Morphological and Phylogenetic Analysis of *Raillietina* sp. Infecting Domestic Pigeons (*Columba livia domestica)* in Al-Qassim Region, Saudi Arabia

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ABSTRACT

Common tapeworms in poultry digestive tracts are called *Raillietina* species. The present study's objective is to characterize the morphological and genetic relationships between the cestode parasites that infect domestic pigeons (*Columba livia domestica*) in Saudi Arabia's Al-Qassim Region. Using optical and scanning electron microscopes, the general morphology and surface characteristics of the collected cestode parasites were examined. Using sequences from the ND1 and 18S rDNA gene areas, molecular analysis was used to confirm the taxonomy of this parasite based on its morphological characteristics. This demonstrated a high degree of similarity—up to 98.0% and 74.7%, respectively between the 18S rDNA and ND1 gene regions and other cestode species found in GenBank. Using close ties, phylogenetic research validated the placement of this *Raillietina* species within Davaineidae.

INTRODUCTION

One of the birds that coexist with people is the domestic pigeon (Sari et al., 2008). They live alongside humans for food, amusement, and research purposes (Sari *et al*., 2008; Mansur *et al*., 2019). Pigeons may be affected by a variety of health conditions, but parasitic infections are particularly important because they are a major source of disease transmission (Marques *et al*., 2007). One of the most significant diseases affecting birds is endo-parasitism (Dranzoa, Ocaido, and Katete, 1999). Endoparasites include helminths and protozoans (Ghazi *et al*., 2002). Helminth parasites are seen to be the biggest obstacle to profitable pigeon production (Galloway,1972) and have been linked to pigeon sickness and death (Soulsby, 1982). The most common worms that infect *Raillietina* species include pigeons (Adang *et al*., 2008). According to Tadelle and Ogle (2001), they are prevalent in the tropics due to favorable climatic conditions and substandard husbandry practices that facilitate the growth of the parasites. *Raillietina sp.*'s physical characteristics exhibit notable differences both within and across species.

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As such, it is challenging to distinguish them solely by their morphology. Instead, the most reliable and precise technique for identifying helminth parasites is the combination of molecular techniques and morphological investigations. For the identification of helminths, molecular methods combined with morphological investigations worked well (Eom *et al.*, 2002). Thus, the goal of our research plan is to use light and scanning electron microscopes in conjunction with molecular testing to describe *Raillietina sp*. infecting the domestic pigeon (*C. livia domestica*) in Al-Qassim region of Saudi Arabia.

MATERIALS AND METHODS

1-Sample Collection:

In Al-Qassaim region of Saudi Arabia, live *Raillietina* species were obtained from the gastrointestinal tracts of domestic pigeons (*C. livia domestica*) who were infected. All bird handling was done in accordance with the protocol outlined by Al-Hussaini and Demian (1982) and the rules set forth by the Institutional Animal Ethics Committee at King Abdulaziz University's Department of Biological Sciences.

2-Preparation of Permanent Slides of Cestodes:

Live tapeworms were submerged in a saline solution and refrigerated at 4°C to reduce motility and promote total rest. To prevent parasite muscles from contracting, relaxation is required. Large parasites were fixed for 12–24 hours in a petri dish containing the fixative solution, whereas small parasites were fixed for 2-4 hours. Cestodes were fixed separately on a designated slide using a drop of formalin buffer (10%). The worms were then stained with acetocarmine after being repeatedly cleaned in distilled water. The thickness and size of the parasites determine how long they take to stain. As gradually as feasible, the specimens were differentiated using under a light microscope, acid alcohol. Slides with increasing alcohol grades 50%, 70%, 80%, and 90% for five minutes each, and 100% alcohol for ten will be passed to dehydrate the participants. To get rid of cestode worms, use clove oil. The mounting media was a drop of Canada balsam. The specimens were placed in a hot oven set at 40°C and covered with a coverslip. Cestodes were categorized using taxonomic keys like Yamaguti (1961) and Soulsby (1982) based on their morphological features.

