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Ultramorphology and molecular studies of *Contracaecum* larvae (Nematoda: Anisakidae) collected in five Cyprinid fish species from Sulaimani Province, Kurdistan Region-Iraq

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Article info

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Summary

A total of 1134 freshwater fishes belonging to Cyprinidae (*Acanthobrama marmid* (n=20), *Alburnus caeruleus* (n=7), *Alburnus mossulensis* (n=62), *Arabibarbus grypus* (n=123), *Barbus lacerta* (n=7), *Capoeta trutta* (n=222), *C. umbla* (n=161), *Carasobarbus kosswigi* (n=5), *C. luteus* (n=89), *Carassius auratus* (n=54), *Chondrostoma regium* (n=52), *Cyprinion kais* (n=10) and *C. macrostomum* (n=322)) were collected in different water bodies in Sulaimani Province, Kurdistan Region-Iraq for the presence of larval nematode of the genus *Conteacaecum*. This investigation revealed that 17 fishes belonged to five species (*A. marmid*, *A. grypus*, *C. trutta*, *C. luteus* and *C. regium*) were infected with *Contracaecum* larvae with prevalence of 35 %, 0.81 %, 0.90 %, 4.49 % and 5.76 %, respectively. The third- larval stage was morphologically studied by optical microscopy, and the ultrastructure was investigated using scanning electron microscopy (SEM). In addition, molecular analysis was carried out by amplifying, sequencing and comparing different gene loci, including internal transcribed spacers (ITS-1 and ITS-2) and cytochrome oxidase c subunit-II (COX-2), of the different isolated *Contracaecum* larvae. These sequences were also compared with closely related nematode sequences from the GenBank. Fifteen sequences were obtained for this study from the collected *Contracaecum* larvae. ITS-1, ITS-2 and COX-2 were amplified by polymerase chain reaction (PCR) and sequenced. The sequences of ITS-1, ITS-2 and COX-2 revealed that the collected *Contracaecum* larval specimens from all infected fish species represented one species (*Contracaecum rudolphii* B) based on the identity percentage in the GenBank database. The genetic characterisation of the parasite in the present study is available in the GenBank database, and the obtained ITS-1, ITS-2 and COX-2 sequences were deposited in GenBank. The present study provides information on the accurate identification and molecular analysis of *Contracaecum* larvae in the infected fish species in Sulaimani Province, Kurdistan Region-Iraq.

Keywords: *Contracaecum rudolphii* B; Nematode; Cyprinidae; Genetics; Systematics

Introduction

Nematodes from the family Anisakidae are parasites that have a worldwide distribution (Anderson, 2000). The most widespread genera from this family are *Anisakis* and *Contracaecum*, which have similar life cycles. The third larval stage (L3) of *Contracaecum* is usually found in the body cavity, mesenteries and branchial chambers of a wide range of fish species (Norris & Overstreet, 1976). The adult stage is found in the intestine of piscivorous birds and mammals associated with fresh, brackish and seawater

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(Whitfield & Heeg 1977; Anderson, 2000). Anisakid larvae are the causative agent of anisakidosis in humans (Audicana *et al*., 2002; Audicana & Kennedy, 2008), particularly the species belonging to *Anisakis*, *Contracaecum* and *Pseudoterranova* (Oshima, 1987; Yagi *et al*., 1996). This disease has been reported worldwide, and it is endemic in Southeast Asia (Audicana & Kennedy, 2008; Mattiucci & Nascetti, 2008). The knowledge about the life cycle of these species and their geographical distribution is very poor (Shamsi, 2019). The identification of *Contracaecum* larvae from different fish hosts has attracted the interest of scientists in many countries in studying the ultrastructure and genetic characterisation of these larvae using different genetic markers, such as 28S rDNA, 18S rDNA, ITS-1, ITS-2, and mtDNA cox-2 (Garbin *et al*. 2013; Mattiucci *et al*. 2015; Younis *et al*., 2017; Zuo *et al*. 2018; Malviya *et al*., 2018). There are a few publications on the specific identification of the larval stages of *Contracaecum* in fishes from the world (Szostakowska & Fagerholm, 2007; Shamsi & Aghazadeh-Meshgi, 2011; Shamsi *et al.,* 2017; Molnár *et al.,* 2019; Pekmezci & Yardimci, 2019).

In Iraq, *Contracaecum* larvae were known principally from early work by Herzog (1969), Shamsuddin *et al*. (1971), Mhaisen (1986), Ali *et al*. (1987) and Khalifa *et al.* (1987). Recently there are 42 fish host species known for *Contracaecum* larvae in Iraq (Mhaisen, 2019). However, research to date has not yet investigated the specific identification of *Contracaecum* larva in fishes based on molecular approach in Iraqi waters, and this requiring further investigation. Previous studies have shown that specific identification of *Contracaecum* larvae is not possible based solely on morphological description (Shamsi *et al*., 2017). The present study is an investigation toward the molecular genetic characterization of *Contracaecum* nematodes in Iraq by using a combined molecular and ultra-morphological approach based on sequence data of well-identified adults and *Contracaecum* larval types in GenBank. This approach is useful for the reliable identification of *Contracaecum* larvae at the species level (Shamsi *et al*., 2011). The purpose of this study is to know the genetic characterisation and determine the *Contracaecum* larvae among freshwater fish in Iraq.

