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Morphological, ultrastructural, and phylogenetic analysis of *Ascaridia columbae* infecting domestic pigeons (*Columba livia domestica*)

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Summary

Ascaridia species are the most common nematodes infecting pigeons. The current study investigated specific identity of nematode parasites collected from domestic pigeons (*Columba livia domestica*) in Al-Qassim Region, Saudi Arabia. Out of 354 pigeons, 13.3 % were infected with nematode parasites. The morphological structure and genetic relationship of nematode worms were studied using conventional methods (Light and scanning electron microscopes) coupled with the newly introduced molecular method. Microscopical and ultrastructure observations showed that the present nematode worms belong to the genus *Ascaridia* and have all the characteristic features of *Ascaridia columbae*. Moreover, Random Amplifier morphometric (RAPD) PCR analysis revealed that the present *A. columbae* had a close identity of up to 98.3 % to *Ascaridia columbae* JX624729 for Cox-1 gene regions, and up to 98.3 % to *Ascaridia nymphii* LC057210, and *Ascaridia galli* EF180058 for ITS1-5.8s- ITS2 rDNA gene regions. Phylogenetic analysis supported the placement of this *Ascaridia* species within Ascaridiidae family with close relationships to other nematode species obtained from GenBank. Finally, our study recommends using molecular analysis in helminths identification as the main methodology for correct identification especially in closely related species.

Keywords: Phylogenetic; *Ascaridia columbae*; Cox-1; ITS1-5.8s- ITS2 rDNA

Introduction

Pigeons (*Columba livia domestica*) are one of bird species found in ancient times, which has evolved to dwell in both rural and urban locations even in Saudi Arabia (Natala *et al*., 2009). Numerous harmful and potentially fatal blood parasites and helminth parasites are thought to infect pigeons and induce disease in them, (Gilik & Arslan, 2011; Adang *et al*., 2008). According to Cheng (1973) and Adang *et al*. (2008). Helminth infections result in both major harm and financial losses to infected pigeons. They can enter the body through the mouth, skin, or respiratory system, among other routes (Assafa *et al*., 2006). Since the intestine is the best habitat for them, most of them choose to reside there (Matthews, 2001). They receive nourishment and secure shelter from the intestine (Matthews, 2001). Nematodes are thought to be the most significant and widely distributed group of helminth parasites that affect birds. The primary nematode genera which is most common in pigeons are *Ascaridia*, *Heterakis*, *Syngamus*, and *Capillaria* (Matur & Dawam, 2010). *Ascaridia galli* and *Ascaridia columbae* also infect pigeons (Abdel Rahman *et al*., 2019). However, *A. columbae* was frequently discovered in pigeons' digestive systems (Tadelle & Ogle, 2001). According to Caira *et al*. (2014), the morphological criteria of *Ascaridia* species revealed a wide range of variability both within and across species, making it challenging to identify

* – corresponding author

Table 1. List of primers used for PCR amplification of *Ascaridia columbae*.

Gene ID	Direction	Sequencing	References
Cox-1	Cox1-F	5'-TGGTGGTTTAAGTGTTTGACTG-3'	Hamzah. *et al.,* 2020
	Cox1-R	5'-CCAACAACAAAGGCAACATT-3'	
ITS1-5.8s-ITS2 rDNA region	Physa-F	5'-GCGAAC GGC TCA TTA TAA CA-3'	Al Quraishy *et al*., 2020
	Physa-R	5'-AAT TTCACC TCT CAC GCA-3'	

Cox-1; Cytochrome oxidase-1, ITS; rDNA internal transcribed spacer (ITS1-5.8s-ITS2 rDNA)

by morphology. Many molecular methods such as Random Amplifier morphometric (RAPD) PCR, and Restriction Fragment Length Polymorphism (RELF), clarified knowledge on the genus *Ascaridia* (Penner *et al*., 1993). This investigation aims to determine the morphological and genetic relationships of *A. columbae* infecting the domestic pigeon (*C. L. domestica*) in the Al-Qassim region of Saudi Arabia.