3-Scanning Electron Microscope:

Live tapeworms were fixed for 24 hours at 25°C by submerging them entirely in 2.5% glutaraldehyde. Following that, the samples were post-fixed in 1% osmium tetroxide in the same buffer after being cleaned with 0.1 M sodium cacodylate buffer (pH 7.2). The process of dehydration will involve advancing slides with increasing alcohol concentrations: 70%, 80%, 90%, and 100%. After that, samples were fixed to an aluminum stub and allowed to dry for a while. Afterwards, samples were examined with an FEI Quanta FEG 450 Scanning Electron Microscope at 20 KV at King Abdulaziz University after being coated by gold sputtering for 60 s using an Auto Fine Coater (JFC-1600).

Molecular Analysis:

Before being processed, little portions from each individual worm were soaked in sterile distilled water five times. Using a DNeasy tissue kit© (Qiagen, Hilden, Germany) and the manufacturer's instructions, genomic DNA (g DNA) was extracted. Thermo Fischer Scientific, Inc., Wilmington, DE, USA, provided the Nanodrop ND-1000 spectrophotometer, which was used to evaluate the concentration and purity of each DNA sample. The 18S rDNA and ND1 genes were amplified by a particular PCR method employing two pairs of primers (Table 1). Using the Gene JETTM PCR Purification Kit [Thermo (Fermentas)], both the 18S rDNA and the ND1 gene areas were amplified in a total volume of 50 μl, comprising 5 μl of 10 × buffer, 5 μl of each

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dNTP (10 mM), and 10 µl of each primer (1 pmol/ µl), / µl), 2.5 µl MgCl2 (50 mM), 0.3 µl Taq polymerase (5 U/ml), and 2 µl total genomic DNA. Thermocycler settings were as follows for the 18S rDNA gene: 5 minutes at 95°C (initial denaturation), 35 cycles of 1 minute at 95°C, annealing for 30 seconds at 58°C & 1 minute at 72°C (extension), & 7 minutes at 72°C (final extension). ND1 gene annealing was done for 1 minute at 48°C and 1 minute at 72°C (extension), followed by 35 cycles of 1 minute at 95°C, 1 minute at 48°C, and 7 minutes at 72°C (final extension). 1.5% agarose gel electrophoresis in 1X TAE buffer (100 mM Tris-HCl, glacial acetic acid, and 20 mM EDTA) was used to resolve reaction products (10µl). Ethidium bromide was used to stain the gel, and a digital camera was used to take pictures. underneath a UV light source. Sanger sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA) on a 310 Automated DNA Sequencer (Applied Biosystems, Foster City, CA). The analysis of the sequences was done with Geneious Prime® 2022.1.1. For every sequence, a BLAST search was run to identify related sequences. The CLUSTAL-W algorithm (Thompson, Higgins, and Gibson, 1994) was used to create multiple sequence alignments, with a gap opening penalty of 10 and a gap extension penalty of 1. The Tamura_Nei model, 10,000 repetitions, and the Neighbor-Joining method (Saitou and Nei, 1987) were used to generate the phylogenetic tree.

**Table 1.** List of primers used for PCR amplification of *Raillietina* sp.

| Gene ID | Direction | Sequencing | References |
|---------|-----------|------------|------------|
| 18S rDNA | 18S rDNA-F | 5′AAGCCATGCATGTCTCAGTTCAG 3′ | Foronda *et al.,* 2005 |
| | 18S rDNA-R | 5′GCCCTCCAATTGATCCTCGTG 3′ | |
| ND1 | JB11 -F | 5′AGATTCGTAAGGGGCCTAATA-3′ | Butbooncho *et al.,* 2016 |
| | JB12 -R | 5′-ACCACTAACTAATTCACTTTC-3 | |
| *18S rDNA; 18S ribosomal DNA, ND1; (nicotinamide adenine dinucleotide hydrogen) dehydrogenase subunit-1 | | | |

