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Original article

Morphological Description and Molecular Characterization of *Ascaridia columbae* Infecting Domestic Pigeons (*Columba livia domestica*) in Gharbia Governorate, Egypt, Based on ITS rDNA Sequences

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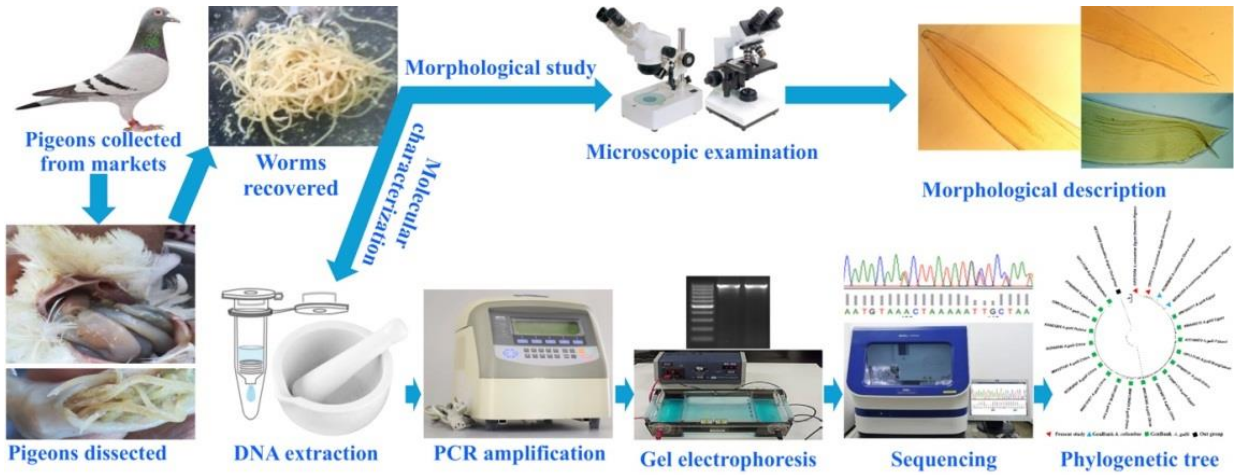
ABSTRACT

Ascaridia columbae is a parasitic nematode commonly infecting domestic pigeons (*Columba livia domestica*), leading to significant health issues and economic losses in avian populations. From April 2018 to March 2019, 130 domestic pigeons from Gharbia Governorate, Egypt, were examined, with 20.7% found to be infected. The high prevalence of infection may be attributed to the selection of weak, lethargic, and poorly growing pigeons. Morphological features of adult worms were studied both macroscopically and microscopically, with a focus on diagnostic traits such as body size, sexual dimorphism, anterior and esophageal structures, and reproductive organs. Detailed morphometric measurements were provided to support species identification. For molecular characterization, DNA was extracted from individual worms, and internal transcribed spacer (ITS) rDNA (ITS1-5.8S-ITS2) was amplified using polymerase chain reaction (PCR). PCR amplification produced a fragment of approximately 900 bp in size. An 897 bp segment of the ITS rDNA sequences was submitted to GenBank with accession numbers OP215354 and OP215355. Phylogenetic analysis indicates that the *A. columbae* samples from the current study are extremely similar to other *A. columbae* sequences, particularly those from Egypt and China. The genetic distinction between *A. columbae* and *A. galli* is evident, supporting the view that they are separate species with unique evolutionary paths despite sharing most morphological features. These findings provide new insights into the morphological and molecular characteristics of *A. columbae*, contributing to a better understanding of its epidemiology and genetic diversity in Egypt.

Graphical abstract



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1. Introduction

Domestic pigeons don't migrate, but because of their excellent homing skills, they can return to their nests from a great distance if permitted. Numerous parasites and diseases can be spread to various flocks by pigeons [1]. They may potentially be a source of various zoonotic illnesses that affect people [2]. Numerous ectoparasites and endoparasites, including acanthocephalans, cestodes, trematodes and nematodes can parasitize birds [3, 4].

Nematodes are the most important and common helminths with a direct or indirect life cycles and pigeons are more tolerant of helminths, especially *Ascaridia* species, than other birds [5, 6]. Pigeons are susceptible to *A. galli* and *A. columbae* infections [7]. Clinical symptoms of *A. columbae* infection in pigeons include losing weight, weakness, diarrhea, anemia, and in cases of severe infestation, death [7,8]. Severe infestations can lead to intestinal blockages and severe nutritional deficiencies, which if untreated, can be fatal. Treatment typically involves the use of broad-spectrum anthelmintic drugs, but resistance to these drugs has been emerging, prompting the need for ongoing research into alternative treatments, including natural remedies like chitosan nanoparticles, which have shown promising in recent studies [9].

Studies on ascarid nematodes show that while the nematodes share gross morphological similarities, also exhibit structural differences across species [10]. Another nematode species (*Hysterothylacium reliquens*), related to *Ascaridia*, revealed significant morphological variation in body length, esophagus size, and spicule arrangement [11]. However, molecular analysis of the ITS regions confirmed these differences as intraspecific, highlighting the limitations of morphology alone for accurate species identification.

