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Morphological description and molecular characterization of *Contracaecum* larvae (Nematoda: Anisakidae) parasitizing market-size hybrid tilapia (*Oreochromis aureus* x *Oreochromis niloticus*) and red drum (*Sciaenops ocellatus*) farmed in Israel

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ABSTRACT

Nematodes belonging to the genus *Contracaecum* (family: Anisakidae) are heteroxenous parasites with a complex life cycle. *Contracaecum* larvae infecting farmed fish and fishery products are economically important causing market rejection in massive infection and may have zoonotic potential. In Israel, *Contracaecum* larvae have been described morphologically in several fish species; however, none of these descriptions were supported by molecular tools. In 2019–2020, hybrid tilapia (*Oreochromis aureus* x *Oreochromis niloticus*) and red drum (*Sciaenops ocellatus*), farmed in polyculture were found to be heavily infected with nematodes referable to *Contracaecum* larvae. Prevalence of infection in hybrid tilapia and red drum was 53.8% and 40.9%, respectively. A combined (morphological and molecular) approach revealed that both infected fish species were parasitized by the same species of *Contracaecum*, although larvae in hybrid tilapia were localized in the pericardial cavity whereas in red drum, they were observed in the abdominal cavity. Genetic analysis of internal transcribed spacer rDNA and *cox*2 mtDNA showed high similarity to unidentified *Contracaecum* larvae detected in several fish species in Ethiopia, Egypt and Kenya. In this study, molecular and morphological analyses place the possible new species in the *C. multipapillatum* complex and was provisionally named *C. multipapillatum* E. Further analyses combining morphological and molecular approaches are required on adult

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specimens collected from piscivorous birds living in the same area to support the identification of a potentially new species.

1. Introduction

The genus *Contracaecum* consists of complexes of sibling species which parasitize piscivorous birds, principally of the family Pelecanidae (Landsberg, 1989; Mattiucci et al., 2010), Phalacrocoracidae, Ardeidae and others (Shamsi, 2019). The life cycle is complex and has yet to be fully clarified for most of the reported species. Shamsi (2019) summarized the general life cycle as follows: eggs are shed in water by the definitive hosts, they embryonate into first-stage larvae (L1) inside eggs and then further develop and molt into L2. Either eggs or larvae can be ingested by a wide variety of aquatic invertebrates which act as intermediate hosts (Mozgovoi et al., 1965; Semenova, 1971, 1979; Norris and Overstreet, 1976), although their role in the natural transmission to fish is still unclear (Anderson, 2000), and a large number of fish species as second intermediate/paratenic hosts. Several species of piscivorous birds and mammals inhabiting marine, brackish or freshwater environments act as definitive hosts by ingesting parasitized fish (Shamsi, 2007). Despite this general information on the life cycle, some researchers have described different patterns based on experimental infection with some *Contracaecum* species (i.e. Huizinga, 1966; Bartlet, 1996). Among others, Moravec (2009) carried out experimental trials to better understand the life cycle of *Contracaecum rudolphii*, by assessing the possible direct transmission of this parasitic species through the ingestion of eggs and free-living larvae with or without passing through paratenic (metaparatenic) invertebrate and fish hosts.

In Israel, Paperna (1964) published the earliest report of *Contracaecum* larvae infecting fish; he described morphologically five types of *Contracaecum* larvae from various fish species in different water bodies (Table 1). Later on, Landsberg (1989) reported that *Contracaecum* larval infections are common in the Israeli pond-cultured tilapia hybrids, with observations in tilapia of 200–350 g of up to 12 worms, which could reach a length of 6 cm and 2–3 mm in diameter, inhabited the pericardial cavity, and remained unencysted (Table 1). Landsberg (1989) claimed that heavy infections by *Contracaecum* larvae had become a problem for the tilapia sector since 1982. Recently, Smirnov et al. (2021) reported the presence of *Contracaecum multipapillatum* larvae in the pericardial cavity of hybrid tilapia, but with no molecular confirmation. Pericardial infections by *Contracaecum* larvae are common (30–70%) in cichlids

Table 1
Contracaecum spp. described in Israel along timeline.