Materials and Methods

Sample collection

A total of 354 pigeons (*Columba livia domestica*) were collected from bird markets in Al-Qassim region, located (between 25° 48 ' 22.68" N, 42° 52' 23.52 E) roughly 400 kilometers (250 miles) northwest of the capital, Riyadh, Saudi Arabia during the period from January to December (2021) and transferred to the health and scientific colleges research center, Majmaah University. Pigeons were humanely anesthetized with an isoflurane-soaked cotton pad and then dissected ethically according to the procedure outlined by (Al-Hussaini & Demian, 1982). All birds handling followed the Institutional Animal Ethics Committee guidelines at the Department of Biological Sciences at King Abdulaziz University. Nematode parasites were collected from infected pigeons and washed several times in saline solution to remove mucous and other host debris.

Preparation of permanent slides of Nemathelminths

After being fixed in 70 % ethanol, the recovered nematodes were cleared using lactophenol and identified using the keys of Soulsby (1982), Ruff (1984); Khalil *et al*. (2014). Nematode size determines how long cleaning takes.

The Scanning electron microscope

Some nematode worms were fixed with 3 % buffered glutaraldehyde in phosphate buffer (PH 7.2). Then samples were dried to CO2 critical point, and gold coated for 60 seconds using an Auto Fine Coater (JFC-1600) after the samples were then examined with an FEI Quanta FEG 450 Scanning Electron Microscope at 20 KV at King Abdulaziz University.

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. Two pairs of unique primers were used in a specialized PCR technique to amplify the rDNA internal transcribed spacer (ITS1-5.8s-ITS2 rDNA) and the Cytochrome oxidase-1 (Cox1) genes (Table 1). Using the Gene JETTM PCR Purification Kit [Thermo (Fermentas)], both gene areas were amplified in a total volume of 50 µl, comprising 5 µl of 10 × buffer, 5 µl of each dNTP (10 mM), and 10 µl of each primer (1 pmol/ µl), 0.3 µl of Taq polymerase (5 U/ml), 2.5 µl MgCl2 (50 mM), and 2 µl of total genomic DNA. A traditional PCR thermocycler technique was used to perform the PCR, as shown in Tables 2 and 3 for the Cox1 and ITS1-5.8s-ITS2 rDNA genes, respectively. 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 of it under a UV lamp. 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. Using the CLUSTAL-W algorithm

Table 2. Optimized cycling condition of Cox -1 gene.

PCR program of Cox -1						
Hold	1 Cycles	35 Cycles			1 Cycles	Cycles
	Final Extention	Extention	Anneling	Denaturation	Initial Denaturation	STEP
4 C	72 C	72 C	52 C	95 C	95 C	TEMP
∞	7 min	45 sec	1 min	30 sec	3 min	TIME

Table 3. Optimized cycling condition of ITS1-5.8s-ITS2 rDNA gene.

PCR program of ITS1-5.8s-ITS2 rDNA						
Hold	1 Cycles	35 Cycles			1 Cycles	Cycles
	Final Extention	Extention	Anneling	Denaturation	Initial Denaturation	STEP
4 C	72 C	72 C	54 C	95 C	95 C	TEMP
∞	7 min	1 min	1 min	30 sec	3 min	TIME

(Thompson *et al*., 1994), multiple sequence alignments were produced with a penalty of 10 for opening gaps and 1 for extending them. The Tamura_Nei model, 10,000 repetitions, and the Neighbor-Joining method (Saitou & Nei, 1987) were used to generate the phylogenetic tree.

Statistical analysis

The Pearson Chi-Square test was used to compare all the qualitative variables between infected and uninfected. Statistical significance was set at "P" <0.05 (two-tailed). The Statistical Package for Social Sciences Version 25 (SPSS) was used.

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).

Results

The nematode infection in *C.L. domestica* was 47 (13.3 %) of the 354 samples examined. The incidence of nematode infection varied considerably between seasons (p=0.004). Of the 67 birds examined, none of the pigeons had nematode infections throughout

