us. Additionally, any Figures and images that do not belong to us have been incorporated with the required permissions for re-publication, which are included with the manuscript.

AUTHOR'S CONTRIBUTION STATEMENT

M.T., A.A., and A.R. sharing equally the conception and design of the study. A.A. was responsible for sample collection and laboratory investigations, while A.R. was responsible for data collection, statistical analysis, and interpretation of the results.

M.T., A.A., and A.R. contribute to manuscript writing and revision.

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تحليل التتابع لجين الـ ITS لطفيليات الدايفينيدي في الحمام البري
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المستخلص

هدفت هذه الدراسة إلى التحقق من فعالية استخدام بادئات محددة لأجل تضاعف الحمض النووي لثلاثة من *Raillietina* spp في الحمام الوحشي (كولومبا ليفيا) المصاب طبيعياً من خلال تقنية تفاعل البلمرة المتسلسل اذ اجري الفحص المعوي الأولي لـ 55 طائراً للكشف عن ديدان Davaineidae عن طريق الصفات بعض الشكلية للطفيلي. تم تصميم البادئات مسبقاً بناءً على منطقة الفاصل الداخلي المنسوخة الثانية (ITS2). وجدت النتائج أن 35 (63.6%) من الطيور التي تم فحصها مصابة مبدئياً. جزيئياً، تم استخلاص الحمض النووي لـ 60 من الديدان ولقد عملت البادئات بنجاح على تضخيم أنواع معينة دون تضخيم الطفيليات أخرى أو المضيف. كانت نتاج تفاعل البلمرة المتسلسل حوالي 473 و 352 و 397 نقطة أساس، تليها عملية تسلسل سانجر الذي يكشف عن التحديد الجزيئي لـ *R. echinobothrida* و *R. tetragona* و *R. cesticillus*، على التوالي. ان البادئات المحددة مفيدة بنجاح للكشف عن الكيسات المذنبة في العوائل الوسيطة أو المراحل البالغة في الحمام والدواجن الأخرى من أجل المسوحات الوبائية وإدارة العدوى ومكافحتها.

الكلمات المفتاحية: لعراق، تفاعل البلمرة المتسلسل، الديدان الخيطية، بادئات خاصة