A study examining *A. columbae* from domestic pigeons in Saudi Arabia utilized the ITS1 and ITS2 regions of rDNA for molecular characterization [12]. Despite morphological similarities to other *Ascaridia* species, these markers enabled precise identification through a phylogenetic approach, underscoring the value of ITS markers in improving taxonomic accuracy. The use of ITS markers for DNA sequencing verified the species identity of *A. galli*, another member of the *Ascaridia* genus, addressing challenges posed by morphological variations [13]. This supports other research showing that ITS sequencing is essential for accurate taxonomic identification in the presence of morphological variability.

A solid understanding of parasitic diseases in pigeons is essential for devising effective control measures that improve pigeon survival and raise public awareness. This study aimed to provide a detailed morphological description of *A. columbae* in domestic pigeons from Gharbia Governorate, Egypt, and to perform molecular characterization based on the ITS regions of rDNA sequences.

2. Materials and methods

A total of 130 domestic pigeons (*Columba livia domestica*), showing signs of weakness, lethargy, and poor growth, were purchased from markets in Al Mahalla Al Kobra, Kutour, Samannoud, and Tanta in Gharbia Governorate, Egypt. This Governorate is located in the Nile Delta region, and the pigeons were collected between April 2018 and March 2019.

The collected birds were transferred to the Laboratory of Biological and Environmental Sciences, Faculty of Home Economics, Tanta, Al-Azhar University, for parasite extraction. Ectoparasites were recovered and published [14], after which the birds were dissected, and their digestive tracts were examined for endoparasites. Nematode specimens were recovered and preserved in 70% ethanol, until processed and examined.

Confirmatory examination and identification were conducted at the Parasitology Laboratory, Zoology Department, Faculty of Science (Boys), Nasr City, Al-Azhar University. The parasites were carefully differentiated into species using both NOVEL (NTB-2B) and XSZ-107T light microscopes. Nematode worms were cleared in lactophenol and mounted on polyvol [15, 16].

According to the manufacturer's instructions, DNA extraction was performed randomly from two worms, separately preserved in ethanol, using the QIAamp DNA Mini Kit (Qiagen, USA). The extracted genomic DNA was utilized for polymerase chain reaction (PCR) amplification of the ITS region (ITS-1, 5.8S, and ITS-2) using the primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) as the forward primer and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) as the reverse primer [17]. PCR amplification was conducted using the Taq PCR Master Mix Kit (Qiagen, USA) according to the manufacturer's protocol in a total reaction volume of 50 µL. This included 20 µL of ddH_2O, 25 µL of Taq PCR Master Mix, 1 µL of each primer, and 3 µL of genomic DNA.

The thermal cycling conditions were as follows: an initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, primer annealing at 55 °C for 30 seconds, and extension at 72 °C for 30 seconds, with a final extension step at 72 °C for 10 minutes.

The PCR products were separated on a 1.5% agarose gel in 1× TBE buffer, stained with ethidium bromide, and visualized under UV light. Bands of the expected size were excised and purified using the QIAquick PCR and Gel Cleanup Kit (Qiagen, USA) following the manufacturer's protocol. Nucleotide sequencing was performed using a 3500 Genetic Analyzer (Applied Biosystems, Foster City, USA) with the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA) at Colors Laboratories (Maadi, Cairo, Egypt).

The obtained sequences were inferred to species based to the data available in GenBank using the NCBI BLAST server (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (National Center for Biotechnology Information /Basic Local Alignment Search Tool). The sequences were then compared with those from other studies using the BioEdit Sequence Alignment Editor [18] to remove unresolved or 'noisy' nucleotide sites at both ends. Subsequently, the sequences were deposited in GenBank.

Sequence alignment was performed using the program Clustal W within MEGA v.11 [19] and it was displayed in Jalview v.2.11.0 [20]. The phylogenetic tree was constructed using the neighbour-joining method [21] within MEGA v.11 program, with the evolutionary distances computed using the Maximum Composite Likelihood method [22].

3. Results and discussion

3.1. Prevalence:

In the current study, 130 digestive tracts of domestic pigeons (*Columba livia domestica*) were examined. Samples were collected from markets in Al Mahalla Al Kobra, Kutour, Samannoud, and Tanta in the Gharbia Governorate, Egypt, a region within the Nile Delta. The study was conducted between April 2018 and March 2019.

The overall prevalence of *A. columbae* was found to be 20.7% (27 out of 130 samples). This prevalence was higher than those reported in previous studies conducted in different governorates of Egypt, including Gharbia (12%) [23], Beni-Suef (3%) [24], Sharkia (10%) [7], Aswan (9.16%) [25], Dakahlia (8.91%) [26] and Gharbia (12.44%) [26]. It also exceeded the prevalence recorded in Nigeria (11.3%) [27].

However, the prevalence observed in this study was lower than those recorded in Ismailia governorate (23.2%) [28] and Giza governorate (63.1%) [9], as well as in Bangladesh (28.33%) [29] and in Brazil (25.3%) [30].