Host	Geographical location	Infection site in host	Measurements (mm)	Identification method	Reference
Kinneret bream (*Mirogrexterraesanctae*), Levantine minnow (*Garra nana*) (formerly: *Tylognathus steinitziorum*), Levantine scraper (*Capoeta damascina*) (formerly: *Varicorhinus damascinus*), longhead barbel (*Luciobarbus longiceps*), North African catfish (*Clarias gariepinus*) (formerly: *Clarias lazera*), redbelly tilapia (*Coptodon zillii*) (formerly: *Tilapia zillii*)	Sea of Galilee	Serosae	Length: 6–8 Width: nr	Morphological	Paperna (1964)
Thinlip grey mullet (*Chelon ramada*) (formerly: *Mugil capito*)	Streams of the coastal Mediterranean plain	Serosae	Length: 6–8 Width: nr	Morphological	Paperna (1964)
Leaping mullet (*Chelon saliens*) (formerly: *Mugil saliens*) and pompano (*Trachinotus ovatus*) (formerly: *Lichia glauca*)	Estuary zone of the streams of the coastal Mediterranean plain	Serosae	Length: 3.5–4 Width: nr	Morphological	Paperna (1964)
North African catfish (*C. gariepinus*)	Sea of Galilee and Hula nature reserve	Serosae	Length: 20–22 Width: nr	Morphological	Paperna (1964)
Flathead grey mullet (*Mugil cephalus*)	Estuary zone of the streams of the coastal plain	Gut	Length: 38 Width: nr	Morphological	Paperna (1964)
Tilapia sp.	Hula nature reserve	Body cavity	Length: 40–46 Width: 4.5	Morphological	Paperna (1964)
Hybrid tilapia (*Oreochromis aureus* x *Oreochromis niloticus*)	A fish farm near Hula nature reserve	Pericardial cavity	Length: 60 Width: 2–3	Morphological	Landsberg (1989)
Great white pelican (*Pelecanus onocrotalus*)	nr	nr	nr	Morphological	Landsberg (1989)
Cyclops	Fish ponds	Intestinal wall and hemocoel	nr	Morphological	Landsberg (1989)
Hybrid tilapia (*O. aureus* x *O. niloticus*)	Fish pond	Pericardial cavity	nr	nr	Smirnov et al. (2021)
Hybrid tilapia (*O. aureus* x *O. niloticus*)	Fish farm in Kfar Ruppin, Valley of the Springs region	Pericardial cavity	Length: 34–46 Width: 11–16	Morphological and molecular	This study
Red drum (*Sciaenops ocellatus*)	Fish farm in Kfar Ruppin, Valley of the Springs region	Caudal part of the abdomen cavity	Length: 29–38 Width: 13–16	Morphological and molecular	This study

nr = not reported.

(*Oreochromis* spp., *Tilapia* spp. and *Haplochromis* spp.) in African lakes (Paperna, 1974; Younis et al., 2017) *Contracaecum* larvae of apparently different species occur in the peritoneum and mesenteries of numerous fish species representing most African fish families (Khalil and Polling, 1997; Barson, 2004; Moravec et al., 2016).

Fish-borne larval nematodes belonging to the family Anisakidae with the three genera *Anisakis*, *Pseudoterranova* and *Contracaecum* are of a cosmopolitan dispersion among wild and farmed fish populations (Aibinu et al., 2019; Al Quraishy et al., 2019; Shamsi, 2019) and the infective third-stage larva (L3) in fish is able to infect humans ingesting raw or undercooked fish products which may elicit severe clinical symptoms (Shamsi and Butcher, 2011; Nagasawa, 2012).

Globally, tilapias and other cichlid species are the second most important group of farmed fish after cyprinids (FAO, 2020). These groups of fish provide an affordable protein source, especially in developing countries. In 2018, global tilapia production (aquaculture and capture) amounted to 5.5 million tons. In Israel, tilapia is the main farmed species (Skornik et al., 2021); the annual production in 2019 was 5.4 thousand tons and 4.9 thousand tons in 2020, equal to 36% and 32% of the total aquaculture production, respectively (data provided by the Food Safety of Animal Products Department, Israeli Veterinary Services).

Red drum (*Sciaenops ocellatus*) is one of the most important marine-cultured fish species in the USA and China because it is fast-growing, has strong resistance to infectious pathogens, is easy to manage and is adaptable to large-scale sea-resistant net-cage aquaculture (Zhou et al., 2010; Li et al., 2013). In Israel, *S. ocellatus* is usually farmed in polyculture in earth ponds with all-male populations of tilapia hybrids (*O. aureus* x *O. niloticus*). Because of its voracious predatory nature, the Israeli fish breeders use red drum also for biological control, to manage the uncontrolled reproduction of tilapia in these ponds. In 2019, the annual production of *S. ocellatus* in Israel was 136 tons, and 150 tons in 2020, equal to 1% of the total aquaculture production (data provided by the Food Safety of Animal Products Department, Israeli Veterinary Services).

Herein, we describe *Contracaecum* larvae parasitizing market-size hybrid tilapia (*O. aureus* x *O. niloticus*) and red drum (*S. ocellatus*) polycultures in Israel, using morphological and molecular approaches.

2. Materials and methods

2.1. Fish sampling

The first sampling was carried out in November 2019 (fish-sorting station A) and was composed of 50 market-size hybrid tilapia (*O. aureus* x *O. niloticus*) (600–800 g) and 3 flathead grey mullet (*Mugil cephalus*) (800–1000 g) from a 6-ton batch of market-size fish. The second sampling was in May 2020 (fish-sorting station B) and included 15 hybrid tilapia (600–800 g), 22 red drum (*S. ocellatus*) (400–600 g), 3 flathead grey mullet (800–1000 g) and 2 black carp (*Mylopharyngodon piceus*) (800–1000 g) from 2 tons of market-size fish. The fish from both shipments originated from the same pond, in a farm located in Kfar Ruppin in the Valley of the Springs region (Fig. 1) of northeastern Israel, where most of the inland aquaculture takes place (Scholz et al., 2021). At the sorting stations, fish were stunned and euthanized by immersion in an ice-water slurry under a public veterinarian's supervision. Randomly selected fish were