## RESULTS

The recovered *Raillietina* species are actively moving and very abundant in the intestine of infected pigeons. The infection rate was comparable in males (35.3%) and female gender (27.4%). Additionally, the season did not have any impact on the infection rate; autumn (30.1%), spring (31%), summer (32.2%), and winter (34.3%). The morphological examination revealed that worms have a body with a traditional tapeworm shape consisting of a series of body segments with a ribbon-like shape. The body is broad at the back, narrowing toward the front with an average length of 12.50 ± 25 cm. The scolex of *Raillietina* sp. is small, oval, and measures about (0.55± 0.40 X 0.45 ± 0.35 mm) in diameter. The rostellum is wide and disc-shaped, nearly rounded or circular, measures about (0.27± 0.22 X 0.21± 0.18 mm) in diameter, and armed with two rows of hooks. Scolex contains four rounded suckers, each one measuring about (0.3 ± 0.1 X 0.2 ± 0.09 mm) in diameter, and armed with many rows of hooks. The neck region is short and unsegmented followed by a segmented body called proglottids, which are arranged to form strobila (Fig. 1 A&B). The mature segment measures about (0.72 ± 0.64 X 0.62 ± 0.57 mm) in diameter, with unilateral and single genital pores arranged at the middle to the posterior region of each segment. The ovary is oval in shape and located in the middle part of each segment, while the testes are small in size, rounded in shape, and 27-37 in number (Fig. 1 C&D). The gravid segment measures about (0.85 ± 0.74 X 0.76 ± 0.68 mm) in diameter and it is filled with egg capsules, each containing about 6-9 eggs (Fig. 1 E&F). The scanning electron microscope (SEM) demonstrated that the scolex is small with four armed suckers,

each sucker with numerous hooks. In addition, the body proglottids of *Raillietina* sp. have a smooth surface with hair-like microvilli called microtriches, all oriented toward the back, giving a silky appearance to the entire body surface (Fig. 2).

**1-PCR Amplification, Sequencing, And Phylogenetic Relationships of *Raillietina* sp. Based on 18S rDNA Gene:**

The gel electrophoresis revealed that the molecular size of the PCR product for samples numbers (1, 2, 3, and 4) of the 18S rDNA gene was 550 bp (Fig. 3), with GC content of 52.5% and a pairwise identity of 99.5%. The BLAST search showed that the four sequences are similar to *Raillietina micracantha* hosted in pigeons (*Columba livia*) from Spain (accession number AJ555178) with 98% pairwise identity and 98% coverage. The multiple sequence alignment of the four 18S rDNA samples in this study with (AJ555178) is shown in (Fig. 5). The four sequences of the 18S rDNA gene from the current study were deposited successfully in GenBank under accession numbers (ON855020, ON85521, ON855022, and ON855023). Using the relevant sequences that are accessible on GenBank, a phylogenetic tree was built using the sequencing data that was acquired for this investigation. The phylogenetic tree of the 18S rDNA samples with other species retrieved from GenBank is shown in (Fig. 7), and *Lyruterina nigropunctata* (accession number AJ555173) was used as an outgroup. The tree showed that the present *Raillietina* species are also related to other species of *Raillietina* within the Davaneidae group such as; *Raillietina* sp. from Nebraska (accession number EU665467), *Raillietina* sp. from Zimbabwe (accession number MH702247), *Raillietina australis* from Australia (accession number AY382311), and *Raillietina chiltoni* from Australia (accession number AY382313).

The *Raillietina* group was monophyletic with moderate nodal support for grouping.