The variations in prevalence may be attributed to differences in sampling timing, host behavior, and local geo-climatic factors. Additionally, sampling methods could have influenced the results. For instance, in this study, most pigeons selected exhibited signs of lethargy, weakness, and poor growth, whereas other study [9] focused on suspected cases from veterinary clinics. Nevertheless, unsanitary living conditions among many of these birds contribute significantly to the high risk of parasite transmission [31].

3.2. Morphological characterization:

In the present study, adult *A. columbae* were elongated, cylindrical worms found predominantly in the small intestine. They were visible to the naked eye due to their creamy white and semi-transparent appearance (Fig. 1). This observation is consistent with previous studies [9, 12]. The location within the host and the coloration of adult *A. columbae* are characteristic of their genus, aiding in their identification.

Macroscopic and microscopic methods were employed to examine the similarities and differences between male and female worms. A distinct size difference was observed, with females being longer and slightly wider than males, consistent with the sexual dimorphism reported in nematodes such as *Ascaridia* species. Females measured 40–55 mm in length, compared to males at 30–45 mm, with slight regional variations in body width. The greatest body width in adult females, observed at the vulvar region, ranged from 0.65 to 0.85 mm, while males measured 0.60 to 0.90 mm. These measurements support prior findings that females of *Ascaridia* species are often larger to accommodate reproductive structures such as the uterus and vulva [32]. The trilobed lips and cephalic alae (wing-like structures extending from the base of the lips) described in this study (Fig. 2 A&B) match previous descriptions of the anterior morphology of *Ascaridia* species. These structures aid in attachment to the host's intestinal wall and feeding [33]. This morphology has served as a distinctive characteristic, helping to differentiate *Ascaridia* from other nematode genera, such as *Ascaris* and *Toxocara*, which have different lip and esophageal structures.

The club-shaped esophagus (Fig. 2 A), which was longer in females (2.6–3.20 mm long, with a maximum width of 0.35-0.55 mm) than in males (2.1–3.0 mm long, with a maximum width of 0.25-0.40 mm), was characteristic of nematodes. Esophageal morphology and measurements were crucial for taxonomy and identification. This elongated esophagus, terminating near the anterior end with a nerve ring, aligns with descriptions in taxonomic studies, highlighting the role of esophageal length in differentiating species within the *Ascaridia* genus [34]. The distance from the nerve ring and excretory pores to the anterior end of the body measured 0.50-0.75 mm and 0.80-1.12 mm, respectively, in females, while in males, the measurements were 0.30-0.50 mm and 0.70-0.85 mm.

The position of the vulva, slightly posterior to the midpoint of the body (with a distance from the anterior end ranging from 18.50 to 35.00 mm), and the conical tail (Fig. 3) with a cuticular spine (the distance from the anus to the tip of the tail ranges from 0.65 to 1.20 mm) were distinguishing features observed in female *A. columbae*. Previous studies also report similar vulva positioning and uterine structures filled with thick-shelled eggs, which are important for species identification [32].

The precloacal sucker, caudal papillae, and equal spicules in males were key diagnostic features (Fig. 4). The structure and positioning of the precloacal sucker and caudal papillae were essential for identifying *A. columbae* males and distinguishing them from closely related nematode species. The precloacal sucker, located 0.22–0.30 mm anterior to the cloaca, was oval to circular, with a diameter of 0.09–0.15 mm and a length of 0.17–0.25 mm. Thirteen pairs of caudal papillae (eight post-cloacal and five pre-cloacal) were observed at the posterior extremity. The cloaca appeared as a prominent, transverse, slightly tongue-shaped slit, positioned 0.40–0.60 mm from the posterior end of the

body. Two identical spicules, 1.42–2.00 mm in length and either straight or slightly curved, extended from the cloacal opening. The gubernaculum was absent. The proximal and distal portions of the spicules measured 0.03–0.06 mm and 0.02–0.04 mm in width, respectively, with blunt and rounded ends. The absence of a gubernaculum, a common characteristic in nematode males, and the presence of equal spicules align with established morphological traits for this species and contribute to its specific identification within *Ascaridia* [35, 36].

The morphological data on *A. columbae* in terms of body size, sexual dimorphism, anterior and esophageal structures, and reproductive organs closely align with prior descriptions of *Ascaridia* species, reinforcing these features as critical for species identification [12, 37].

Studies, such as those in Saudi Arabia have combined morphological analysis with molecular techniques to further elucidate the taxonomy and genetic relationships of *A. columbae* [12]. For example, molecular analyses targeting ITS rDNA regions have clarified its phylogenetic position, revealing a high degree of genetic similarity with other *Ascaridia* species, suggesting close evolutionary relationships within the family.

3.3. Molecular characterization:

PCR amplification with the described primers (NC5 and NC2) yielded a fragment of approximately 900 bp. An 897 bp fragment of the ITS rDNA (ITS1-5.8S-ITS2) sequence was deposited in GenBank under accession numbers OP215354 and OP215355 and used for phylogenetic studies. BLAST results showed that the ITS sequences were 97.22% to 99.33% homologous with *A. columbae*, with query cover ranging from 99% to 100%. The sequences were also 96.01% to 98.63% homologous with the closest species, *A. galli*, with query cover ranging from 89% to 100%.