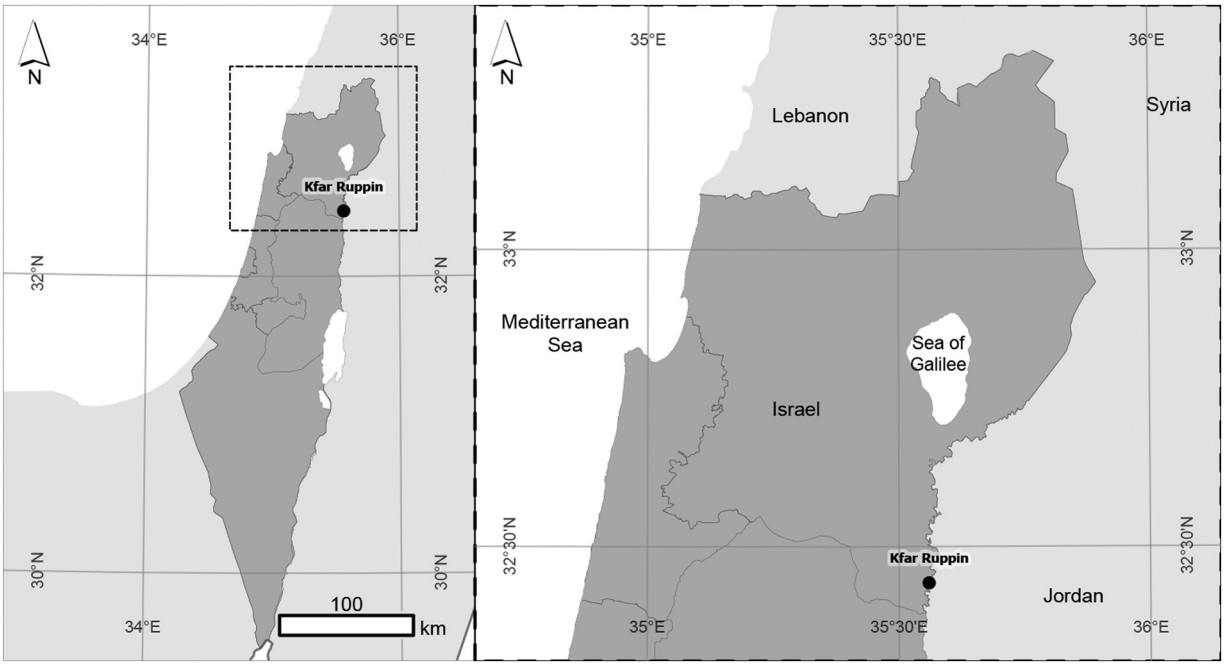

Fig. 1. Map of Israel. Fish specimens originated from a farm located in Kfar Ruppin (Valley of the Springs region) in northeastern Israel (inset right map).

then subjected to routine visual inspection, including macroscopic examination for the presence of zoonotic parasites as specified in the procedure for premarketing control of locally grown edible fish (Israeli Veterinary Services, 2017).

All of the collected parasites were preserved in 70% ethanol for both morphological and molecular analyses. Mean intensity (MI) and mean abundance (MA) were calculated following Bush et al. (1997).

2.2. Morphological examination

Twenty larvae, 10 from hybrid tilapia and 10 from red drum, were observed under a dissection microscope to evaluate gross morphology, then under a light microscope (Leica Microsystems, Wetzlar, Germany) to record total length (TL) with the aid of a digital Nikon DS-Fi1 camera and image-acquisition software (Nikon Nis-Elements D3.0), also used for further morphometrics. A small part, about 5 mm, was dissected from the central portion of the nematodes, where taxonomical features are not present, for molecular studies. Anterior and posterior portions of the parasite body were clarified in Amman's lactophenol to measure the internal taxonomical structures by light microscope. Morphometric analysis was conducted following Berland (1961), Anderson (2000) and Younis et al. (2017). Measures are given in micrometers unless otherwise indicated.

For scanning electron microscopy (SEM), anterior and posterior portions of 4 nematodes were dehydrated through a graded ethanol series, subjected to critical point drying, sputter-coated with gold palladium, and observed using a Phenom XL G2 Desktop SEM (Thermo Fisher Scientific, Eindhoven, The Netherlands) operating at 5 kV.

2.3. Molecular identification

For molecular analysis, genomic DNA was extracted from 20 central pieces of larvae (10 from hybrid tilapia and 10 from red drum) using a PureLink® Genomic DNA Kit (Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions. Amplification of the ITS rDNA region was performed with the primers NC5_f (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2_r (5′-TTAGTTTCTTCCTCCGCT-3′) (Zhu et al., 1998). The *cox*2 mtDNA was also amplified, with primers 211_f (5′-TTTTCTAGTTATATA-GATTGRTTTYAT-3′) and 210_r (5′-CACCAACTCTTAAAATTATC-3′) of Mattiucci et al. (2008), following the same protocol. The PCR products were electrophoresed on a 1% agarose gel stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, Carlsbad, CA,