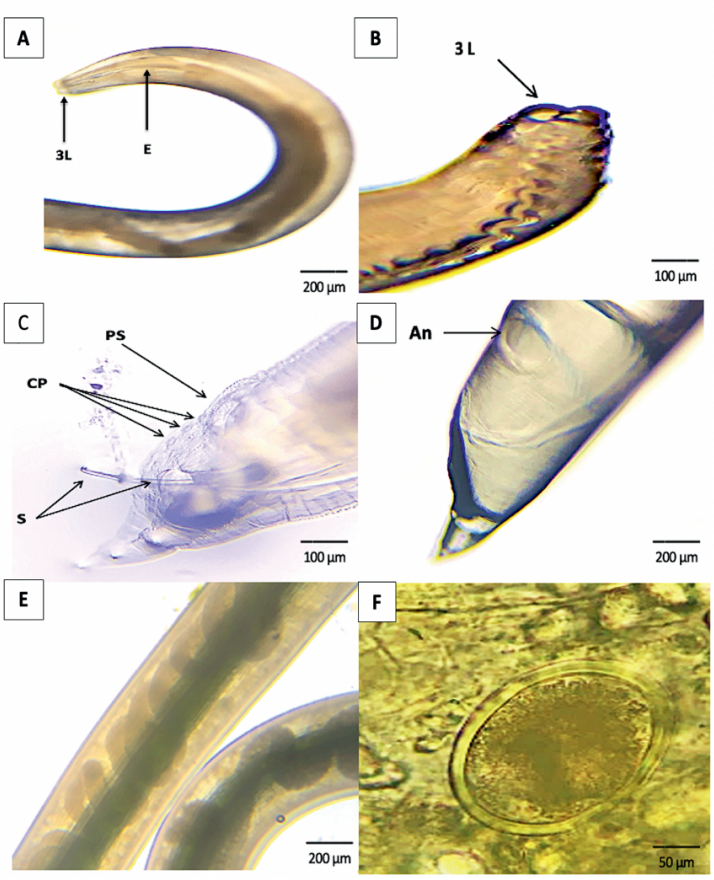

Fig. 1. (A & B) The anterior end of *A. columbae* showing a mouth with three trilobed lips (L) and a cylindrical esophagus (E) (Lactophenol) (Scale bar=200 µm) (Scale bar=100 µm).(C) The posterior end of *A. columbae* male showing 2 spicules (S), precloacal suker (PS), and caudal papillae (CP) (Lactophenol) (Scale bar=100 µm). (D) The posterior end of *A. columbae* female with anus (An) (Lactophenol) (Scale bar=200 µm). (E) The middle part of *A. columbae* female showing the uterus with eggs (Lactophenol) (Scale bar=200 µm). (F) The egg of *A. columbae* in feces of infected pigeon without stain (Scale bar=50 µm)

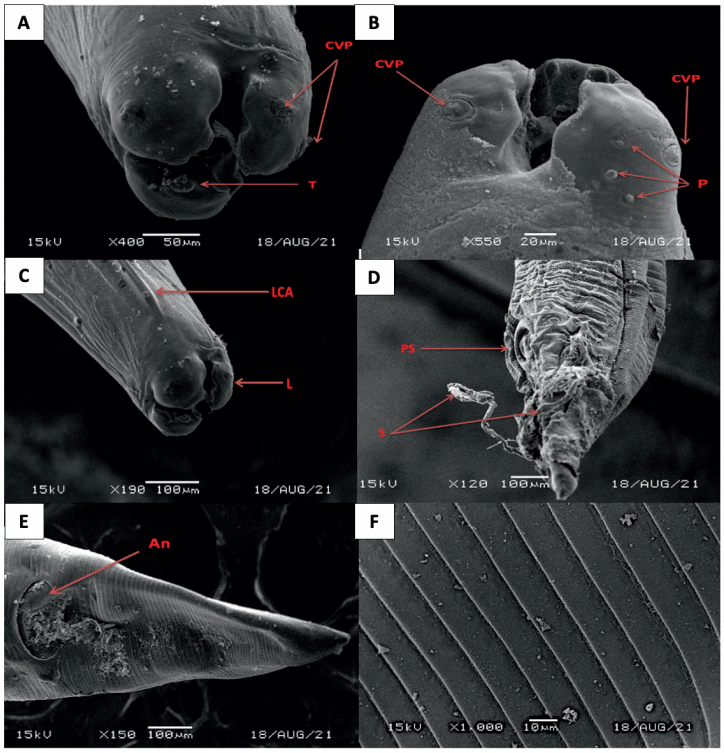

Fig. 2. SEM micrographs of *A. columbae*; (A). The anterior end showing three lips with teeth (T) and two cervical papillae behind each lip (CVP). (B). The anterior end showing cervical papillae (CVP) and amphidial pores (P). (C). The anterior end showing three lips (L) and lateral cephalic alae (LCA). (D). The posterior end of the male showing pre-cloacal sucker (PS) and two spicules (S). (E). The posterior end of the female showing anus (An). (F). The body surface is ornamented with cuticular transverse striations.

