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Occurrence and molecular identification of *Contracaecum* larvae (Nematoda: Anisakidae) of the marine fish, *Dicentrarchus labrax* in Egypt

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ABSTRACT

Globally, fish-borne nematodes are a major public health hazard. Anisakidosis is a zoonotic infection caused by members of the family Anisakidae. *Contracaecum* (Railliet and Henry, 1912) is one of the most common genera in this family. Anisakid larvae in fish pose a risk for humans and reduce their marketability. The present study found anisakid nematodes of the genus *Contracaecum* as third-stage larvae in the European Seabass *Dicentrarchus labrax* collected from the Mediterranean Sea, Egypt. The larvae were observed encapsulated on the surface of various abdominal organs and embedded in fish muscles. The prevalence of the parasite was 52.72% (29 out of 55 fishes), with a mean intensity of 7.86 ±0.69. Morphological examinations of the studied larva using light and scanning electron microscopy revealed a head region with a prominent boring tooth, inconspicuous lips, and a distinct protruded cylindrical mucron. These findings are supported by phylogenetic analysis based on ITS region using maximum likelihood, corroborating the evidence that L3 larvae parasitizing the *D. labrax* belong to the species *Contracaecum quadripapillatum*. This study is the first to identify this parasite in the marine fish European Seabass, *D. labrax*, from Egyptian waters using morphological and molecular approaches.

INTRODUCTION

Contracaecum **(Railliet and Henry, 1912)** is the most abundant and diverse genus of the family Anisakidae, with approximately 140 species and a global distribution **(Shamsi, 2019; Angeles-Hernández *et al*., 2020)**. These nematodes can infect a wide variety of aquatic animals **(Di Azevedo *et al*., 2017)**. Third-stage larvae (L3) were commonly found in fish body cavities, branchial chambers, mesenteries, and muscles, whereas, adults were found in the guts of marine mammals and fish-eating birds **(Mattiucci and Nascetti, 2008)**. The high incidence of *Contracaecum* in fish may affect their health, and as a result, it may impact on the commercial worth of fish, especially when discovered in the muscles **(Angot and Brasseur, 1995)**. Anisakidosis can potentially be transferred to humans by raw or undercooked fish and seafood **(Shamsi and Butcher, 2011; Eiras *et al*., 2018; Martínez-Rojas *et al*., 2021)**. Contracaecosis is a

zoonotic infection caused by *Contracaecum* spp, characterized by stomach pains, fever, diarrhea, and vomiting **(Palm, 2004; Buchmann and Mehrdana, 2016; Mattiucci *et al*., 2018)**. The first documented case of human infection by those nematodes in Egypt was recorded by **Cocheton *et al*. (1991)**. Identification of fish-borne helminths species in clinical cases is thus critical for understanding zoonotic species and aiding in the prevention and treatment of diseases caused by them **(Shamsi, 2019)**.

Morphological identification of larval stages of *Contracaecum* is insufficient to differentiate species **(Mattiucci and Nascetti, 2008; Mattiucci *et al*., 2008; Garbin *et al*., 2013)**; hence molecular genetic approaches have become crucial tools for such species. There are few publications on the molecular identification of the larval stages of *Contracaecum* in fishes around the world **(Szostakowska and Fagerholm, 2007; Shamsi and Aghazadeh-Meshgi, 2011; Shamsi *et al*., 2017; Molnár *et al*., 2019; Pekmezci and Yardimci, 2019; Hamouda and Younis, 2022)**. Also, several authors in Egypt have described *Contracaecum* nematodes from freshwater fishes with little molecular data on them **(Al-Bassel 1990; Garo 1993; Al-Bassel 2003; Younis *et al*., 2017; Hamouda *et al*., 2018; Saad *et al*., 2018; Hefnawy *et al*., 2019; Taha 2020; Thabit and Abdallah, 2022)**. As a result, the purpose of this study was to identify the morphometric and morphological characteristics of the third-stage larva of *Contracaecum* infecting the commercial fish European Seabass *D. labrax* from the Mediterranean Sea, Egypt using light and scanning electron microscopy (SEM), in combined with molecular phylogenetic analysis, in order to confirm its taxonomic status.