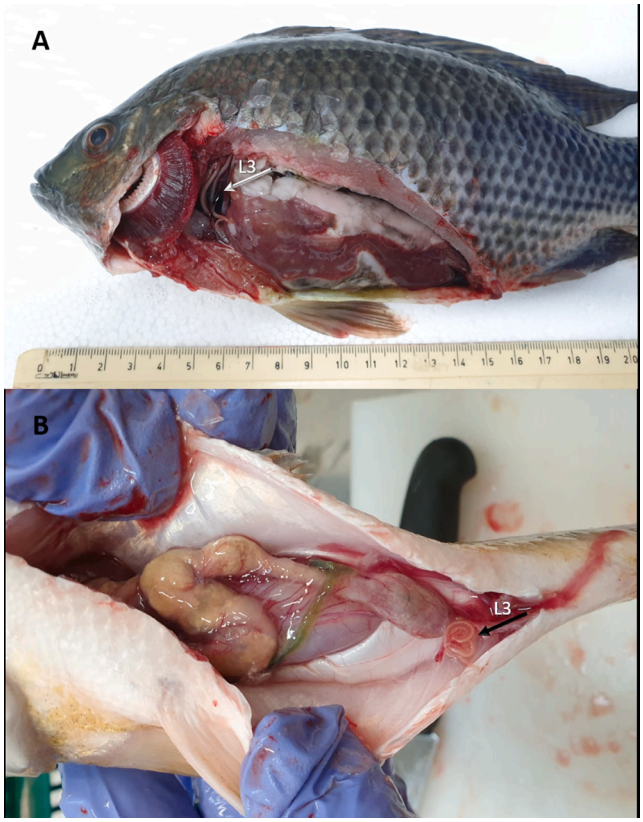

Fig. 2. *Contracaecum* sp. third-stage larvae (L3) in: (A) the pericardial cavity (arrow) of hybrid tilapia (*Oreochromis aureus* x *O. niloticus*); and (B) the abdominal cavity (arrow) of red drum (*Sciaenops ocellatus*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

USA) in 0.5× TBE. For sequencing, the amplicons were excised and purified by Nucleo-Spin Gel and PCR Clean-up (Mackerey-Nagel, Düren, Germany), and sequenced with an ABI 3730 DNA analyzer (StarSEQ, Mainz, Germany). The DNA trace files were assembled with Contig Express (VectorNTI Advance 11 software, Invitrogen, Carlsbad, CA, USA), and the consensus sequences of the ITS after separating the two regions (ITS1 and ITS2) and *cox*2 were compared with previously published data by BLAST tools (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Multiple sequence alignments were performed using BioEdit 7.2.5, p-distance and maximum-likelihood (ML) tree (K2 + G + I substitution model for ITS and KHY + G + I for *cox*2, bootstrap of 1000 replicates for both genes) were obtained using MEGA 7. The ITS1 and ITS2 rDNA sequences were then concatenated and used to build a ML tree together with the sequences of *Contracaecum* spp. reported by Mattiucci et al. (2020). The *cox*2 gene was also aligned with the sequences reported by Mattiucci et al. (2020).

The sequences generated in this study have been deposited in GenBank under accession numbers OL830790-OL830809 for ITS rDNA and OL809970-OL809986 for *cox2*.

3. Results

Overall, 35 out of 65 (53.8%) hybrid tilapia examined showed unencysted nematodes referable to *Contracaecum* larvae in the pericardial cavity (Fig. 2A) for a total of 83 larvae (MI = 2.4 and MA = 1.3); 9 out of 22 (40.9%) red drum specimens were infected with 18 *Contracaecum* larvae (MI = 2 and MA = 0.8) in the abdominal cavity around the distal part of the intestine (Fig. 2B). No nematodes were observed in any of the other fish species examined. Table 2 summarizes the results of both samplings.

3.1. Morphology

Larvae from tilapia and red drum showed overlapping morphological features; the main morphometric parameters of 10 subjects from each fish species are detailed in Table 3.

The larvae had a TL of 3.6 ± 0.5 (2.23–4.6) cm, with a stout body thinning at the extremities where the cuticular ridges, extending along the whole body, were more evident. Internal anatomy after clarification showed typical structures, such as the intestinal caecum (Fig. 3A) and the ventricular appendix (Fig. 3B) together with the subterminal position of the excretory pore, characterizing the genus *Contracaecum* among the Anisakidae.

Description (n = 20): cephalic end with three labial primordia, presence of a short and weakly evident boring tooth-like, 8.1 ± 1 (6.8–10) long, subterminal excretory pore very close to the oral opening (Fig. 4A); cuticle striated transversely; cuticular ridges more evident in the distal part of the cephalic end (Fig. 4B), interrupted by narrow lateral lines (Fig. 4C); narrow esophagus, 4426.3 ± 541 (2786.6–5452.3) long, ending in a small roundish ventriculus; intestinal caecum, 3372.8 ± 370 (2786.6–3926.2) long, extending beyond the nerve ring, the latter slightly visible; ventricular appendix, 1164.0 ± 370 (266.7–1547.3) long, much shorter than the caecum; length ratio of intestinal caecum and ventricular appendix 1:0.4; tail, 27.9 ± 8 (15.4–42.3) long, conical, ending with a pointed tip (Figs. 3C, 4D).

3.2. Molecular analysis

All of the nematodes, from both fish species, were successfully amplified and their ITS rDNA was identical (p-distance = 0%), confirming the morphological observations. Concerning the sequences of the *cox*2 mtDNA, the p-distance was 0.1–0.2%, confirming the intraspecific variability typical of this gene.

The sequences of the ITS rDNA were analyzed as ITS1 and ITS2, deleting the 5.8S rDNA. For ITS1 rDNA, the BLAST search gave 99.8% identity with a *Contracaecum* sp. from Ethiopia (MT450699–MT450702); unfortunately, the fish species from which the parasites were collected were not reported. Moreover, our ITS1 rDNA showed 99.5% identity with a *Contracaecum* sp. from *O. niloticus* sampled in Egypt (KX580603) and a *Contracaecum* sp. 1 from *Hydrocynus forskahlii* collected in Kenya (KF990491). ITS2 showed 100% identity with a *Contracaecum* sp. from *Sarotherodon galilaeus* (syn. *Tilapia galilaea*) and *O. niloticus* from Egypt (KX580608–09). All of the matching sequences were from L3. The ITS2 sequences from Ethiopia were not included in the concatenated tree as they are not available in GenBank. The ML tree (Fig. 5) showed our sequences forming a well-supported separate cluster together with *Contracaecum* sp. from Egypt. Moreover, the most closely related species, as sister taxa, were adults of *C. multipapillatum* (p-distance 0.10%;

Table 2
Fish specimens analyzed and number of specimens parasitized by *Contracaecum* larvae with prevalence, mean intensity and mean abundance.