the winter (0 %). The prevalence of nematode infection in summer was (16.7 %), spring (14.3 %), and fall (17.7 %). Male and female infection rates were 14.7 % and 11.6 %, respectively, among the two genders. All four cities had the same infection rate: Unaizah (17.2 %), Buraydah (11.1 %), Ar-Rass (9.1 %), and Al-Bukairiyah (15.2 %). The prevalence rate of nematode infection was 13. 3 %. The body of the present nematode species of both male and female appeared cylindrical and creamy white-colored. Males ranged from (20 ± 35 mm) in length and (0.5 ± 0.9 mm) in width, while females ranged from (25 ± 45 mm) in length and (1.2 ± 1.8 mm) in width. The mouth is surrounded by three globular, trilobed, and equal lips. The esophagus is cylindrical without a posterior bulb and slightly extended towards the posterior end (Fig.1 A&B). The posterior end of male worms showed two strong and equal spicules protruded out from the cloacal opening and measures about (1.1 – 1.4 mm) long. Pre-cloacal sucker is located a short distance before the cloacal opening and measures about (0.18 – 0.20 mm) in diameter. It is circular to oval in shape with a strong chitinous-rimmed wall. There are thirteen pairs of caudal papillae, including (8 pairs postcloacal and 5 pairs precloacal) (Fig. 1 C). The female worms have a long, pointed tail with an anus (Fig. 1 D). The middle part of female shows a uterus filled with eggs (Fig. 1 E). The egg is oval to circular in shape and enveloped by a thick and smooth shell measured about (58 x 30µ) (Fig. 1 F). The scanning electron microscope (SEM) of the anterior end of nematode worms showed three large trilobed lips, and the inner surface of each lip carried two triangular teeth (spoon-like). Behind each lip, two cervical papillae and amphidial pores were also seen. Two cephalic alae extending from the anterior end on both lateral sides of the body surface were also observed. The cuticular surface is wrapped with faint transverse striations and lacks any cuticular vesicles (Fig. 2).

PCR amplification, Sequencing, and phylogenetic relationships of Ascaridia columbae based on Cox-1 gene

The gel electrophoresis revealed that the molecular size of the PCR product for samples number (1, 2, 3, and 4) of the Cox-1 gene was (240 bp) (Fig. 3), with Guanine-Citosine (GC) content of 34.1 – 35.6 % and pairwise identity of 99.1 %. The BLAST search showed that the four samples had similarities to *Ascaridia columbae* (accession number JX624729) hosted in pigeons from China, with 98.3 % pairwise identity and 99.4 % coverage. The multiple sequence alignment of the four sequences of the Cox-1 gene with (JX624729) is shown in (Fig. 5). The four sequences of the Cox-1 gene from the current study were deposited successfully in GenBank under accession numbers (ON844188, ON844189,

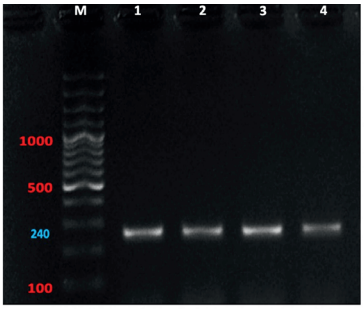

Fig. 3. Gel electrophoresis of PCR products of *Cox-1* gene for *Ascaridia columbae* (n=4) on 1.5 agarose gel. The molecular size of the ladder is 1500 bp (M)

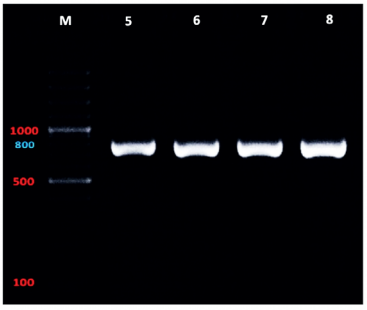

Fig. 4. Gel electrophoresis of PCR products of *ITS1-5.8s- ITS2 rDNA* gene for *Ascaridia columbae* (n=4) on 1.5 agarose gel. The molecular size of the ladder is 1500 bp (M)

ON844190, and ON844191) for the first time from Saudi Arabia. Using related sequences that are accessible on GenBank, a phylogenetic tree was built using the data sequence from this study. The phylogenetic tree of the four-sequence of the Cox-1 gene sequences in this study with other species retrieved from GenBank is shown in (Fig. 6), and *Thelazia callipaeda* (accession number AB538282) was used as an outgroup. The tree showed the present *Ascaridia* species are also related to *Ascaris suum* (accession number HQ704901) and *Ascaris ovis* (accession number KU522453) from China. The *Ascaridia* group was monophyletic with strong nodal support for grouping.