Ten SNPs (single nucleotide polymorphisms) were identified when the *A. columbae* ITS sequences from the current study were aligned with the most similar sequence in the Chinese GenBank (KC905082). These SNPs were caused by additions, deletions, and substitutions at the nucleotide positions (464, 497, 519, 526, 563, 590, 630, 668, 692, and 878), as shown in the Jalview v.2.11.0 program (Fig. 5).

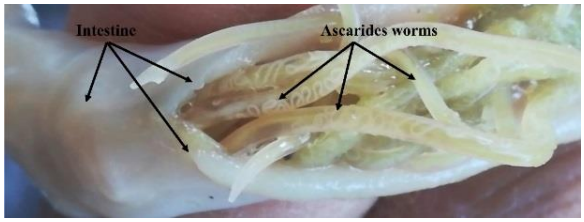

Fig. (1): Photograph of *Ascaridia columbae* in the intestine of domestic pigeons (*Columba livia domestica*), showing the creamy white color and semi-transparent appearance.

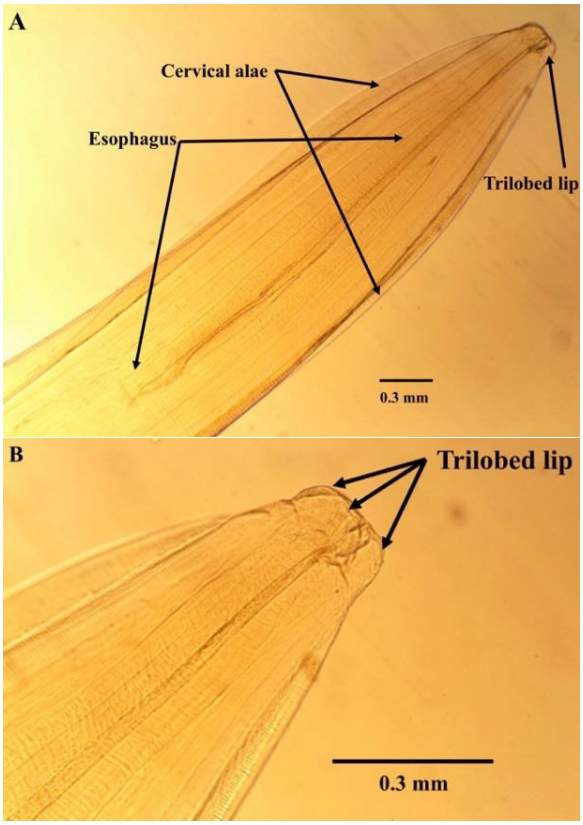

Fig. (2): Photomicrographs of the anterior end (A) Showing the cephalic alae and esophagus and (B) Enlarged anterior tip showing the trilobed lip.

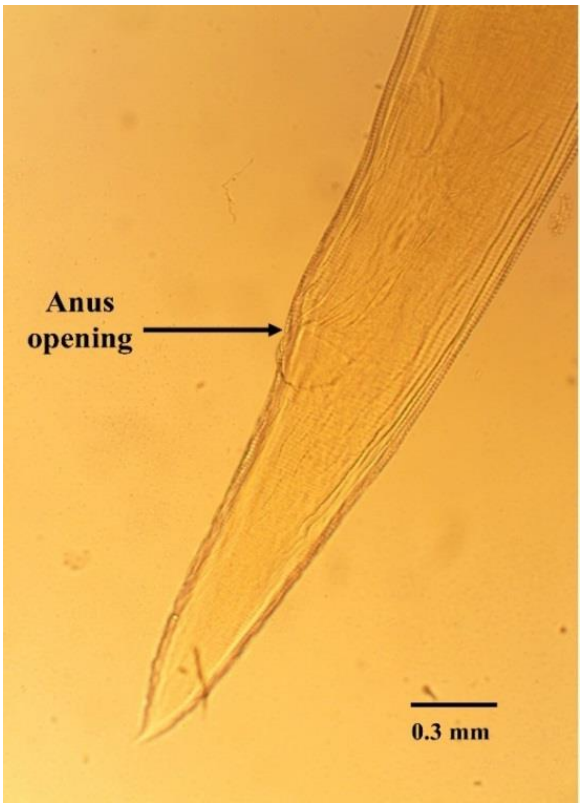

Fig. (3): Photomicrographs of the posterior end of an adult female showing the anus opening.