Sampling date	Fish species	No. of PS/AS	NL	P (%)	MI[a]	MA[b]
November 2019	*O. aureus* x *O. niloticus*	24/50	63	48	2.6	1.2
	Mugil cephalus	0/3	0	0	0	0
May 2020	*O. aureus* x *O. niloticus*	11/15	20	73.3	1.8	1.3
	Sciaenops ocellatus	9/22	18	40.9	2	0.8
	M. cephalus	0/3	0	0	0	0
	Mylopharyngodon piceus	0/2	0	0	0	0

AS = Specimens analyzed; PS = Parasitized specimens; NL = number of larvae; P = Prevalence; MI = Mean Intensity; MA = Mean Abundance.
[a] Mean intensity, calculated as n. of larvae/n. parasitized fish.
[b] Mean abundance, calculated as n. of larvae/total n. of fish analyzed.

Table 3
Measurements (in μm) of *Contracaecum* larvae ($n = 20$) from hybrid tilapia (*O. aureus* × *O. niloticus*, 1–10) and red drum (*Sciaenops ocellatus*, 11–20).

Fish species	TL	MW	BT	Oes	IC	VA	Tail
1	46,182,52	1465.06	8.91	4761.78	3711.26	n.a.	23.21
2	39,418,81	1468.83	7.04	4021.32	2703.41	1242.61	30.68
3	39,289,33	1209.52	9.64	4035.45	3095.44	1281.08	22.82
4	43,593,09	1608.00	8.92	4390.23	3317.41	1435.02	26.49
5	44,415,14	1447.77	8.40	4856.47	3469.42	1343.17	30.68
6	34,743,2	1364.63	9.10	3686.73	2968.34	1390.42	31.54
7	34,320,9	1408.37	8.28	3948.59	2841.73	1138.31	33.73
8	36,617,95	1197.76	9.73	4471.47	3555.89	1762.62	29.57
9	35,626,51	1296.92	8.60	4505.18	3528.68	1162.42	33.35
10	41,448,11	1496.89	8.48	4229.76	3370.69	1426.4	37.05
11	25,387,17	1250,81	8,92	3540,08	2786,57	266,68	13,19
12	32,412,68	1520,72	8,58	4284,98	3378,88	1233,71	20,55
13	33,671,2	1423,66	8,15	5452,3	3479,88	1229,67	22,23
14	30,051,97	1364,16	7,59	4708,11	3926,25	n.a.	29,9
15	31,217	1351,74	7,11	3674,62	2906,25	1188,67	28,09
16	32,304,05	1395,34	8,08	4594,76	3629,51	1547,32	15,39
17	29,782,2	1379,94	9,97	4293,79	3041,62	1436,79	34,77
18	32,263,62	1605,26	9,21	4453,41	3417,98	1401,11	42,32
19	38,796,41	1670,13	7,59	4573,02	3366,94	1282,97	26,45
20	36,903,42	1406,18	6,79	4687,77	3793,97	889,47	31,54
Mean	35,922,26	1436,794	8199	4426,284	3372,785	1164,043	26,443
Min	25,387,17	1250,81	6,79	3540,08	2786,57	266,68	13,19
Max	46,182,52	1670,13	9,97	5452,3	3926,25	1547,32	42,32
SD	5396,104	126,0646	0,986,187	541,504	370,3129	384,3914	889,662

TL = total Length; MW = maximum width; BT = boring tooth; Oes = esophagus; IC = intestinal caecum (length); VA = ventricular appendix (length).

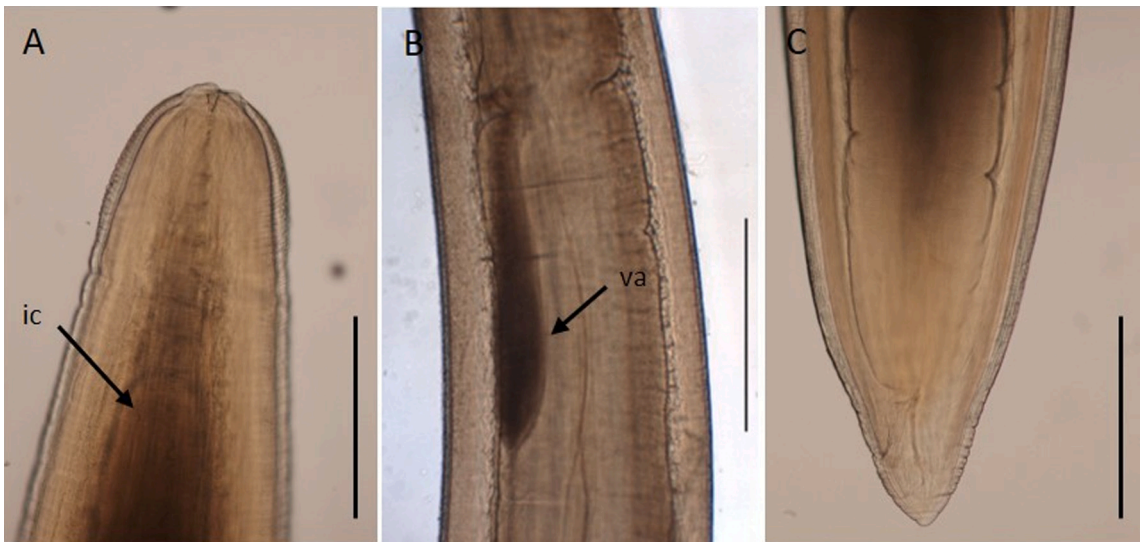

Fig. 3. *Contracaecum* larva from hybrid tilapia: (A) Cephalic region with distal part of intestinal caecum (ic); (B) caudal end; (C) ventricular appendix (va); scale bar = 500 μm.