Materials and Methods

Description of Study Area

Sulaimani Province is located in the northeast of Iraq; it is situated between latitudes 35° 05' and 36° 30' and between longitudes '44° 25' and 46° 20'. It is located close to the Iraqi-Iranian border. There are many water bodies in this province in addition to the two large rivers (the Lesser Zab River and the Sirwan River) that pass through this province (Fig. 1).

Specimen Collection and Preservation

During the period from January to the end of December 2018, 1134 fish were collected and examined for infection with anisakid larvae (*Contracaecum*). The fishes comprised 13 species of Cyprinidae as follows: *Acanthobrama marmid* (n=20), *Alburnus caeruleus* (n=7), *A. mossulensis* (n=62), *Arabibarbus grypus* (n=123), *Barbus lacerta* (n=7), *Capoeta trutta* (n=222), *C. umbla* (n=161), *Carasobarbus kosswigi* (n=5), *C. luteus* (n=89), *Carassius auratus* (n=54), *Chondrostoma regium* (n=52), *Cyprinion kais* (n=10) and *C. macrostomum* (n=322). They were caught by a pulsed DC electro-shock device (SAMUS 1000). The fish were identified based on their morphometric and meristic characteristics (Coad, 2010) and the scientific names for fishes were identified according to Froese and Pauly (2019). The fish were immediately transported to the laboratory for parasitological examinations, where they were dissected from the ventral side. The body cavity, stomach, spleen, liver, kidneys, heart, muscles, swim bladder and gonads were all examined for anisakid cysts. The gastrointestinal tract was removed, from the rectum to the esophagus, opened longitudinally, and carefully examined under stereoscope (Amlacher, 1970). The cysts were collected and washed with physiological solution (saline solution 0.9 %) in a glass Petri dish. The cysts were opened under a stereoscope with the aid of a fine needle to release the *Contracaecum* larva, and then washed with saline solution and preserved. The prevalence and intensity of infection were calculated for each fish species based on Margolis *et al.* (1982). The infection level was calculated as prevalence (percentage of investigated fish infected) and mean intensity (mean number of *Contracaecum* larva per infected fish).

Morphological identification

Light microscopy

After opening the cysts, the *Contracaecum* larvae were washed with saline solution (0.9 %), fixed in hot 4 % formaldehyde solution (60°C) to relax the body, and preserved in 70 % ethanol. A small piece of the mid-body of each nematode was excised for molecular study (Shamsi & Aghazadeh-Meshgi, 2011), and the rest of the nematode was cleared with increasing concentrations of glycerine (5 %, 10 % and 50 %, each for 1 hour), followed by 100 % glycerine for 1 – 2 hours; they were then mounted in glycerine jelly (Moravec *et al*., 2009; Moravec & Yooyen, 2011). All measurements of the parasites were made with an Olympus ocular micrometre eyepiece and are given in millimetres. Photos were taken with a Sony Optical Steady Shot digital camera (model DSC-W570, 16.1 mega pixels). The morphology of detected third larval stage of the Anisakid were identified according to the key features and descriptions of Bykhovskaya-Pavlovskaya *et al*. (1962), Hoffman (1998), Anderson (2000) and Shamsi *et al*. (2011).

Scanning electron microscopy

The larvae were removed from the cysts for scanning electron microscopy (SEM) study. The specimens were fixed in 4 % (v/v) hot formaldehyde solution (60°C), preserved in 70 % (v/v) ethanol, and post-fixed in 1 % osmium tetroxide (in phosphate buffer). The samples were then dehydrated by incubating in a graded series of acetone–ethanol concentrations ((1:1), (1.5 – 0.5) and abso-

Fig. 1. Map of north Iraq showing the study area.

lute acetone, 15 min each) (Moravec *et al.*, 2012). A critical-point method was used for drying by shaking the samples for 24 hr. in centrifuge to remove the acetone; they were then embedded on the targets and sputter-coated with gold (Moravec *et al.*, 2009; Moravec & Yooyen, 2011). The specimens were examined using a FEI Quanta 400 scanning electron microscope at an accelerating voltage of 25 kV.

Molecular study

DNA extraction

Genomic DNA was isolated from mid piece of individual larvae after being preserved directly in absolute ethanol (99 %). The genomic DNA was extracted by using a QIAamp® DNA Mini Kit with slight modifications. In brief, the mid piece of individual larval parasite digested for 1 – 3 h at 56 °C with proteinase K in ATL buffer and eluted in 50 µl of AE buffer (QIAamp® DNA Mini Kit).