PCR amplification, Sequencing, and phylogenetic relationships of Ascaridia columbae based on ITS1-5.8s- ITS2 rDNA gene

The gel electrophoresis revealed that the molecular size of the PCR product for samples number (5, 6, 7, and 8) of the ITS1-5.8s-ITS2 rDNA gene was (800 – 810 bp) (Fig. 4), with GC content of 45.8 – 46.2 % and pairwise identity of 97.8 %. The BLAST search showed that the samples have similarity to *Ascaridia nymphii* (accession number LC057210) from Japan, with 98.3 % pairwise identity and 100 % coverage. The results also showed similarity to *Ascaridia galli* (accession number EF180058) from California with 98.3 % pairwise identity and 100 % coverage. For sample 8, no significant similarity was found. The multiple sequence alignment of the three sequences with (LC057210) and (EF180058), respectively are shown in (Figs. 7 and 8). The three- sequences of the ITS1-5.8s- ITS2 rDNA gene from the current study were deposited successfully in GenBank under accession numbers (ON855017, ON855018, and ON855019). Using similar sequences that are accessible on GenBank, a phylogenetic tree was constructed using the data sequence from this study. The phylogenetic tree of the three ITS1-5.8s- ITS2 rDNA sequences in this study with other species retrieved from GenBank is shown in (Fig. 9), and *Thelazia callipaeda* (accession number AB538282) was used as an outgroup. The tree showed the present *Ascaridia* species are also related to *Ascaridia galli* (accession number OK501308) from Israel and a close association between the present *A. columbae* and *Heterakis dispar* (accession number MG 763171) from Poland. The *Ascaridia* group was monophyletic with moderate nodal support for grouping.

Discussion

According to Lichtenfels *et al*. (1997), helminth parasites are typ-

Fig. 5. Multiple sequence alignment of the four sequences of the *Cox-1* gene in this study aligned with *Ascaridia columbae* (JX624729)

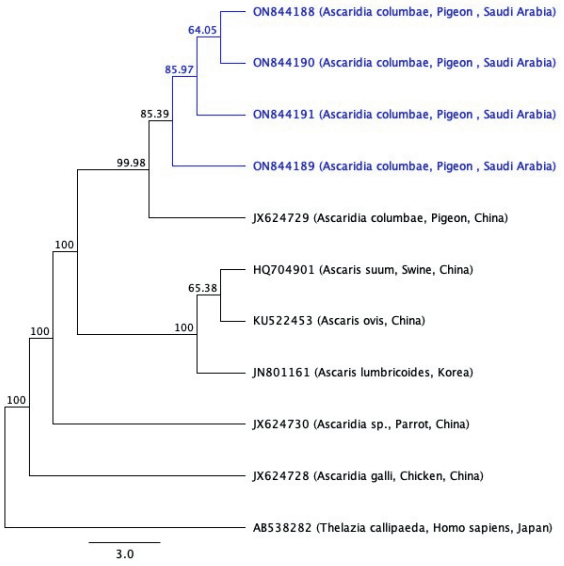

Fig. 6. Maximum likelihood tree of the four sequences of the *Cox-1* gene in the current study with other species downloaded from GenBank.