**2-PCR amplification, Sequencing, and Phylogenetic Relationships of *Raillietina* sp. Based on ND1 Gene:**

The gel electrophoresis revealed that the molecular size of the PCR product for samples number (5, 6, 7, and 8) of ND1 gene was 510 bp (Fig. 4), with GC content of 31.4% and pairwise identity of 96.3%. The BLAST search showed the three sequences (numbers 5, 6, and 7) were similar to *Raillietina australis* (accession number EU665484) from Australia with a pairwise identity of 74.7% and coverage of 94%. For sample number 8, there was no significant similarity. The multiple sequence alignment of the three ND1 samples in this study with EU665484 is shown in (Fig. 6). The three sequences of the ND1 gene from the current study were deposited successfully in GenBank under accession numbers (OP029038, OP029039, and OP029040). The sequence of the data obtained in this study was used to construct a phylogenetic tree utilizing the related sequences available on GenBank. The phylogenetic tree of the three ND1 samples with other species retrieved from GenBank is shown in (Fig. 8), and *Dibothriocephalus nihonkaiensis* (accession number HQ 423296) was used as an outgroup. The tree showed that the present *Raillietina* sp. is also related to other species of *Raillietina* within the Davaneidae group such as; *Raillietina tunetensis* and *Raillietina* sp. from Costa Rica (accession number EU665487 and EU665486, respectively), *Raillietina sonini* from Bulgaria (accession number EU665490), and *Raillietina* sp. (accession number EU665487 and EU665486) from Nebraska. The *Raillietina* group was monophyletic with moderate nodal support for grouping.

**Fig. 1.** Photomicrographs of the adult *Raillietina* species. **(A & B)** The scolex showing; rostellum (R) armed with hooks (H), 4 suckers with hooks (S), and a narrow neck (NC). (A) without stain, (B) with acetocarmine stain (Scale bar=100 µm). (C & D) Mature proglottids showing; unilateral genital pore (GP), ovary (OV), and testes (TE) (Acetocarmine) (Scale bar= 100 µm. (E) Gravid proglottid filled with egg capsules (Acetocarmine) (Scale bar=100 µm). (F) Egg capsules with 6-9 eggs (Acetocarmine) (Scale bar=50 µm).

**Fig. 2.** SEM micrographs of the adult *Raillietina* species. (A). The scolex showing rostellum (R) and one of the 4 suckers (SC). (B). One of the 4 suckers showing numerous hooks (H). (C). The body proglottids of. *Raillietina* sp. (D). The body proglottids showing numerous hair-like on the body surface.



**Fig. 3.** Gel electrophoresis of PCR products of 18S rDNA gene for *Raillietina* sp.(n=4) on 1.5 agarose gel. The molecular size of the ladder is 1500 bp (M).

**Fig. 4.** Gel electrophoresis of PCR products of ND1 gene for *Raillietina* sp.(n=4) on 1.5 agarose gel. The molecular size of the ladder is 1500 bp (M).



**Fig. 5.** Multiple sequence alignment of the four sequences of the 18S rDNA gene in this study aligned with *Raillietina micracantha* (AJ555178).

**Fig. 6.** Multiple sequence alignment of the three sequences of the ND1 gene in this study aligned with *Raillietina australis* (EU665484).

**Fig. 7.** Maximum likelihood tree of the four sequences of the 18S rDNA gene in the current study with other species downloaded from GenBank.

**Fig. 8.** Maximum likelihood tree of the three sequences of the ND1 gene in the current study with other species downloaded from GenBank.

## DISCUSSION

The anatomy of cestode parasites in the genus *Raillietina* that infect domestic pigeons has not been well documented in Saudi Arabia (Magzoub *et al.,* 1980; Al Quraishy *et al*., 2019; Ali *et al*., 2020; Al Quraishy *et al*., 2021). Reliability in the morphological traits that form the basis of cestode classification has not been extensively studied. Few researchers have tried to ascertain the frequencies at which these variants occurred in the species exhibiting them, despite the fact that numerous reports have detailed the variation in the morphological characters of cestode species (Yamaguti 1958; Soulsby 1982; Khalil, 1994; Jatoi *et al*., 2013;