DNA amplification

The PCR was used to amplify the ITS-1, ITS-2, and COX-2 regions. The specific primer sets SS1F/NC13R, SS2F/NC2R (Shamsi *et al.*, 2008) and 210F/211R (Nadler & Hudspeth, 2000) were used to amplify the two nuclear ribosomal markers (ITS-1 and ITS-2) and cytochrome oxidase-II (COX-2), respectively. The PCR reaction (in a volume of 30 µl) was performed in 25 mM Tris-HCl, pH 9.0 at 25°C and contained 50 mM KCl, 2 mM MgCl2, 0.1 mg/ml gelatin, 200 µM dATP, dGTP and dTTP, 100 µM [α32-P] dCTP (0.05 µCi/nmol), 12.5 µg of activated salmon sperm DNA, 10 pmol of each primer and 1.5 U *Taq* polymerase (Canvax Biotech, S.L.). The PCR reactions were carried out in a thermocycler (Applied Biosystems 2720, USA) using the following cycling instructions: 94°C for 5 min (initial denaturation), 35 cycles of 94°C, 30 sec (denaturation), 55°C, 30 sec (annealing), 72°C, 30 sec (extension) and a final extension of 72°C for 7 min, followed by holding at 4°C. Two microliters of genomic DNA (20 – 40 ng) in nuclease-free deionised distilled water were added to each PCR reaction. Samples with fish genomic DNA (extracted from muscle) were included in the PCR as negative controls; no amplicons were produced from these samples. Five microliters of each PCR product was examined on a 1.5 % w/v agarose gel, stained with DNA stain (Good View™ SBS Genetech Beijing, China) and photographed using a gel documentation system. A 1000 bp DNA ladder (Vivantis, Malaysia) was used. The amplicon were then purified using the EasyPure® Quick Gel Extraction Kit (TransGen Biotech), according to the manufacturer's protocols. The resulting products were sent to Macrogen in South Korea for nucleotide sequence analysis by a dideoxy termination method using a Genetic Analyzer 3500 DNA sequencer (Applied Biosystems, USA) in both directions (forward and reverse) using the same PCR primers.

Table 1. Prevalence of *Contracaecum* larva and mean of intensity among infected fish species.

Host	Fish		Prevalence %	Mean intensity
	Examined	Infected		
Acanthobrama marmid	20	7	35.00	5.57
Arabibarbus grypus	123	1	0.81	7.00
Capoeta trutta	222	2	0.90	3.00
Carasobarbus luteus	89	4	4.49	4.25
Chondrostoma regium	52	3	5.76	9.00

Sequence and phylogenetic analysis

The resulted ITS-1, ITS-2 and COX-2 sequences (forwards) were compared with their complements (reverses) and then adjusted using online software tool (bioinformatics.org\sms\rev_comp.html) to obtain reverse complement. Then the resulted sequences were aligned to each other using multiple sequence alignment program by using the online software tool CLUSTALW (genome.jp/tools-bin/clustalw) to get the most homologous sequences (one sequence). Subsequently, the obtained sequences were compared with previously published sequences data for identification by using the Basic Local Alignment Search Tool (BLAST) from GenBank database (http://www.ncbi.nlm.nih.gov/) (Altschul *et al*., 1990). Sequences of ITS-1 and ITS-2 from individual *Contracaecum* larvae in the different fish host species in the present study were aligned by using the online computer program CLASTALAW (https://www.genome.jp/tools-bin/clustalw) and then adjusted manually for searching nucleotide variations among *Contracaecum* larvae in different fish host species.

For the phylogenetic study, the sequence data of ITS-1, ITS-2 and COX-2 fragments obtained from *Contracaecum* larvae collected from all different fish host species were installed into the MEGA X version 10.7.1 software program (Kumar *et al*., 2018). To unify the length of the sequences, the common 447, 268 and 475 bp length of ITS-1, ITS-2 and COX-2 segments respectively were selected and used for phylogenetic analysis to determine the most appropriate sequence evolution model for the given data, treating gaps and missing data with the partial deletion option. The sequences were aligned using CLUSTALW alignment for constructing the trees of evolutionary development. The trees of all isolated species were constructed based on the Maximum Likelihood (ML) method and Tamura-Nei model (Tamura & Nei, 1993).

Ethical Approval and/or Informed Consent

The care of experimental animals was consistent with Rebuplic of Iraq animal welfare laws, guidelines and policies approved by University of Sulaimani Local Ethics Committee (Permit reference number 122/2020). All fishes were collected from both Lesser Zab and Sirwan River drainages, with required permissions of the Directorate Police of Forest and Regional Sulaimani Province (reference number 1060/2018).

Results and Discussion

Prevalence of larval Contracaecum nematodes

A total of 1134 fish were examined for parasitic larval *Contracaecum* nematodes. Only 17 fishes (belonged to five species) were infected with this parasite. The parasitic larvae were found in the intestinal wall, liver, ovaries, swim bladder, gallbladder and mesenteries of 1.49 % (17\1134) of fishes. The prevalence of *Contracaecum* larvae and the mean of intensity per infected fish varied among the different fish species (Table 1).