ically identified through the examination of their morphological features, pathogenic potential, or modes of transmission. But frequently, these standards fall short of allowing for precise identification (Chilton, 1999). The variation in infection rates of helminth species associated with habitat alteration explain such changes in relation to parasite, host, and environmental features (Carrera-Játiva & Acosta-Jamett, 2023) Molecular diagnostics has emerged as the most sensitive and reliable identifying method. According to Ibrahim *et al*. (2018a); Safi-Eldin *et al*. 2019; Al Quraishy *et al*. (2020), it improves knowledge and data for recognition, identification, and phylogenetic relationships among the species. The physical traits of the nematode worms in this study verified that they are all members of the Ascaridiidae family, which is subdivided into the genus *Ascaridia*, as proposed by Dujardin (1845) the presence of two cephalic alae on opposite sides of the body, a club-shaped esophagus without a posterior bulb, and three globular, trilobed, identically sized lips in the mouth; Two spicules, a pre-cloacal sucker with a chitinous rim, and the quantity and arrangement of caudal papillae are present in males; a pointed tail and thick-shelled eggs are present in females. These results corroborated those reported by Ibrahim *et al*. (2018a), Abdel Rahman *et al*. (2019), Salem *et al*. (2022), Banaja *et al*. (2013), and Al Quraishy *et al*. (2020). The *Ascaridia* species examined in this study were contrasted with other *Ascaridia* species found in various bird hosts in various parts of the world. Our findings verified that all the differentiating features and host-specific species of the present *Ascaridia* species were highly comparable to those of the previously identified *Ascaridia columbae*. The most prevalent nematode found in domestic pigeons is *A. columbae*, according to reports from various researchers across the globe: Bangladesh (Begum & Shaikh, 1987); Brussels (Bernard & Biesman, 1987); Spain (Martinez *et al*., 1989); Yugoslavia (Kulisic, 1989); Italy (Tacconi *et al*., 1993); Egypt (Ibrahim *et al*., 1995; Ibrahim *et al*., 2018a; Salem *et al*., 2022); Pakistan (Hayat *et al*., 1999); Tanzania (Msoffe *et al*., 2010); Saudi Arabia (Banaja *et al*., 2013; Ali *et al*., 2020; Al Quraishy *et al*., 2020). Furthermore, the present study's description of *A. columbae* ultrastructure features is consistent with that provided by Banaja *et al*. (2013), Abdel Rahman *et al*. (2019), and Al Quraishy *et al*. (2020). Among the most variable characteristics of the male *Ascaridia*

Fig. 7. Multiple sequence alignment of the three sequences of the ITS1-5.8s- ITS2 rDNA gene in this study aligned with *Ascaridia nymphii* (LC057210)

Fig. 8. Multiple sequence alignment of the three sequences of the ITS1-5.8s- ITS2 rDNA gene in this study aligned with *Ascaridia galli* (EF180058)

species is the quantity and orientation of caudal papillae. There were thirteen pairs of caudal papillae on the current *A. columbae*. This finding is consistent with other research from Egypt (Abdel Rahman *et al*., 2019) and Ibrahim *et al*., 2018a), which discovered 13 pairs of caudal papillae grouped as 5 precloacal and 8 postcloacal. In contrast, 10 pairs of papillae were observed in the prior Saudi Arabian study (Al Quraishy *et al*., 2020), which were divided into 3 pairs of pre-anal, 1 pair of ad-anal, 3 pairs of post-anal, and 3 pairs of sub-terminal caudal papillae. It also differs from *A. platyceri* (Mines, 1979), *A. dissimilis* and *A. nicobarensis* (Soota *et al*., 1971), and *A. galli* (Ramadan & Abou Znada, 1992) in terms of the quantity and arrangement of caudal papillae, with the latter species having 10 pairs. Furthermore, other investigations have documented differences in the number of caudal papillae in other *Ascaridia* species, such as *A. galli* (5 – 10) (Dehlawi, 2007), *A. amblymoria* (Von Drasche, 1883), and others, *A. francolina* (Von Linstow, 1899), *A. cordata* (Von Linstow, 1901), *A. dolichocerca* (Stossich, 1902), (9) in *A. longecirrata* (Von Linstow, 1879), *A. cristata* (Von Linstow, 1901), *A. magnipapilla* (Barus, 1966), *A.compar* (Barus, 1966), (12) in *A. orthocerca* (Stossich, 1902), *A. magalhaesi* (Travassos, 1913), (12 – 13) in *A. sergiomeirai* (Pereira, 1933), (13 – 16) in *A. hermaphrodita* (Travassos, 1913), 13 in *A. australis* (Von Linstow, 1898), and (18) in *A. catheturina* (Johnston, 1912). The variation seen in the quantity and location of papillae among *Ascaridia* species could potentially be linked to the characteristics of the insemination process within the *Ascaridia* genus. Furthermore, using the Cox-1 and ITS1-5.8s-ITS2 rDNA genes, we examined the sequencing and phylogenetic connections of *Ascaridia columbae* and other related species in this work. Saudi Arabia deposited the Cox-1 gene's four sequences into GenBank for the first time. The Cox-1 gene's phylogenetic tree revealed that the four *Ascaridia columbae* sequences under investigation were only clustered with their closely related species in GenBank, with a high bootstrap of 99.98 %. This finding was corroborated by Berry and Gascuel's (1996) assertion that high bootstrap values near 100 % indicate uniform support, if the bootstrap value for a certain clade is close to 100 %, it means that nearly all the species of this clade have uniform characters and considered as a group.