Franzese and Ivanov 2018; Al Quraishy *et al*., 2019). Butboonchoo *et al*. (2016), Schmidt (1986), and Yamaguti (1958) came to the conclusion that some morphological criteria were extremely beneficial and verified the detection of cestodes. When the recovered cestode from this study was compared to other tapeworm species from around the world, it was shown to share similarities with other comparable species that inhabit the same host species (*C. L. domestica*) and share generic characteristic traits. In line with similar studies conducted around the world, which discovered that worms of the genus *Raillietina* are the most common internal parasites infecting domestic pigeons (Dede and Richards 1998;

Ghazi *et al.*, 2002; Dehlawi 2006; Adang *et al.*, 2008; Ali *et al.*,2020), the morphological observation in the current study confirmed that all obtained cestode worms belong to the *Raillietina* species. The current cestode also bore a striking resemblance to both *Raillietina canabia*, *Raillietina zahratis* (Magzoub *et al.*, 1980) and *Raillietina saudiae* (Al Quraishy *et al.*, 2019) in that they shared the same climatic circumstances and host place, which is the central region of Saudi Arabia. It is impossible to pinpoint the precise identity of the *Raillietina* sp. studied here. It matched several traits of *Raillietina echinobothrida* and *Raillietina tetragona* that were isolated from Egyptian domestic pigeons (Safi-Eldin *et al.*, 2019). The long body (25 cm) and spherical scolex, short and unsegmented neck, round and disc-shaped rostellum with two rows of hooks, four rounded suckers armed with six to eight rows of hooks, unilateral posterior genital pore, and oval ovary of the present *Raillietina sp.* were similarities to those of the *Raillietina echinobothrida*; however, there were differences in the number of testes (23–26 in *Raillietina echinobothrida* compared to 27–37 in the present *Raillietina sp.*) and the number of eggs per egg capsule (4–7 in *Raillietina echinobothrida* compared to 6–9 in the present *Raillietina sp.* Additionally, the gravied proglottid of the current *Raillietina sp.* resembled that of *Raillietina tetragona*, containing six to nine eggs per capsule. However, it was different, possessing a large, disc-shaped rostellum with double rows of hooks and four rounded suckers instead of *Raillietina tetragona's* rostellum with a single row of hooks and four oval suckers. Additionally, the present study's description of *Raillietina sp.* was similar to that of *R. echinobothrida* as reported by Lalchhandama (2009) and Butboonchoo *et al.* (2016) in that it possessed a unilateral posterior genital pore, an armed rostellum with two rows of hooks, and a rounded armed sucker; however, there were differences in the number of eggs per egg

capsule, While Butboonchoo *et al.* (2016) stated that worms had eight to twelve eggs in each egg capsule, Lalchhandama (2009) reported that *R. echinobothrida* had a single egg in each egg capsule. Additionally, the recovered *Raillietina sp.* was relatively similar to *R. echinobothrida* described by Ibrahim *et al.* (2018), but it differed from *R. echinobothrida* in that it had a smooth surface with hair-like microvilli as they appeared in the SEM, whereas *R. echinobothrida* had longitudinal folds. The number of testes in the present *Raillietina sp.* was 27–37, whereas in *Raillietina echinobothrida*, there were 20–25. Additionally, the current *Raillietina sp.* was not the same as the *Raillietina cesticillus* reported by Butboonchoo *et al.* (2016), which said that the former is distinguished by a broad, armed rostellum with 400–500 tiny hooks, Four unarmed suckers, one egg per capsule, and sporadic, alternating genital pore opening. Frequently, these standards are not enough for precise identification. *Raillietina* species recognition, identification, and evolutionary relationships have been better understood and data gathered through the use of molecular tools for species identification.