MATERIALS AND METHODS

Ethical Approval

The current study was carried out following the guidelines approved by the Cairo University Institutional Animal Care and Use Committee (CU-IACUC), under the relevant document (No. CU/I/F/32/19).

1. Collection of fish

Fifty-five European Seabass, *Dicentrarchus labrax* (Family: Moronidae) were purchased from local fish markets in Alexandria, Egypt, on the Mediterranean Sea. The body cavity, digestive tract, and visceral organs of fish were dissected and examined for nematode parasites. Under white light, the musculature was sliced into thin slivers (1.0–2.0 mm thick) and visually inspected for parasites. Larvae were removed from the surrounding host tissues using a stereomicroscope, noting the site of infection, then washed n physiological saline, counted, and preserved in 70% ethanol until use.

2. Morphological examination

2.1. Light microscopy

The larvae were fixed in hot 70% ethanol, cleared with lactophenol **(Pritchard and Kruse, 1982)**. Identification was done at the genus level using available systematic keys based on morphological features of larval anisakids such as boring tooth or lips at the

anterior end, ventriculus length, postanal tail shape, and the presence or absence of a terminal mucron **(Yamaguti 1961; Gibbons 2010)**. All measurements were taken with an ocular micrometer, presented as a range with the mean ± S.E. in parentheses, and photographed with a LEICA DM 750 microscope.

2.2. Scanning electron microscopy

Nematode larvae were fixed in 2.5% glutaraldehyde. After 24 h, samples were post-fixed in 1% osmium tetroxide (OsO4) in phosphate buffer for another 24 h, then dehydrated through a graded ethanol series (50%, 60%, 70%, 80%, 90%, and 100%), and dried at 30°C for 30 min using a critical point drier (LEICA, EM CPD300). Dried specimens were mounted with carbon tape on aluminum stubs, coated with gold, and examined with a JEOL JSM-5200 SEM (Tokyo, Japan) at an accelerating voltage 25kV (**Guo *et al.*, 2014**).

3. Molecular analysis

3.1. DNA extraction

Following the manufacturer's protocol using a QIAamp® DNA Mini Kit (Qiagen), genomic DNA was extracted from individual larvae (25 specimens) after preservation in 70% ethanol.

3.2. PCR and DNA sequencing

The internal transcribed spacer (ITS1-5.8S-ITS2) region of ribosomal DNA (rDNA) was amplified using two universal primers; NC5 (forward; 5′-GTAGGTGAACCTGCGGAAGGATCATT-3′ and NC2 (reverse; 5′-TTAGTTTCTTTTCCTCCGCT-3′) **(Zhu *et al.*, 1998)**. The following PCR reactions were carried out in a total volume of 50 µl, as follows: 25 µl PCR Super-Mix (Genetech) containing dNTP, MgCl2, buffer, and Taq-polymerase,1µl of 10 Pmol of both forward and reverse primers; and 3 µl parasite genomic DNA; then it followed by 20 µl of nuclease-free water. Thermocycling conditions were as follows: an initial denaturation step at 94°C for 5 min, then 35 cycles of denaturation step at 94°C for 30 seconds, primer annealing at 58°C for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 7 min, according to **Costa *et al.* (2018)** with some modifications using a Thermal Cycler, Model FTC3/20 (TC-3000X, TECHNE, Bibby Scientific, and United Kingdom). PCR products were visualized by UV transilluminator (Cedex 1, France), then purified using a gel purification kit (Genedirex. Inc) and sequenced using an automated sequencer, ABI PRISM model 377, version 3.3.1 (Clinilab, Egypt).

3.3. Sequence alignment and phylogenetic analysis

The nucleotide sequence obtained in this study was deposited in GenBank under accession number OP750050. To detect sequence similarities, BLAST searches were done at the National Center for Biotechnology Information (NCBI) database (http:// www.ncbi.nlm. nih.gov) to find sequence similarities. The query sequence and those obtained from GenBank were aligned using Bioedit version 3.3.19.0. The phylogenetic tree was constructed using the maximum likelihood method based on the Kimura 2-

parameter model by the MEGA software version 11.0.10 **(Tamura *et al*., 2021)** with bootstrapping of 1,000 replications. *Procamallanus fulvidraconis* (DQ076698) was used as an outgroup.