Furthermore, the identification of the present *Ascaridia* species was validated by Physa-F/Physa-R primer-based PCR amplification and sequencing of the ITS1-5.8s-ITS2 rDNA gene region. Gomes *et al*. (2015) used a similar primer to classify and differentiate three different nematode species that infect pigeons (*C. L. domestica*) from Brazilian Pantanal Wetlands: *Ancylostoma buckleyi*, *Pterigodermatites pluripectinata*, and *Ascaridia galli*. Additionally, Al Quraishy *et al*. (2020) employed a similar gene region to perform molecular phylogenetic analysis to clarify the taxonomic status of *Ascaridia* species that infect *C. L. domestica*, adopting a comparable strategy for the first time in Saudi Arabia. The current *A. columbae* is firmly buried in the *Ascaridia* genus and highly linked to *A. nymphii* and *A. galli*, according to the phylogenetic tree of the ITS1-5.8s-ITS2 rDNA gene. Bootstrapping revealed moderate nodal support for grouping. In line with previous research by Kim *et al*. (2014) and Šnábel *et al*. (2014), the tree also revealed a close association between the current *A. columbae* and *Heterakis dispar* (MG 763171) from Poland. This suggests that the *Ascaridia*

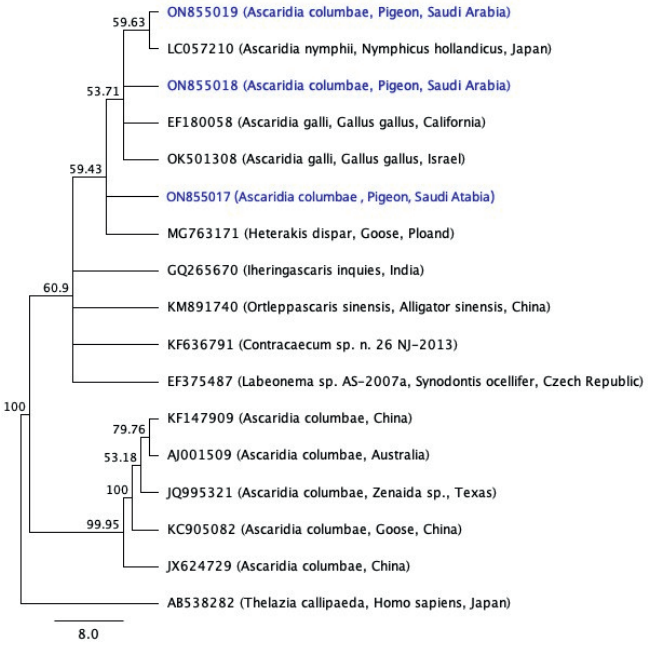

Fig. 9. Maximum likelihood tree of the three sequences of the ITS1-5.8s- ITS2 rDNA gene in the current study with other species downloaded from GenBank.

genus is a sister genus of the *Heterakis* genus and supports the theory that the Heterakidae and Ascaridiidae families are closely linked to the Ascaridomorpha suborder. Our research shows that the ITS1-5.8s-ITS2 rDNA gene is a highly variable area that may particularly discriminate between closely related species. It also verified that molecular identification of nematode species is a very successful method in separating morphologically similar species. The findings we obtained corroborated the statements made by Park *et al*. (2007) and Engelmann *et al*. (2009) that ITS spacers are thought to be the most variable and informative region. It can describe various closely related species. Nonetheless, every primer utilized in this research was helpful and unique to the species being examined.

In the present study, the pigeons were collected from one region. As mentioned earlier, Saudi Arabia has several areas that significantly differ in climate conditions and geographical landforms. This can substantially impact the intermediate host and the prevalence of infection as a result. A national study with pigeons collected from different regions is required to accurately assess the prevalence of infection in Saudi Arabia. Further investigations should focus on the analysis of different genes to clarify the phylogenetic relationships of Ascaridiidae

Conclusion

One extremely effective method for distinguishing physically similar species from one another has been the molecular identification of species. Consequently, the primary methodological tool for precise helminth identification is advised to be molecular methods. The PCR primer used in the molecular approach is very unique to the *Ascaridia columbae* species being studied, making it more accurate in identifying hybrid and cryptic species.

Conflict of interest

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

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