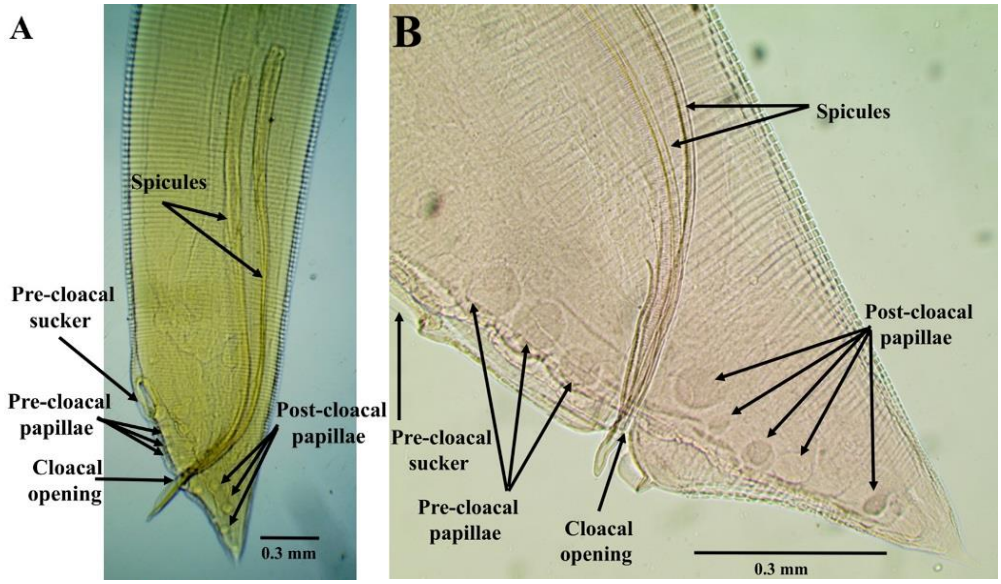

Fig. (4): Photomicrographs of the posterior end of an adult male showing the spicules, pre-cloacal sucker, cloacal opening and caudal papillae.

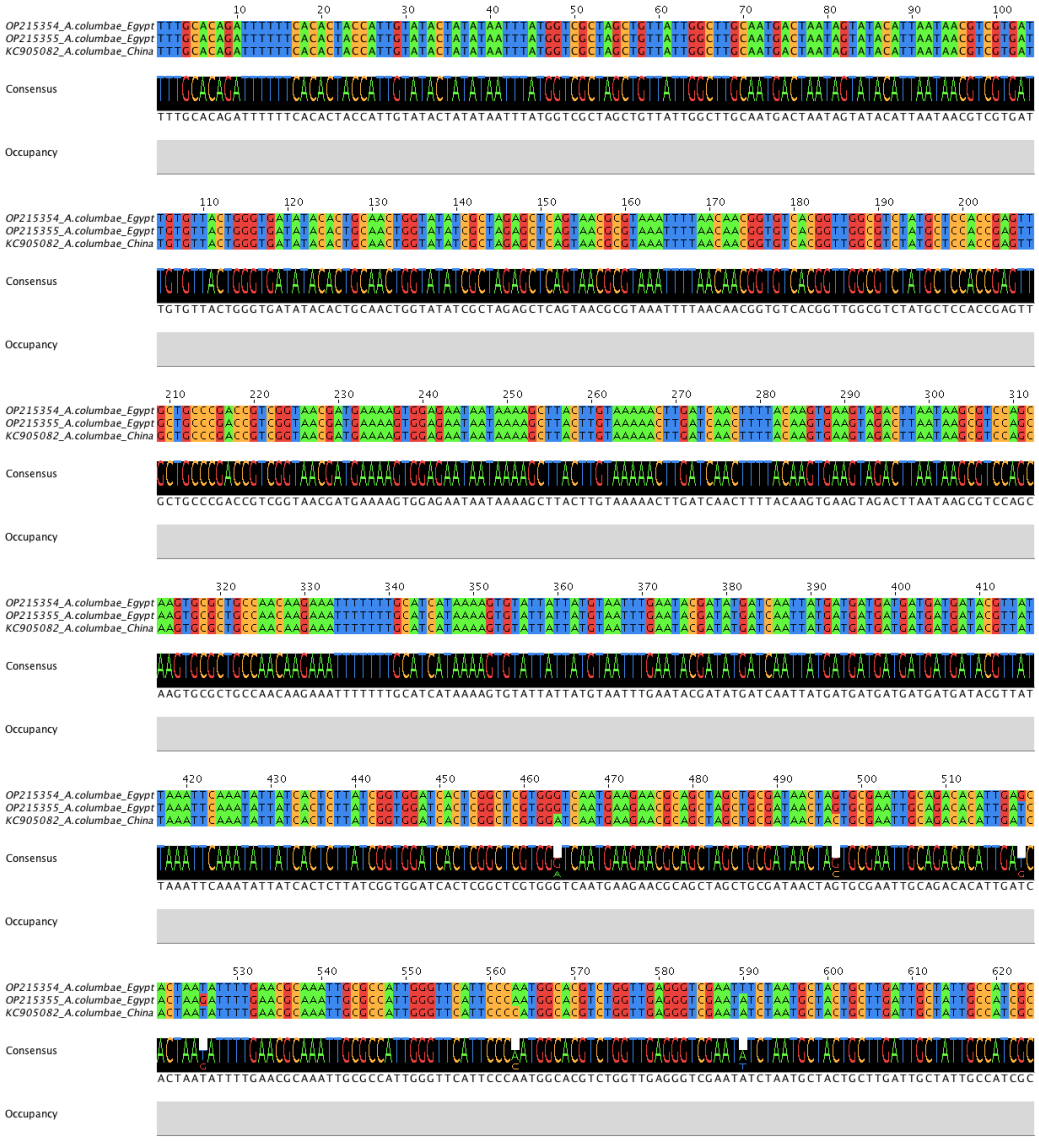

Fig. (5): Alignment of nucleotide sequences for the ITS rDNA region (5′ to 3′) of *A. columbae* of the present sequences from Egypt and most homologous sequence in the GenBank (KC905082) from China.