RESULTS

A total of 194 nematode specimens were detected in 29 (52.72%) out of 55 *Dicentrarchus labrax* fish examined with a mean intensity of 7.86±0.69. All samples obtained belong to *Contracaecum* L3. The larvae were found encapsulated on the surface of various organs in the abdominal cavity or embedded in fish muscles.

1. Morphological description: (based on 5 specimens), (Figs. 1, 2)

The larvae had a cylindrical body that was attenuated at both ends and measured 19±0.2 (8–25) long and 0.68±0.1 (0.5–0.9) wide. Lips were inconspicuous, with a prominent boring tooth 0.02±0.004 (0.014-0.023) mm at the anterior extremity, and four small papillae (two dorsolateral and two ventrolateral) surrounding the triradiate mouth opening. The excretory pore was anteriorly located beneath the boring tooth, which opened ventrally. The worm's esophagus had a long anterior muscular part measured 1.0±0.1 (0.8–1.2) mm and a long ventriculus with an oblique esophago-intestinal junction. The cuticle had annular striations covering the surface of the juveniles with longitudinal and parallel striations that start from the cephalic region and extend to the slit-shaped anus. Most of the body was striated longitudinally, with transverse striation in the tail region. No intestinal caecum was observed. The tail was short-ended with a distinct mucron measured 0.06±0.001 (0.05–0.065) mm.