For the genetic characterisation and identification of parasites, particularly helminths, molecular markers based on DNA analysis have been employed (Vilas *et al.*, 2005). Under the title "DNA barcoding," Li *et al.* (2011) stated that the DNA sequence provided an effective method for species-level identifications. According to Trivedi *et al.* (2015), DNA barcoding has emerged as one of the most significant scientific trends of the past ten years. In this study, two genetic markers—the ND1 gene and 18S rDNA were utilized to identify particular cestodes. Foronda *et al.* (2004, 2005) and Al Quraishy *et al.* (2021) used the same molecular strategy and reported sequencing the 18S rDNA gene using PCR amplification. and the same molecular method for the detection of cestodes has already been documented by

Butboonchoo *et al*. (2016) and Al Quraishy *et al*. (2019) employing the PCR amplification and sequencing of the ND1 gene. Due to their high evolutionary rates at the species and genus levels, the ribosomal DNA gene is often employed to detect and explore phylogenetic connections among nearby species (Wolf *et al*. 2005; Xiao *et al*. 2005; Ghobashy and Taeleb 2015). The four current *Raillietina* species sequences based on the 18S rDNA gene were shown to be homologous with a maximum identity of 98% to *Raillietina micracantha* hosted in *Columba livia*, from Spain, according to the BLAST hits on the query sequences, while the three sequences based on the ND1 gene recorded a maximum identity of 74.7% to *Raillietina australis* from Australia. Additionally, it was demonstrated by the phylogenetic trees constructed from the ND1 and 18S rDNA gene areas that each *Raillietina* species was divided based on their definitive host and physical characteristics. This outcome agrees with Littlewood *et al*. (2008) and O'Callaghan, Davies, and Andrews (2000). Consequently, the morphological identification of the current cestode samples was validated by genetic analysis. Our findings also revealed that the 18S rDNA and ND1 gene regions trees from the *Raillietina* species seemed to be monophyletic. Khalil *et al*. (1994) investigated the monophyly of *Raillietina* species based on the query sequences of the ITS2 and ND1 gene regions, with bootstrapping of both phylogenetic trees indicating significant support for species grouping. This is consistent with the previous studies of Butboonchoo *et al*. (2016), Al Quraishy *et al*. (2019), and Al Quraishy *et al*. (2021). Additionally, the query gene sequences of the present *Raillietina sp*. were aligned and connected to other *Raillietina* species found in GeneBank's Davaineidae group. With a distinct genetic sequence that is firmly ingrained in a genus that comprises *R. micracantha*, *R. australis*, *R. chiltoni*, *R. tunetensis*, and *R. sonini*, the current work

supports the taxonomic position of the rediscovered Davaineidae species as a probable sister taxon. Al Quraishy *et al*. (2019, 2021) earlier revealed the evolutionary relationships of *Raillietina* species in domestic pigeons from Saudi Arabia. However, the short sequence used to build the phylogenetic tree hindered the previously published phylogenetic study. The number of places in our analysis with gaps and insufficient sequence data limits the sequence. To fully utilize the 18S rDNA and ND1 for reliable molecular characterisation of these avian parasites, additional validation must be well-established.

**Conclusion**

Our findings show that domestic pigeons (*C. L. domestica*) should be taken into consideration as a potential natural reservoir for several cestode parasite species. In order to shed light on the evolutionary relationships of Davaineidae, additional research should concentrate on analyzing various genes.

**Declarations**:

**Ethical Approval and/or Informed Consent:**All procedures in the present study were conducted and authorized according to the King Abdulaziz University animal ethics committee (protocol no. 327-19).

**Conflict of Interest:**The authors have indicated that they have no conflict of interest regarding the content of this article.

**Authors Contributions:** I hereby verify that all authors mentioned on the title page have made substantial contributions to the conception and design of the study, have thoroughly reviewed the manuscript, confirm the accuracy and authenticity of the data and its interpretation, and consent to its submission.

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**Availability of Data and Materials:** All datasets analysed and described during the present study are available from the corresponding author upon reasonable request.

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