A total of 7 fish (prevalence 35 %, mean intensity 5.57) out of the 20 *Acanthobrama marmid* specimens examined were infected with *Contracaecum* larvae, which represented the highest prevalence among the collected fishes. While, only one fish (prevalence 0.81 %, mean intensity 7) out of 123 *Arabibarbus grypus* specimens examined was infected with this larva, which represented the smallest prevalence among the collected fishes in the present study. These results agree with Abdullah and Mhasein (2011), who recorded *Contracaecum* larvae in *Acanthobrama marmid* and *Chondrostoma regium* with prevalence of 16.6 % and 36.4 %, respectively, among ten fish species in the Lesser Zab River. This variation in the prevalence may be related to the temperature, water level, intensity of both the intermediate host and migratory bird (final host), and the types of food and feeding habits of the fish (Younis *et al*., 2017).

The present investigation shows that prevalence and intensity of *Contracaecum* larvae in the examined fishes are relatively low. This may be because only visual examination was used to isolate *Contracaecum* larvae in this study. The prevalence and number of larvae may have been higher than *Contracaecum* larvae found in the collected fishes in the present study if an incubation method had been used (Shamsi & Suthar 2016; Buchmann, 2007). Third larval stage of the anisakid nematode *Contracaecum rudolphii* Hartwich, 1964, are commonly infect a range of fish species and mainly cyprinids particularly in area where the final host (piscivorous birds such as cormorants) are found (Moravec, 1994).

Morphological identification

Morphological examination and measurements were conducted by optical microscopy and identified that the Anisakid larvae in the present study were *Contracaecum* larvae (L3), as described by

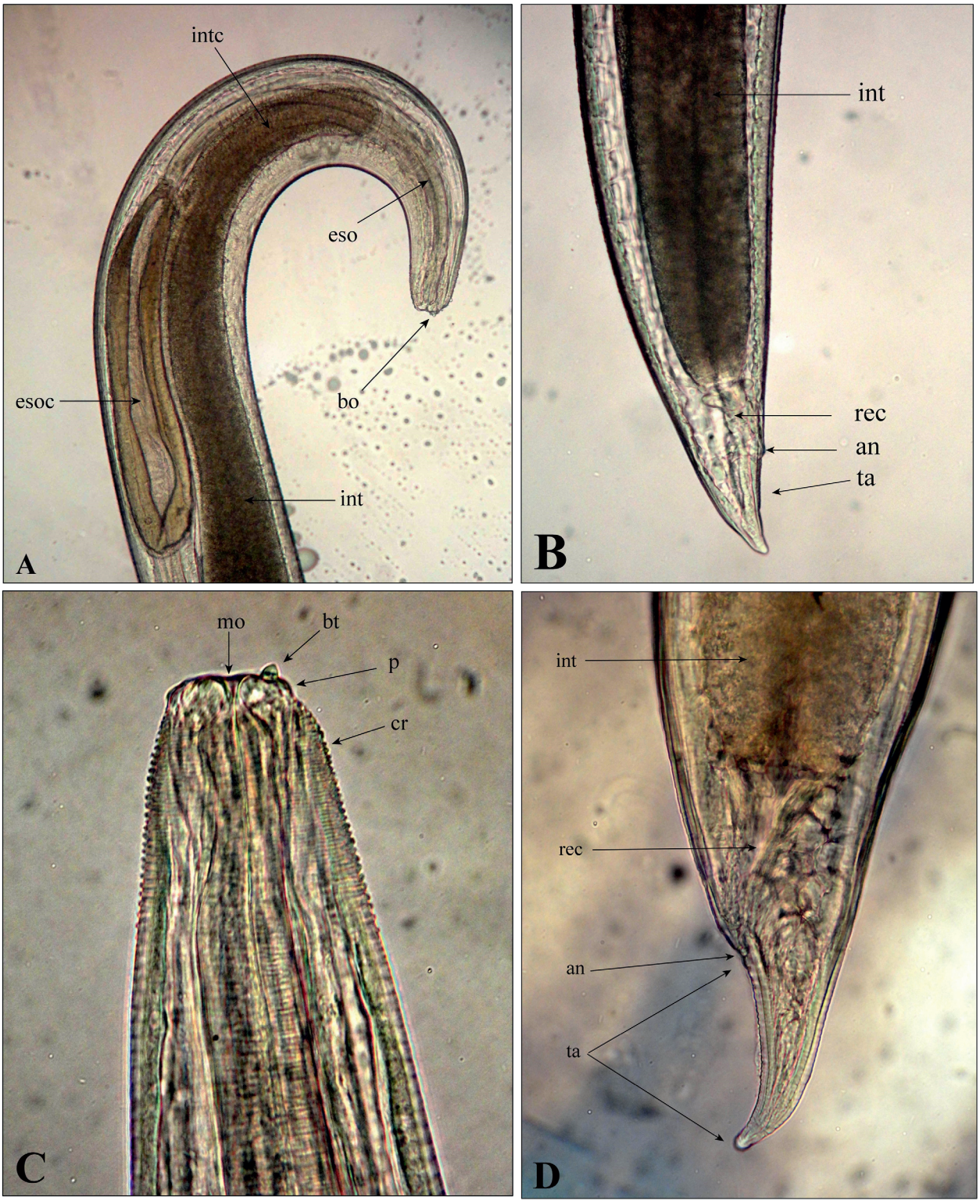

Fig. 2. Photo micrograph of *Contracaecum* larva in *Carasobarbus luteus.*
A - Anterior region of the larva (I00×); B - Posterior region of the larva (I00×); C - Mouth region of the larva (400×); D - Tail region of the larva (400×)
an = anus; bt = boring tooth; er = cuticle ridges; eso = esophagus; esoc = esophageal caecum; int = intestine; intc = intestinal caecum; mo = mouth opening; p = papillae; rec = rectum; ta = tail.