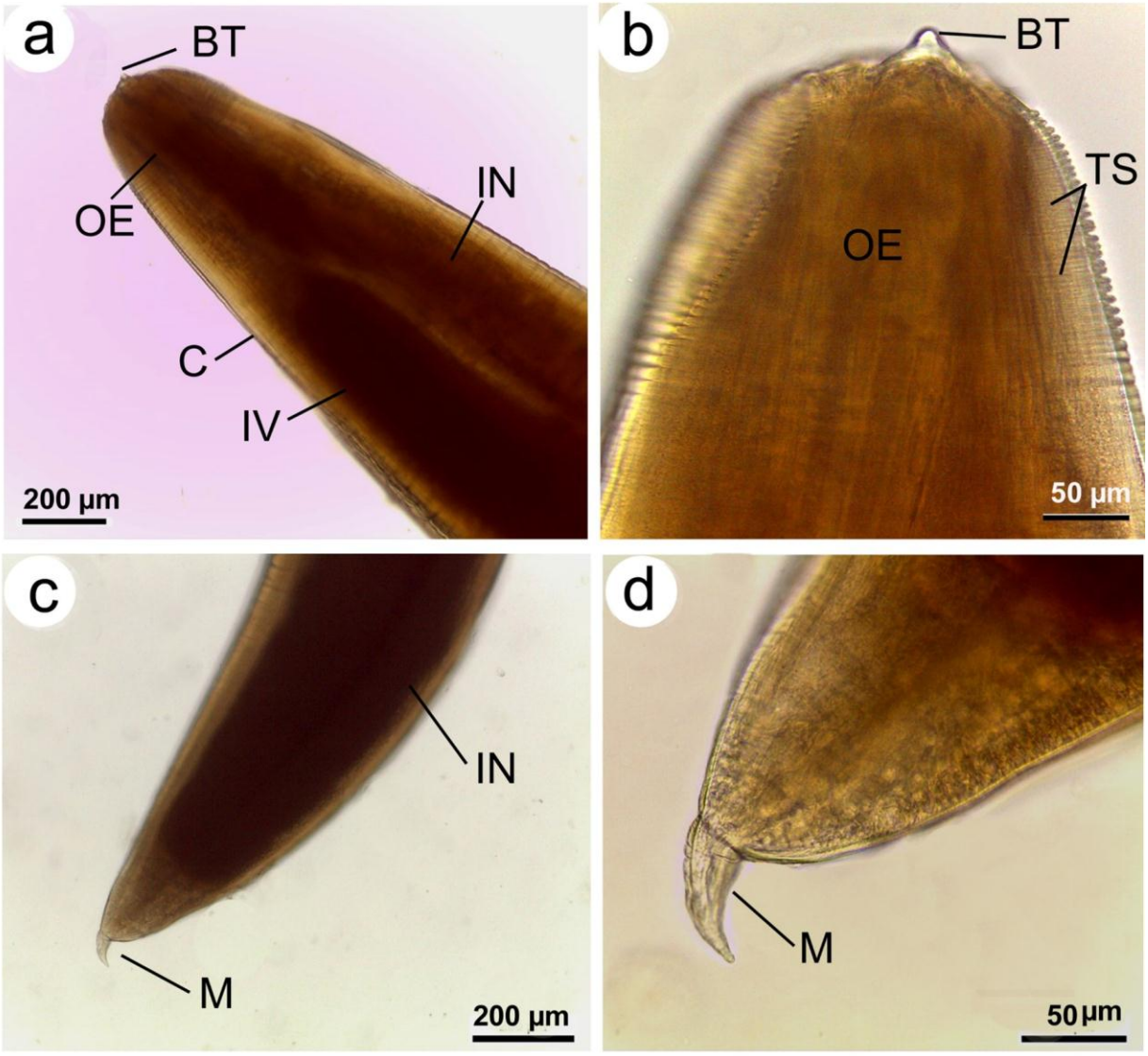

Fig. 1. Photomicrographs of Anisakid larvae *C. quadripapillatum* parasitizing the European Seabass *Dicentrarchus labrax* showing**: a)** Cephalic region with boring tooth (BT), esophagus (OE), ventriculus (IV), the distal part of the intestine (IN) and the body is covered with cuticle (C), scale bar = 200 µm**, b)** High magnification of the anterior part of the body showing boring tooth (BT), esophagus (OE) and transverse striations (TS) of body cuticle scale bar = 50 µm, **c)** Tail region with the posterior part of the intestine (IN) and the mucron (M), scale bar = 200 µm, **d)** Posterior extremity showing the characteristic mucron (M), scale bar = 50 µm