AM940056) and *C. pyripapillatum* (p-distance 0.11%; AM940062), both from *Pelecanus conspicillatus* collected in Australia.

BLAST search of the *cox*2 mtDNA gene gave maximum similarity (87.9%) with *C. multipapillatum* (AF179910, Nadler and Hudspeth, 2000) from an unknown host, and 87.6–87.3% similarity with an undescribed *Contracaecum* sp. from *Mirounga leonina* (KF718924). The ML tree of the *cox*2 mtDNA gene (Fig. 6) showed our sequences forming a separated cluster with *C. gibsoni* (syn. *C. multipapillatum* A), *C. overstreeti* (syn. *C. multipapillatum* B) and *C. multipapillatum* (*s.l.*) as sister taxa to our sequences. The p-distance between our specimens and these latter three species was 0.11–0.12%, 0.11% and 0.11–0.12%, respectively.

4. Discussion

Larval stages of *Contracaecum* are widespread in fish species from freshwater, euryhaline and marine environments, but a lack of morphological features of taxonomic relevance hampers their proper identification to the species level without associating them to

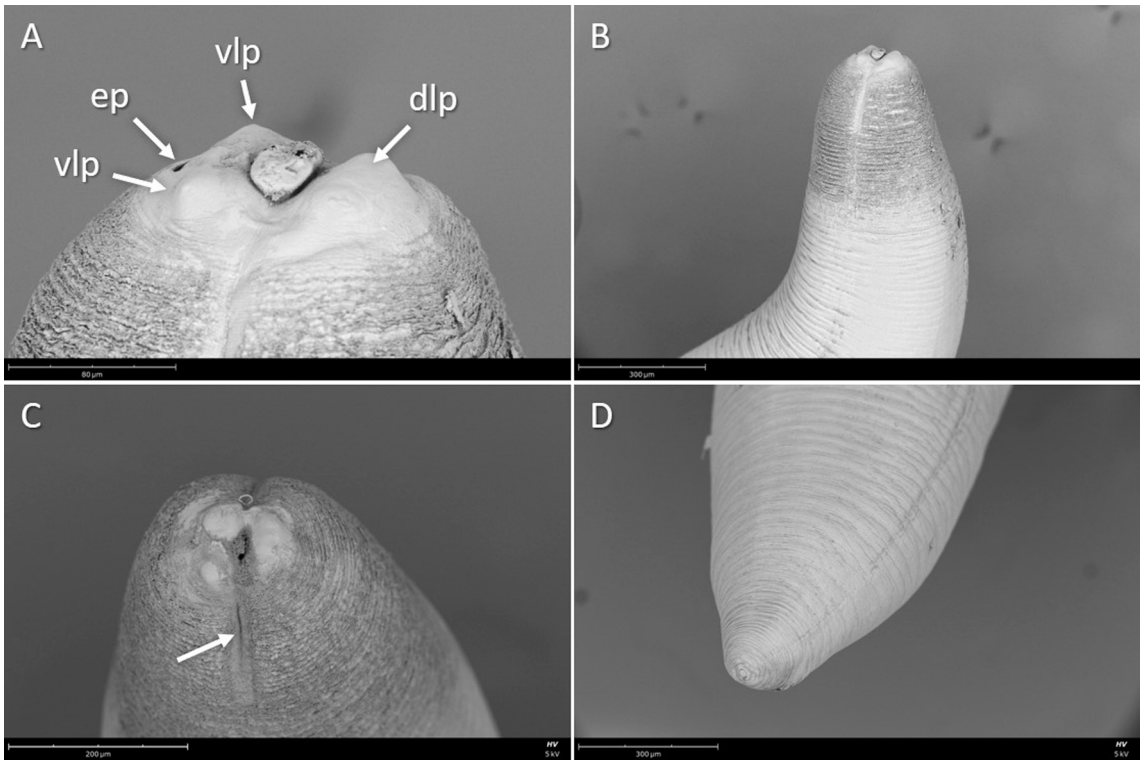

Fig. 4. *Contracaecum* third-stage larva (L3), SEM micrographs: (A) cephalic end, sublateral view; (B) caudal end, sublateral view. bt: boring tooth; ep: excretory pore; dlp: dorsal lip primordium; vlp: ventral lip primordium; ll: lateral line; pt.: pointed tip.