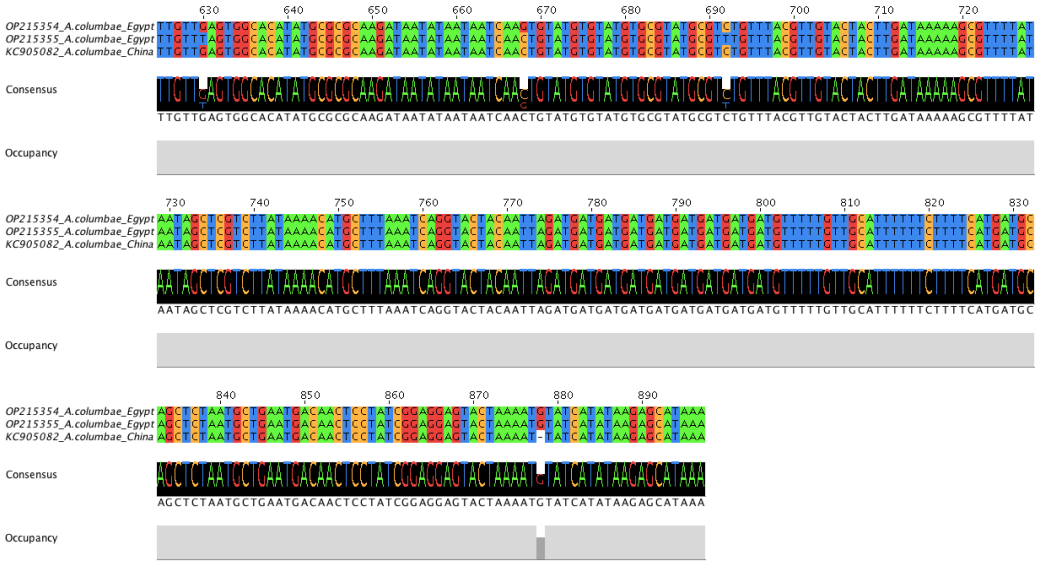

Fig. (5): Continued.