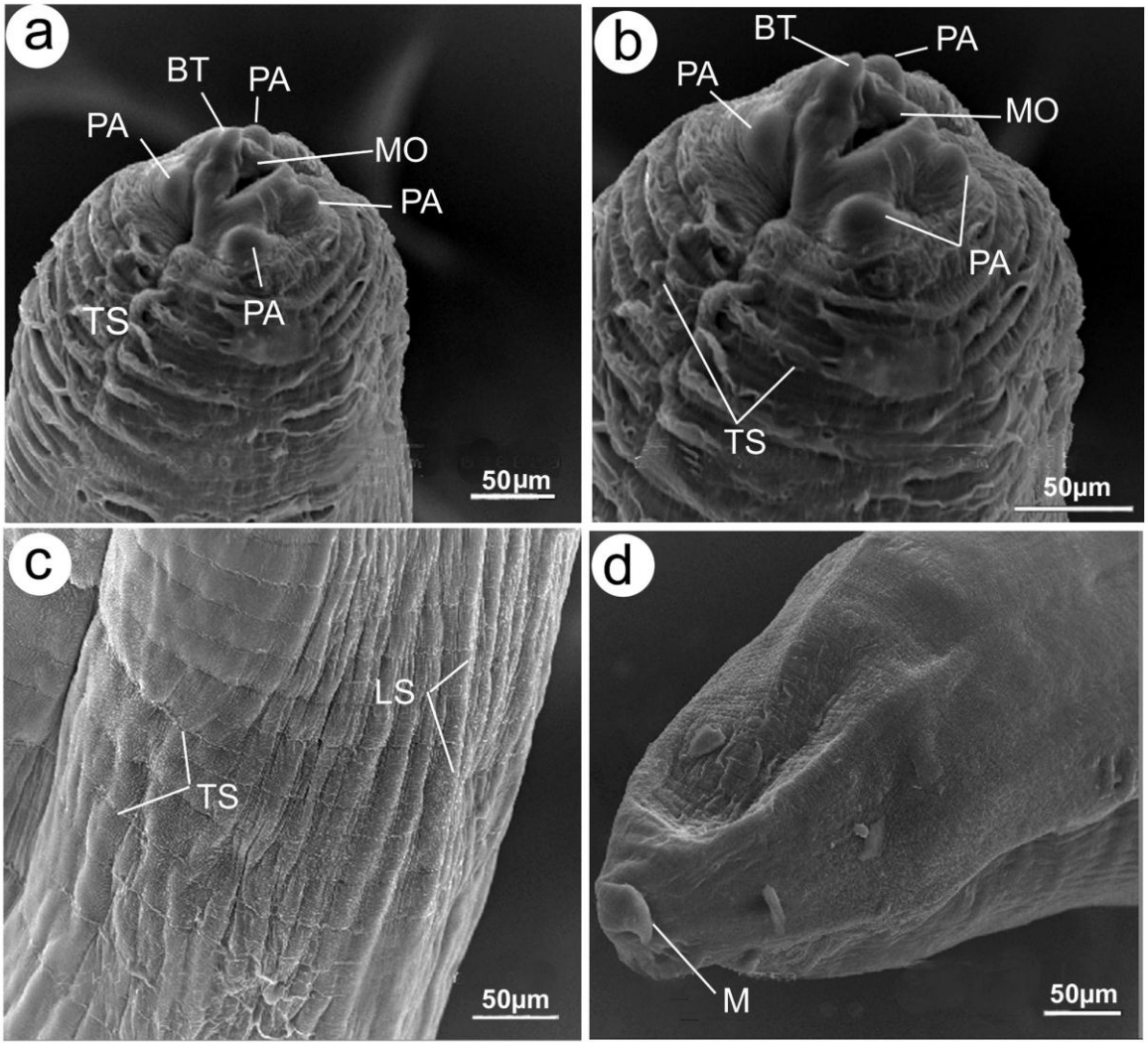

Fig. 2. Scanning electron micrograph showing larvae of *C. quadripapillatum* showing: **a & b)** Anterior extremity of the body provided with triangular mouth opening (MO), a boring tooth (BT), four cephalic papillae (PA), and transverse striations (TS) of the body cuticle, scale bar = 50 µm **c)** Cuticle with transverse striations (TS) and longitudinal striations (LS), scale bar = 50 µm, **d)** Tail region showing characteristic mucron (M), scale bar = 50 µm.

2. Molecular analysis

Nucleotide sequencing of the ITS region of rDNA (ITS-1, 5.8S, and ITS-2) yielded 776 bp and was deposited in the GenBank under the accession number (OP750050). Compared to previously published sequences, the current ITS sequence revealed the highest similarity to *Contracaecum quadripapillatum* with the accession numbers (ON714985, 97 %; query coverage), (ON714982, 96.01; query coverage 100%) and (OK138878, 97.54%; query coverage 52%) as shown in **(Fig. 3).** Moreover, the phylogenetic tree of ITS region of different *Contracaecum* larvae was constructed

using maximum likelihood (ML) method inferred a topology strongly supported the monophyly of *Contracaecum* spp. In terms of clades, the nematode larvae examined in this study were clustered with *C. quadripapillatum* based on 100 % bootstrap value, as presented in **(Fig. 4).**