Table 2. Comparison of measurements in *Contracaecum* larvae in different infected fish species in the present study (in millimeter).

Host	Total length	Maximum width	Tail length	Rectum length	Boring teeth length	Esophagus length	Esophageal caeca length	Intestinal caeca length
A. marmid	4.10	0.20	0.07	0.07	0.005	0.480	0.50	0.38
A. grypus	5.07	0.25	0.07	0.07	0.010	0.650	0.30	0.20
C. trutta	4.20	0.23	0.08	0.07	0.005	0.450	0.48	0.33
C. luteus	4.62	0.25	0.08	0.07	0.005	0.600	0.55	0.40
C. regium	7.75	0.35	0.08	0.09	0.010	0.600	0.60	0.45

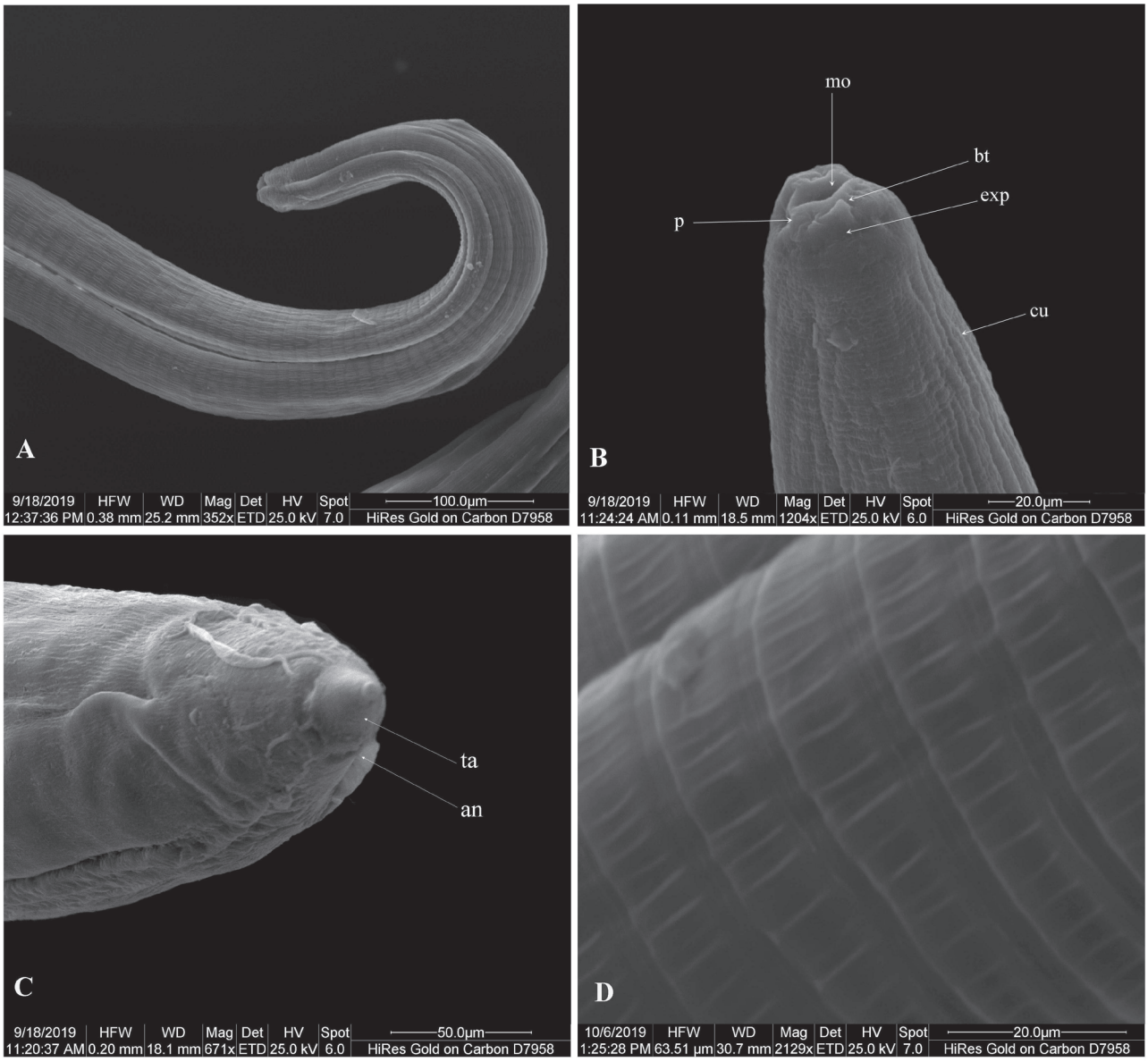

Fig. 3. Scanning electron micrograph of *Contracaecum* larva in *Carasobarbus luteus*.
A - Anterior region of the larva; B - Head region of the larva; C - Tail region of the larva; D - Cuticle of the larva
an = anus; bt = boring tooth; cu = cuticle; exp = excretory pore; mo = mouth opening; p = papillae; ta = tail.