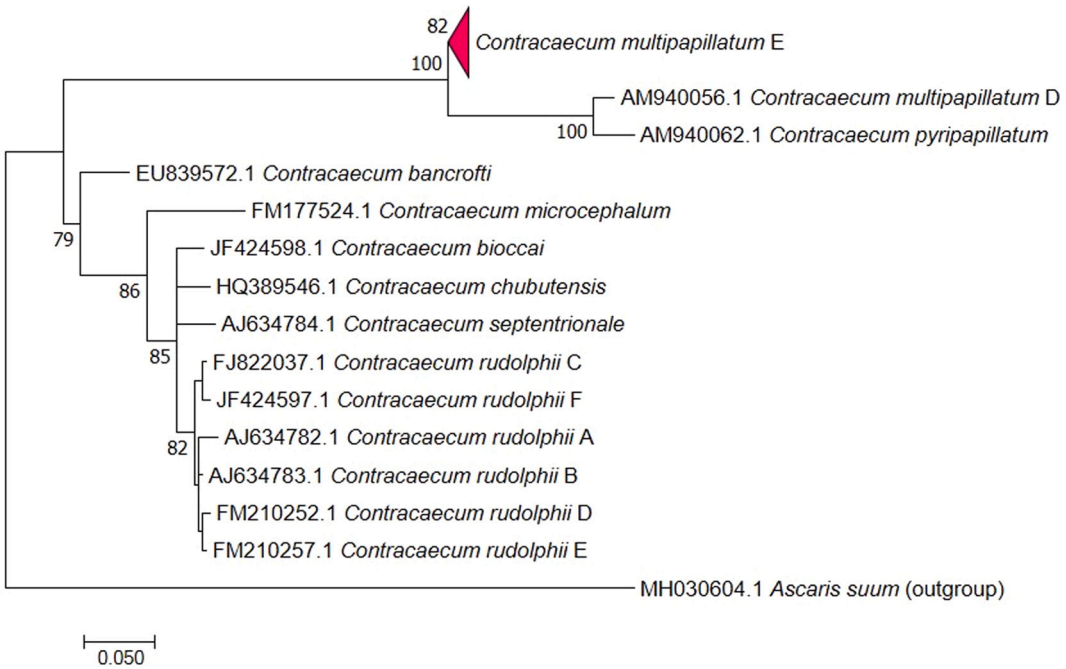

Fig. 5. Maximum-Likelihood tree based on the concatenated ITS1-ITS2 rDNA sequences showing the relationship between the *Contracaecum multipapillatum* E (condensed, containing 20 sequences from the two fish species, GB acc. n. OL830790-OL830809 plus KX580602-03 from Younis et al., 2017) described in the present paper and the other congeneric *Contracaecum* species. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

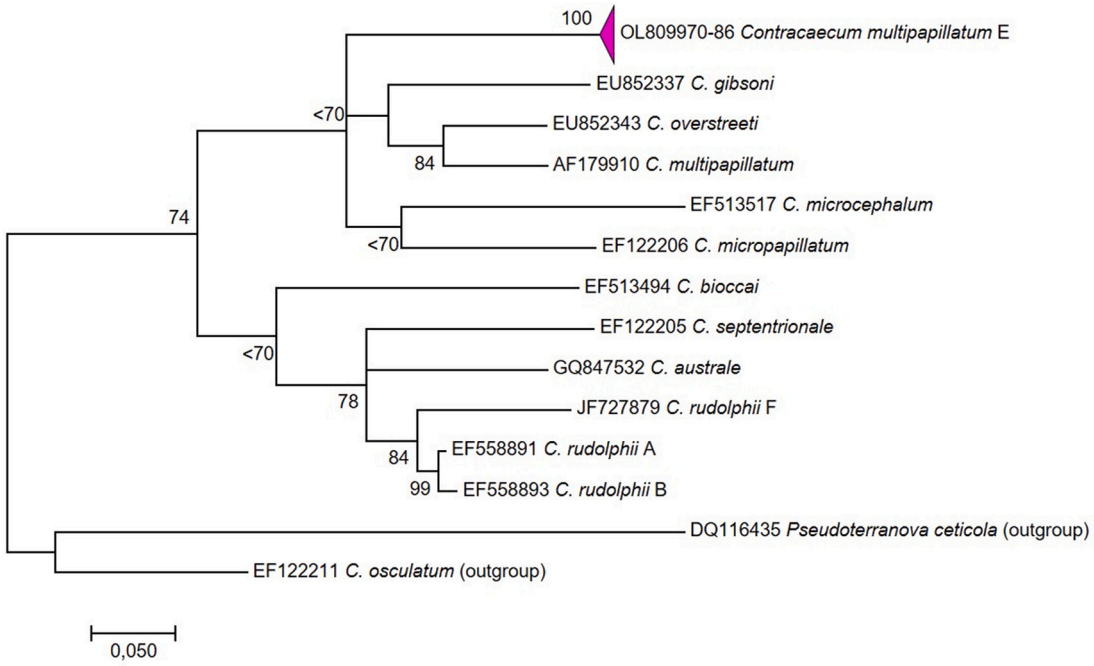

Fig. 6. Maximum-Likelihood tree based on the cox2 mtDNA sequences showing the relationship between the *Contracaecum multipapillatum* E (condensed, containing 17 sequences from the two fish species, GB acc. n. OL809970-OL809986) described in the present paper and the other congeneric *Contracaecum* species. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

morphological and molecular analyses on the adult stages in fish-eating birds or mammals (Moravec et al., 2016).