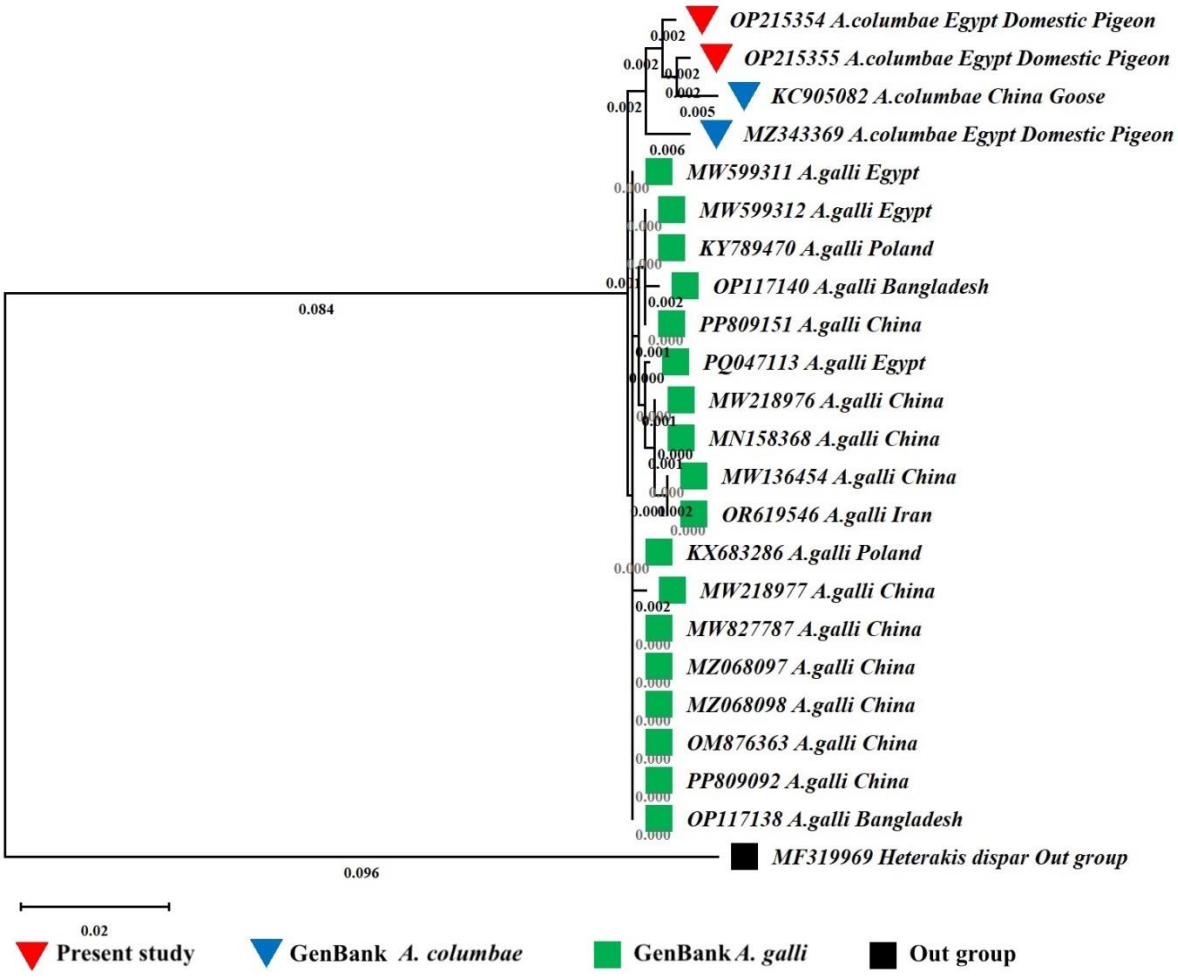

Fig. (6): Diagrammatic representation of the phylogenetic tree showing the relationships among ITS sequences of *A. columbae*, *A. galli*, and the outgroup *H. dispar*. The tree was constructed using the Neighbour-Joining method within the MEGA 11 program.

The phylogenetic tree of the ITS sequences was constructed using Neighbour-Joining (NJ) method [21], which is commonly used for analyzing evolutionary relationships based on genetic distance data. This analysis included 23 nucleotide sequences representing the present *A. columbae* and available sequences from GenBank database, as well as sequences from different studies of the closest species *A. galli* and the out group *Heterakis dispar* (Table 1). All ambiguous positions were removed for each sequence pair, resulting in a final dataset containing 566 positions.

The phylogenetic tree (Fig. 6) of the present study sequences showed that the sequences were closely related, with only a small branch length separating them. This indicates a high degree of similarity between the two samples, which clustered with other *A. columbae* sequences, particularly (KC905082) from China, isolated from geese, and (MZ434369) from Egypt, isolated from domestic pigeons. This clustering suggests that the *A. columbae* strains in the present study are genetically similar to both other Egyptian samples and a Chinese sample, indicating a possible common lineage or recent divergence within the species. The *A. galli* sequences form a separate cluster, distinct from the *A. columbae* group, supporting the notion that *A. galli* and *A. columbae* are genetically distinct, despite sharing some similarities. These findings align with [38], who revealed that gene flow among nematode populations is limited over large distances and that genetic structure and cryptic speciation vary significantly between nematode species in the same area. The sequence MF319969 of *H. dispar* was used as an outgroup to root the tree. Its placement at the base of the tree suggests that it is more distantly related to both *A. columbae* and *A. galli*, helping to define the evolutionary relationships among the other sequences.

Table 2 presents the estimates of evolutionary divergence among sequences based on genetic distances. A comparison of the genetic distances between the sequences from the current study and other available sequences highlights the degree of divergence from other *A. columbae* or *A. galli* samples. The low genetic distance between the two samples in the present study indicates that they are almost identical or very closely related, confirming their origin from the same or closely related pigeon populations in Egypt (Gharbia Governorate).

Comparisons with other *A. columbae* samples indicate some genetic diversity within *A. columbae* across different regions (e.g., Egypt, Giza Governorate vs. China). The greater genetic distances to *A. galli* samples confirm the species distinction between *A. columbae* and *A. galli*, as these values suggest more substantial evolutionary divergence.

The grouping of the *A. columbae* samples from the current study with other *A. columbae* samples from various regions, along with the clear separation between the *A. columbae* and *A. galli* clusters, supports species-level differentiation. This suggests that the ITS rDNA gene is an effective marker for phylogenetic analysis at both the species and genus levels [39, 40]. Furthermore, the current results are consistent with previous studies [12, 41, 42].

Table 1: GenBank sequences used for phylogenetic analysis

	Accession number	Place of origin	Isolate identification
1	OP215354*	Egypt	*A. columbae*
2	OP215355*	Egypt	*A. columbae*
3	MZ343369	Egypt	*A. columbae*
4	KC905082	China	*A. columbae*
5	MW599311	Egypt	*A. galli*
6	MW599312	Egypt	*A. galli*
7	PQ047113	Egypt	*A. galli*
8	KX683286	Poland	*A. galli*
9	KY789470	Poland	*A. galli*
10	MW827787	China	*A. galli*
11	MW218976	China	*A. galli*
12	MW136454	China	*A. galli*
13	MN158368	China	*A. galli*
14	MW218977	China	*A. galli*
15	MZ068097	China	*A. galli*
16	MZ068098	China	*A. galli*
17	OM876363	China	*A. galli*
18	PP809092	China	*A. galli*
19	PP809151	China	*A. galli*
20	OP117138	Bangladesh	*A. galli*
21	OP117140	Bangladesh	*A. galli*
22	OR619546	Iran	*A. galli*
23	MF319969	Out group	*Heterakis dispar*

* Sequences obtained in the present study.