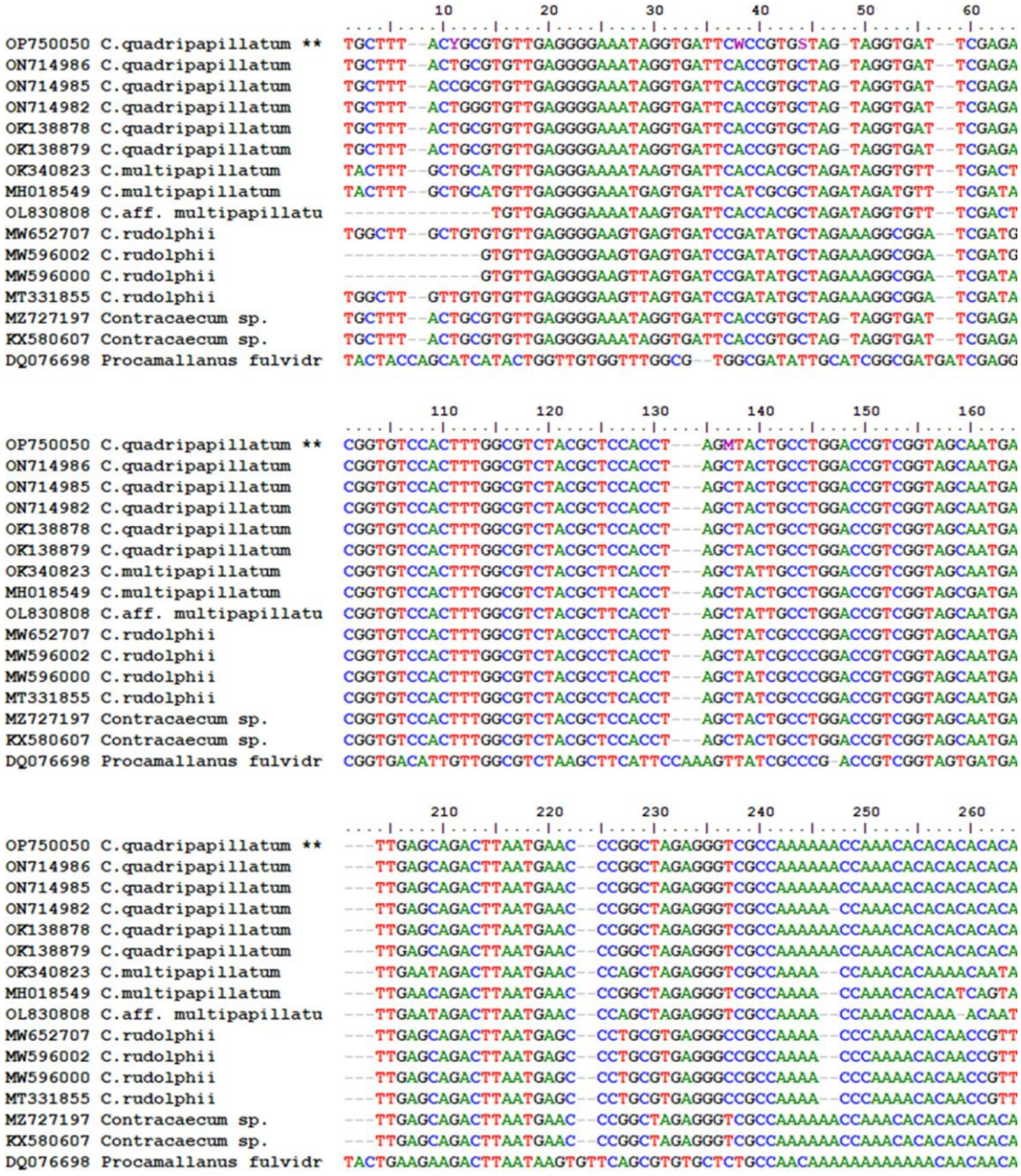

Fig. 3. Multiple sequence alignment of ITS region of *C. quadripapillatum* ** larvae in the present study (accession number: OP750050 **) with the most similar sequences in the GenBank database using Bioedit (version 3.3.19.0).