Zoonotic and potentially zoonotic parasites such as *Contracaecum* larvae can be present, in addition to wild fish also in intensively farmed fish under poor feeding or farming management that drives the underfed fish into the natural trophic chain, or in semi-intensive and extensive systems where farming conditions are much closer to natural living conditions (Younis et al., 2017; Shamsi, 2019; Tesfaye et al., 2020). The latter is the case for the present study, in which the finding of *Contracaecum* larvae of the same species in both tilapia and red drum could be easily explained by the polyculture semi-intensive farming system, leading to involvement of the two fish species as intermediate/paratenic (the former) and paratenic (the latter) hosts at two consecutive steps of the same trophic web, and probably providing all of the required players for establishment of the *Contracaecum* spp. life cycle.

In Israel, Paperna (1964) described a *Contracaecum* sp. larva in *Tilapia* with morphology similar to the ones described herein, although the maximum width of those specimens was much higher (4.5 vs. 1–1.16 mm) and the ventricular appendix much shorter than the specimens from our study. Later on, Landsberg (1989) reported *Contracaecum* sp. larvae from hybrid tilapia reaching dimensions of 60 mm vs. 46 mm in our study, without any further morphological description or line drawings; thus, any comparison would only be speculative, because the TL of larval nematodes is affected by several in-vivo, fixation and postprocessing artifacts. Finally, the most recent report of *Contracaecum* in Israel was by Smirnov et al. in 2021; those authors provided an update on the diffusion of *C. multipapillatum* larvae in hybrid tilapia farmed in Beit She'an Valley (Israel). Unfortunately, the authors did not provide any morphological descriptions but only some pictures of the larvae, useless to confirm the species identification.

Contracaecum larvae from the pericardial cavity of tilapias have also been reported in fish from African countries such as Kenya (Paperna, 1974; Malvestuto and Ogambo-Ongoma, 1978; Florio et al., 2009; Otachi et al., 2014), Ethiopia (Yimer and Enyew, 2004; Florio et al., 2009; Gulelat et al., 2013; Reshid et al., 2015; Ageze and Menzir, 2018; Mitiku et al., 2018) and Egypt (Younis et al., 2017). Among these, nematodes in the pericardial cavity are generally identified only to the genus level, and reports analyzing the parasites morphologically are scarce. *Contracaecum* larvae examined in the present study showed gross morphological similarities with those reported by Paperna (1974) in *T. nilotica* from East African lakes, Florio et al. (2009) in *O. niloticus* from Kenya and Ethiopia, and Younis et al. (2017) in *O. niloticus*, *S. galilaeus* and *Lates niloticus* form Lake Nasser in Egypt.

The larvae described by Florio et al. (2009) were tentatively identified morphologically as *C. multipapillatum*. This species was also described in the USA in the visceral organs and mesentery of red drum (Overstreet, 1974, 1983; Deardorff and Overstreet, 1980). In the present study, the larvae were much longer than those reported in those three papers and also compared to the length ranges of *C. multipapillatum* larvae previously described by Shamsi and Aghazadeh-Meshgi (2011) and Motamedi et al. (2019), and even compared to the *Contracaecum* larvae I–IV described by Shamsi et al. (2011) from several Australian fish species.

SEM showed the morphology of the anterior end with transversal cuticular ridges, more pronounced in the distal part of the cephalic end, and interrupted by narrow lateral lines. This feature, already reported in Moravec et al. (2016), is also visible in several *Contracaecum* specimens described by Younis et al. (2017). Furthermore, SEM micrographs allowed a detailed observation of the

morphology of the dorsal and ventral labial primordia and the position of the excretory pore, situated below the ventral boring tooth, and a finer characterization of the caudal end, showing a pointed tip.

Genetic analysis of the ITS rDNA showed that our specimens are closely related to the unidentified African *Contracaecum* larvae (100% similarity with Kibet and Zhao, unpublished; 99.8% with Younis et al., 2017; 99.5% with Otachi et al., 2014). The most closely related species are *C. multipapillatum* and *C. pyripapillatum* (p-distance 0.10% and 0.11%, respectively), as also observed by Younis et al. (2017) for the former. These observations are further confirmed by the ML tree of the *cox*2 mtDNA gene, showing that our specimens are most closely related to *C. gibsoni* (syn. *C. multipapillatum* A), *C. overstreeti* (syn. *C. multipapillatum* B) (Mattiucci et al., 2010) and *C. multipapillatum* (*s.l.*) (Nadler and Hudspeth, 2000). Mattiucci et al. (2010) reported that *C. multipapillatum* is a complex of species (at least four) that are indistinguishable by their morphological traits but discernible by genetic analysis and by their different geographical distribution. So far, this species complex has been described in Europe (Mattiucci et al., 2010), Central and South America (see Mattiucci et al., 2010), USA (D'Amelio et al., 2007) and Australia (Shamsi et al., 2008). We could hypothesize that our specimens belong to a possible new species in the *C. multipapillatum* complex, tentatively named *C. multipapillatum* E, expanding the geographical area of *C. multipapillatum* (*s.l.*).

In this study, the molecular analysis based on two genes (ITS rDNA and *cox*2 mtDNA) was coupled with a morphological description of the collected larvae. Unfortunately, this approach did not help us identify the nematodes at the species level because no morphological description or sequences of the related adults are currently available. However, we were able to examine one adult *Contracaecum* female collected in a great white pelican (*Pelecanus onocrotalus*, sampled under permit 2020/42659 from the Israel Nature and Parks Authority) from another fish farm in the Valley of the Springs region, matching the DNA of the larvae herein described. Unfortunately, females lack taxonomic characteristics useful to characterize a species, so further analyses combining morphological and molecular approaches on adult specimens, including males, from fish-eating birds living in the same area are required to support the identification of a potentially new species.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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