Table 2: Estimates of evolutionary divergence among ITS sequences of *A. columbae*, *A. galli*, and the outgroup *H. dispar*. Shaded cells represent the sequences obtained in the present study. Analyses were conducted using the Maximum Composite Likelihood method within the MEGA v.11 program, with an overall distance of 0.0212

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	OP215354_*A.columbae*_Egypt_Domestic_Pigeon																							
2	OP215355_*A.columbae*_Egypt_Domestic_Pigeon	0.005																						
3	MZ343369_*A.columbae*_Egypt_Domestic_Pigeon	0.011	0.013																					
4	KC905082_*A.columbae*_China_Goose	0.009	0.007	0.013																				
5	MW599311_*A.galli*_Egypt	0.007	0.009	0.009	0.013																			
6	MW599312_*A.galli*_Egypt	0.009	0.011	0.011	0.015	0.002																		
7	PQ047113_*A.galli*_Egypt	0.009	0.011	0.011	0.015	0.002	0.004																	
8	KX683286_*A.galli*_Poland	0.007	0.009	0.009	0.013	0.000	0.002	0.002																
9	KY789470_*A.galli*_Poland	0.009	0.011	0.011	0.015	0.002	0.000	0.004	0.002															
10	MW827787_*A.galli*_China	0.007	0.009	0.009	0.013	0.000	0.002	0.002	0.000	0.002														
11	MW218976_*A.galli*_China	0.011	0.013	0.013	0.016	0.004	0.002	0.002	0.004	0.002	0.004													
12	MW136454_*A.galli*_China	0.013	0.014	0.014	0.018	0.005	0.004	0.004	0.005	0.004	0.005	0.002												
13	MN158368_*A.galli*_China	0.011	0.013	0.013	0.016	0.004	0.002	0.002	0.004	0.002	0.004	0.000	0.002											
14	MW218977_*A.galli*_China	0.009	0.011	0.011	0.015	0.002	0.004	0.004	0.002	0.004	0.002	0.005	0.007	0.005										
15	MZ068097_*A.galli*_China	0.007	0.009	0.009	0.013	0.000	0.002	0.002	0.000	0.002	0.000	0.004	0.005	0.004	0.002									
16	MZ068098_*A.galli*_China	0.007	0.009	0.009	0.013	0.000	0.002	0.002	0.000	0.002	0.000	0.004	0.005	0.004	0.002	0.000								
17	OM876363_*A.galli*_China	0.007	0.009	0.009	0.013	0.000	0.002	0.002	0.000	0.002	0.000	0.004	0.005	0.004	0.002	0.000	0.000							
18	PP809092_*A.galli*_China	0.007	0.009	0.009	0.013	0.000	0.002	0.002	0.000	0.002	0.000	0.004	0.005	0.004	0.002	0.000	0.000	0.000						
19	PP809151_*A.galli*_China	0.009	0.011	0.011	0.015	0.002	0.000	0.004	0.002	0.000	0.002	0.002	0.004	0.002	0.004	0.002	0.002	0.002	0.002					
20	OP117138_*A.galli*_Bangladesh	0.007	0.009	0.009	0.013	0.000	0.002	0.002	0.000	0.002	0.000	0.004	0.005	0.004	0.002	0.000	0.000	0.000	0.000	0.002				
21	OP117140_*A.galli*_Bangladesh	0.011	0.013	0.013	0.016	0.004	0.002	0.005	0.004	0.002	0.004	0.004	0.005	0.004	0.005	0.004	0.004	0.004	0.004	0.002	0.004			
22	OR619546_*A.galli*_Iran	0.013	0.014	0.014	0.018	0.005	0.004	0.004	0.005	0.004	0.005	0.002	0.000	0.002	0.007	0.005	0.005	0.005	0.005	0.004	0.005	0.005		
23	MF319969_*Heterakis_dispar*_Out_group	0.187	0.192	0.192	0.200	0.184	0.186	0.186	0.184	0.186	0.184	0.189	0.186	0.189	0.186	0.184	0.184	0.184	0.184	0.186	0.184	0.189	0.186	

5. Conclusion

The combination of detailed morphological and molecular studies provides a comprehensive understanding of *A. columbae*, confirming its taxonomic placement and highlighting its unique structural traits that facilitate accurate identification and classification. Molecular markers, particularly the ITS regions, have proven essential for precise and consistent species identification across various studies. ITS sequencing not only enables reliable identification but also uncovers genetic diversity that may be overlooked in morphological analysis. After identifying these worms, further investigations should focus on developing effective and safe anti-worm treatments to combat such parasitic infestations.

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