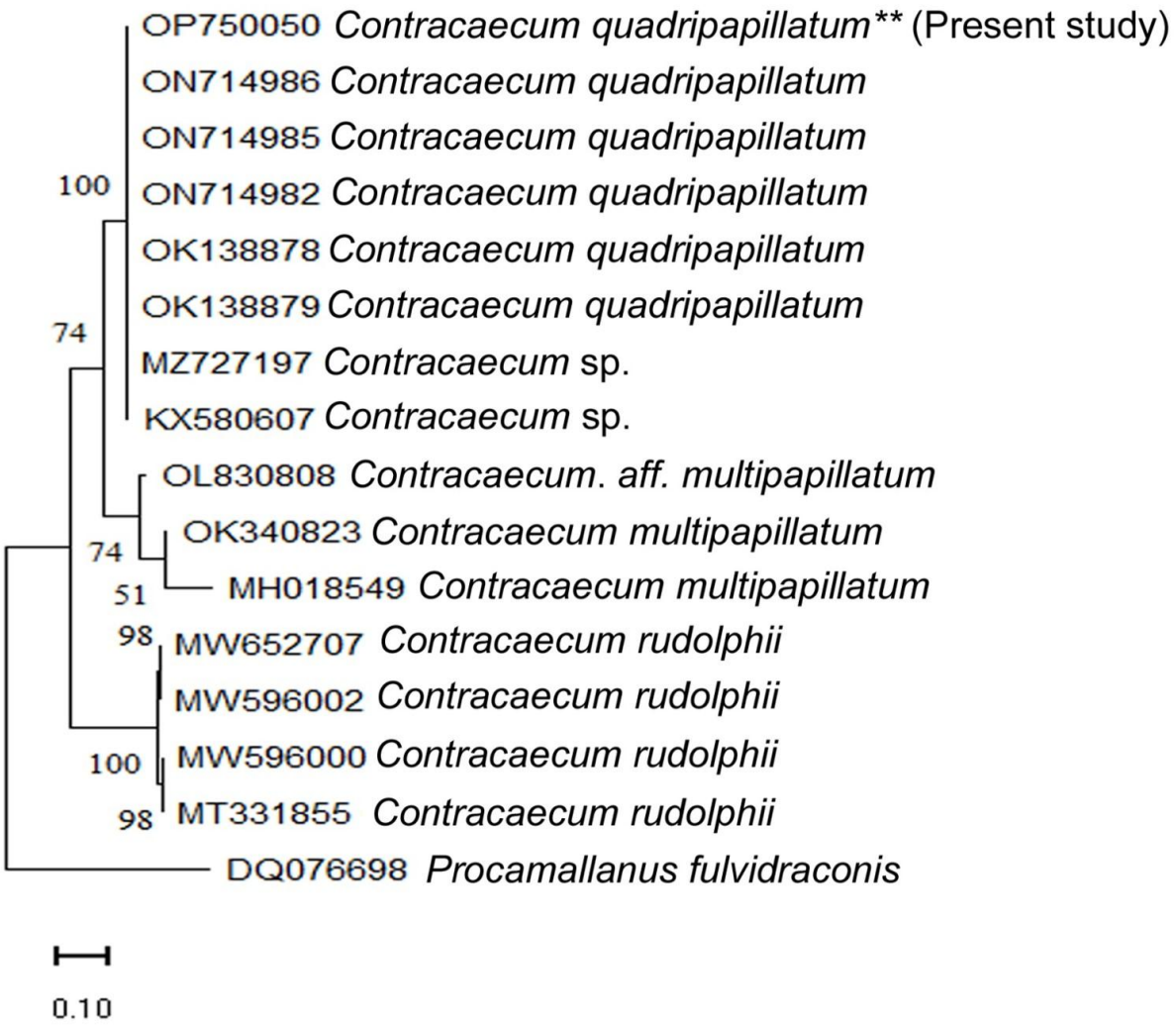

Fig. 4. Maximum likelihood showing the phylogenetic relationships of *C. quadripapillatum* larva reported herein based on ITS of rDNA with other different species using Kimura 2-parameter model and 1,000 boostrap replications with a complete deletion. Bootstrap support values are indicated above the nodes and *Procamallanus fulvidraconis* was used as an outgroup. Asterisks represent the present sample (accession number: OP750050 **).

DISCUSSION

The zoonotic anisakid larvae are not host-specific and infect a wide variety of marine teleost species from the Atlantic to the Antarctic, via the Mediterranean and the Pacific **(McClelland and Martell, 2001; Buchmann and Mehrdana, 2016)**. In Egypt, little is known about the distribution of *Contracaecum* larvae in fish, especially those of commercial importance and that are consumed primarily by humans **(Taha, 2020).** *Dicentrarchus labrax* is a popular commercial marine fish in Egypt, but the presence of

Contracaecum larvae decreases its marketability, highlighting the significance of these fish as sources of human anisakiasis **(Dorny *et al.*, 2009; Shih *et al.*, 2010)**. The current study found a high prevalence of infection (52.72%) by the third stage larvae of *Contracaecum* that were encapsulated on the visceral organs and musculature of *D. labrax* fish, using visual inspection without incubation. Nevertheless, **Shamsi and Suthar (2016)** stated that both visual examination and incubation were more successful ways for detecting anisakid nematodes in fish. **Smith and Wootten (1975)** reported that these parasites can migrate from the fish's internal organs to its flesh, explaining their presence in the muscles.