Moravec (2009). Additionally, there were no significant morphological differences among the larvae that were recorded in different fishes. The *Contracaecum* larvae were light brownish-yellow in colour. They had an elongated cylindrical body and short tail with conical and pointed tip. The bodies had fine, dense transverse striation of the cuticle. The larvae were encapsulated within a slender body, they have distinct boring tooth. Excretory pore situated anteriorly, cuticular striations were observed through the whole length of the body. The esophagus consisted of a long muscular part and a short glandular ventriculus. The esophageal caecum was extended posteriorly and the intestinal caecum was extended anteriorly. The intestine was filled with numerous small brownish granules, and the gonads and other parts of the reproductive system were not developed (Fig. 2).

The total length of the larvae was 3.5 – 11 mm, with a width of 0.10 – 0.25 mm. The esophagus length was 0.7 – 1.3 mm, the intestinal caecum length was 0.20 – 0.35 mm and the esophageal caecum length was 0.25 – 0.40 mm (Table 2). Since this parasite was in the larval stage and lacked the reproduction system, it was difficult to morphologically determine the exact classification at the species level. The specimens showed a close resemblance in both measurements and characteristics to those recorded by Moravec (2009) in *Cyprinus carpio* from the Czech Republic. In the microscopy studies, there were no significant morphological differences among the *Contracaecum* larvae in the five different fish species; the photomicrograph of the third larval stage of *Contracaecum* in *Carasobarbus luteus* is only an example (Fig. 2).

Scanning electron microscopy

The SEM study revealed a regular striation pattern in the cuticular striations of larvae from all fishes. The striations were narrow in the anterior of the larvae and in the pre-tail region, and they became gradually wider when extended posteriorly. There were no significant different ornamentations among the larvae. There were transverse striations between the striated cuticular rings (Fig. 3). The mouth was transverse in shape the lips are not developed and provided by four papillae, a well-defined boring tooth present. Excretory pore is anteriorly located just below the mouth. Anal opening is located near the posterior end. Tail is short. The body was free of any projections, such as a spine or papillae. The cloacal regions had no papillae because they were in the larval stage and not yet mature.

The ultrastructural characteristics of the present specimens showed great similarity with the specimens (*Contracaecum* larva) of Younis *et al.* (2017), who collected them from different freshwater fishes in Lake Nasser, Egypt. Furthermore, the ultrastructural features of the present *Contracaecum* larvae were similar to those of Rahemo and Nawab Al-Din (2009), who used SEM to study larvae from *Acanthobrama marmid* collected in the Tigris River. The SEM study revealed no significant ultra-morphological differences among the *Contracaecum* larvae recorded in the present investigation in these five different fish species. The scanning electron photomicrograph of the third larval stage of *Contracaecum* in *Carasobarbus luteus* is used as an example (Fig. 3).

PCR results

Agarose gels analyses demonstrated the same size for each ITS-1, ITS-2 and COX-2 region. The amplicons were ~530 bp, ~430 bp and ~630 bp for ITS-1, ITS-2, and COX-2 respectively, confirming that all sequences were of the same genus.

Sequence analyses

Alignment of the resulting sequences revealed that there was no significant variation in the ITS-1, ITS-2 and COX-2 regions, which indicated the presence of only one type of larva. Based on percentage identities of nucleotides from GenBank, the on-line BLAST tool showed the ITS-1 sequences obtained from larvae-infected *A. marmid*, *A. grypus*, *C. trutta*, *C. luteus* and *C. regium* matched 99.78 %, 100 %, 99.76 %, 100 % and 100 %, respectively to the previously reported reference gene sequences for the ITS-1 in *Contracaecum rudolphii* B (Zhang *et al*., 2009) from the stomachs of great cormorant *Phalacrocorax carbo sinensis* from the Guangzhou Zoo in Guangdong in China, which was examined previously and deposed in GenBank (FJ467618) (Zhang *et al*., 2009). The ITS-2 sequences obtained from larvae-infected *A. marmid*, *A. grypus*, *C. trutta*, *C. luteus* and *C. regium* matched 100 % to the previously reported reference gene sequences for the ITS-2 in *Contracaecum rudolphii* B (Li *et al*., 2005) from the stomachs of great cormorant *Phalacrocorax carbo sinensis* from the Venice Lagoon in northeastern Italy and from Monaci Lake

Table 3. Accession numbers provided by NCBI for the collected *Contracaecum* larvae in different fish hosts in the present study.