The current species belongs to the genus *Contracaecum* based on the following morphological characteristics such as, the presence of inconspicuous lips with a prominent boring tooth on the anterior end, four cephalic papillae, the cuticle with clearly transverse striations, and the tail is terminated by a distinct mucron, which are consistent with the previous descriptions by **Moravec and Justine (2015)** and **Moravec *et al.* (2016)**. With slight differences in morphometric data, all retrieved larvae were found to share most of the morphological features reported by **Thabit and Abdallah (2022)** from the Nile perch *Lates niloticus*. Also, it revealed body dimensions that were much greater than those described by **Abdullah *et al.* (2021)**, including total body length, maximum width, and esophageal length. Some morphometric features overlapped **(Table 1),** indicating that morphological analysis may not be a good tool for distinguishing larval species; this emphasizes the need of relying on modern molecular methods in distinguishing larvae species **(Jorge *et al.*, 2014)**.

Table 1. Morphometric comparison (in mm) of the present *Contracaecum quadripapillatum* with some previously described species

Species / Aspects	***Contracaecum* sp.**	***C. quadripapillatum***	***C. quadripapillatum***
Host	*Cyprinus carpio*	Nile perch *Lates niloticus*	*Dicentrarchus labrax*
Regional distribution	Sulaimani Province, Kurdistan Region, Iraq	The Nile River, Assiut Governorate, Egypt.	The Mediterranean sea, Egypt
Authors	Abdullah *et al*. (2021)	Thabit and Abdallah (2022)	Present study
Total body length	3.20-3.80 (3.50)	12–36 (25.7 ± 0.9)	19±0.2 (8–25)
Maximum body width	0.22-0.28 (0.25)	0.41– 1.08 (0.81 ± 0.02)	0.68±0.1 (0.5–0.9)
Boring tooth length	0.004-0.006 (0.005)	0.01–0.02 (0.010 ± 0.0004)	0.02±0.004 (0.014-0.023
Tail process length (mucron)	--	0.01–0.09 (0.05 ± 0.002)	0.06±0.001(0.05–0.065)
Esophagus length	0.60-0.80 (0.70)	1.7–3.5 (2.5 ± 0.06)	1.0±0.1 (0.8–1.2)

Several genes, including the internal transcribed spacer region (ITS), cytochrome oxidase subunits cox1 and cox2, have been used to identify *Contracaecum* larvae at the species level **(Mattiucci *et al.*, 2015; Younis *et al.*, 2017; Zuo *et al.*, 2018; Zhang *et al.*, 2018).** The ITS1-5.8S-ITS2 region of ribosomal DNA has been useful as genetic marker in confirming species identification **(Jabbar *et al.*, 2013; Mattiucci *et al.*, 2014; Abdullah *et al.*, 2021),** and allowing precise diagnosis **(Kim *et al.*, 2006; Liu *et al.*, 2015)**, as this region displays higher nucleotide sequence differences between species. Because some *Contracaecum* species with similar morphology differ genetically **(Mattiucci *et al.*, 2020)**, ITS sequence analysis was used to support morphological larval identification in this investigation. The highest genetic resemblance was found to be 97 % and 96.01 % to specimens (ON714985 and ON714982), respectively from Italy **(Caffara, 2022)** and 97.54% (ON138878) from Lake Nasser, Egypt **(Hamouda and Younis, 2022)**. Furthermore, the phylogenetic tree revealed that *Contracaecum* larvae were closely related to *C. quadripapillatum* based on comparisons with other published species. Finally, unlike previous research that documented *Contracaecum* larvae in Egyptian freshwater fishes, this study established the first molecular identification of *Contracaecum* from marine fish.

CONCLUSION

The current work highlights the significance of combining morphological and molecular techniques in validating species identification of *Contracaecum* third larval stage. The European Seabass, *D. labrax*, is a novel transport host, and Egypt's Mediterranean Sea coasts are a new location for this parasite. The high infection level of the *Contracaecum* larvae indicates a substantial risk of contracaecosis and hence a potential health threat for human infected fish must be gutted and properly cooked before consumption.

CONFLICT OF INTEREST

The authors declared that there is no conflict of interest.

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