Host of *Contracaecum rudolphii*	Accession numbers for ITS-1 sequences	Accession numbers for ITS-2 sequences	Accession numbers for COX-2
Acanthobrama marmid	MN557376	MN526259	MN589997
Arabibarbus grypus	MN557377	MN563727	MN589998
Capoeta trutta	MN557378	MN563728	MN589999
Carasobarbus luteus	MN557379	MN563729	MN590000
Chondrostoma regium	MN557380	MN563730	MN590001

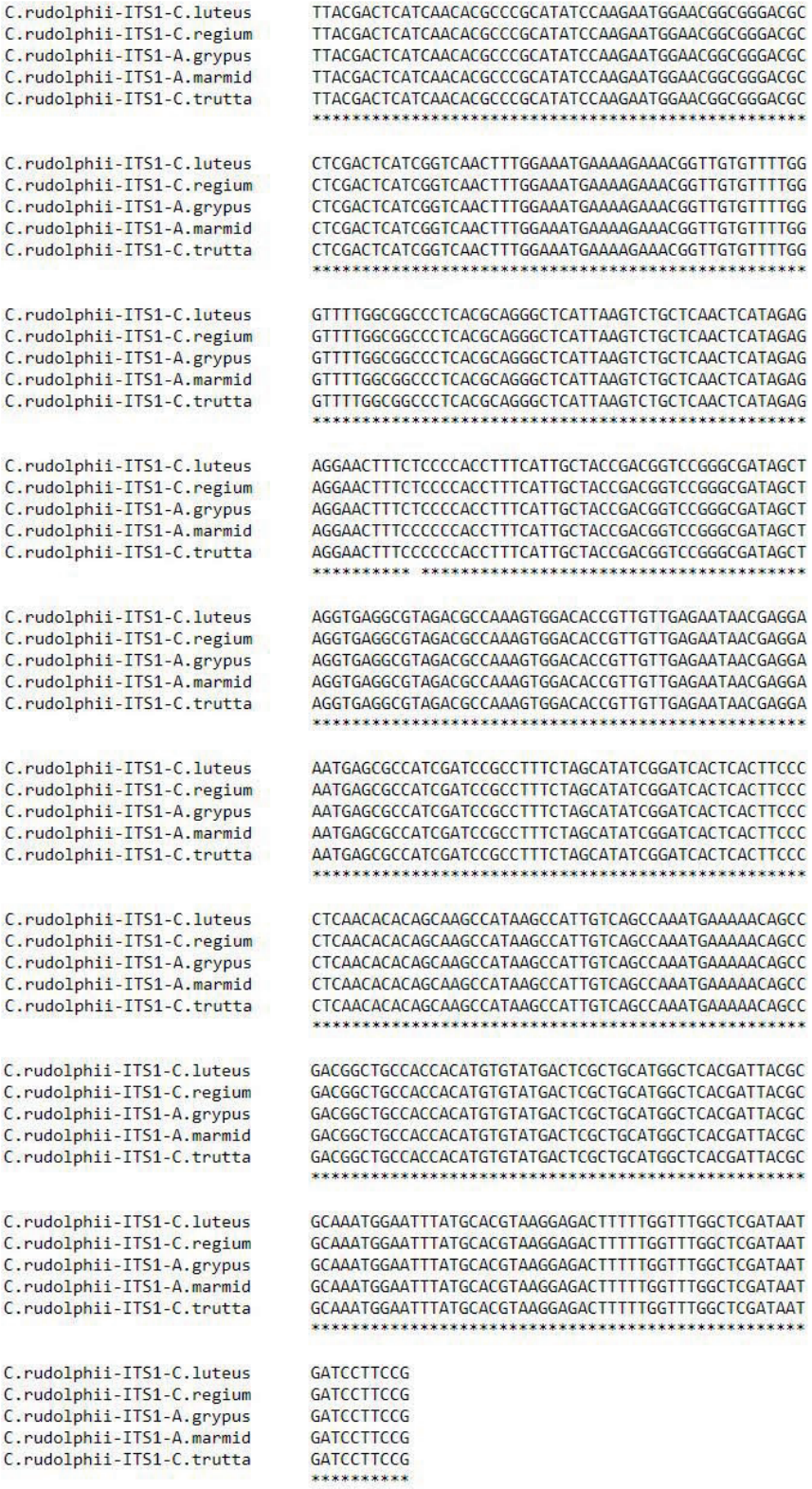

Fig. 4. Multiple sequence alignment for ITS-1 in *C. rudolphii* B in the five different fish species.

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C.rudolphii-ITS2-A.marmid       CGCTGGCACGTCTGGCTGAGGGTCGAAATATTCAATACTATCCGCACAAT
C.rudolphii-ITS2-A.grypus       CGCTGGCACGTCTGGCTGAGGGTCGAAATATTCAATACTATCCGCACAAT
C.rudolphii-ITS2-C.trutta       CGCTGGCACGTCTGGCTGAGGGTCGAAATATTCAATACTATCCGCACAAT
C.rudolphii-ITS2-C.regium       CGCTGGCACGTCTGGCTGAGGGTCGAAATATTCAATACTATCCGCACAAT
C.rudolphii-ITS2-C.luteus       CGCTGGCACGTCTGGCTGAGGGTCGAAATATTCAATACTATCCGCACAAT
                                **************************************************

C.rudolphii-ITS2-A.marmid       GCTTCAGACGAAGCGTGTGGTGCTTTCGACAAGCAGTGTCCCTTTGGGGC
C.rudolphii-ITS2-A.grypus       GCTTCAGACGAAGCGTGTGGTGCTTTCGACAAGCAGTGTCCCTTTGGGGC
C.rudolphii-ITS2-C.trutta       GCTTCAGACGAAGCGTGTGGTGCTTTCGACAAGCAGTGTCCCTTTGGGGC
C.rudolphii-ITS2-C.regium       GCTTCAGACGAAGCGTGTGGTGCTTTCGACAAGCAGTGTCCCTTTGGGGC
C.rudolphii-ITS2-C.luteus       GCTTCAGACGAAGCGTGTGGTGCTTTCGACAAGCAGTGTCCCTTTGGGGC
                                **************************************************

C.rudolphii-ITS2-A.marmid       GCTCCTTGTTTGGTTTGAACGGCAACTTATTGCAAAGATTTACTCGGTAA
C.rudolphii-ITS2-A.grypus       GCTCCTTGTTTGGTTTGAACGGCAACTTATTGCAAAGATTTACTCGGTAA
C.rudolphii-ITS2-C.trutta       GCTCCTTGTTTGGTTTGAACGGCAACTTATTGCAAAGATTTACTCGGTAA
C.rudolphii-ITS2-C.regium       GCTCCTTGTTTGGTTTGAACGGCAACTTATTGCAAAGATTTACTCGGTAA
C.rudolphii-ITS2-C.luteus       GCTCCTTGTTTGGTTTGAACGGCAACTTATTGCAAAGATTTACTCGGTAA
                                **************************************************

C.rudolphii-ITS2-A.marmid       GCAGCAATAATGGCCGTAAGTGTGTGAGTGATTGTGTACGTCCCTCGATG
C.rudolphii-ITS2-A.grypus       GCAGCAATAATGGCCGTAAGTGTGTGAGTGATTGTGTACGTCCCTCGATG
C.rudolphii-ITS2-C.trutta       GCAGCAATAATGGCCGTAAGTGTGTGAGTGATTGTGTACGTCCCTCGATG
C.rudolphii-ITS2-C.regium       GCAGCAATAATGGCCGTAAGTGTGTGAGTGATTGTGTACGTCCCTCGATG
C.rudolphii-ITS2-C.luteus       GCAGCAATAATGGCCGTAAGTGTGTGAGTGATTGTGTACGTCCCTCGATG
                                **************************************************

C.rudolphii-ITS2-A.marmid       CGGCCCCCAGTATTTGTTGACTGCCTCTGGTGGTGACTGGGGGTTAAGTA
C.rudolphii-ITS2-A.grypus       CGGCCCCCAGTATTTGTTGACTGCCTCTGGTGGTGACTGGGGGTTAAGTA
C.rudolphii-ITS2-C.trutta       CGGCCCCCAGTATTTGTTGACTGCCTCTGGTGGTGACTGGGGGTTAAGTA
C.rudolphii-ITS2-C.regium       CGGCCCCCAGTATTTGTTGACTGCCTCTGGTGGTGACTGGGGGTTAAGTA
C.rudolphii-ITS2-C.luteus       CGGCCCCCAGTATTTGTTGACTGCCTCTGGTGGTGACTGGGGGTTAAGTA
                                **************************************************

C.rudolphii-ITS2-A.marmid       TCGGATTATCGAAAGAATGTGACATGTCTTATACGGTTATGTGCTTTTGA
C.rudolphii-ITS2-A.grypus       TCGGATTATCGAAAGAATGTGACATGTCTTATACGGTTATGTGCTTTTGA
C.rudolphii-ITS2-C.trutta       TCGGATTATCGAAAGAATGTGACATGTCTTATACGGTTATGTGCTTTTGA
C.rudolphii-ITS2-C.regium       TCGGATTATCGAAAGAATGTGACATGTCTTATACGGTTATGTGCTTTTGA
C.rudolphii-ITS2-C.luteus       TCGGATTATCGAAAGAATGTGACATGTCTTATACGGTTATGTGCTTTTGA
                                **************************************************

C.rudolphii-ITS2-A.marmid       CCTCAGCTCAGTCGTGATTACCCGCTGAATTTAAGCATATAATTAAGCGG
C.rudolphii-ITS2-A.grypus       CCTCAGCTCAGTCGTGATTACCCGCTGAATTTAAGCATATAATTAAGCGG
C.rudolphii-ITS2-C.trutta       CCTCAGCTCAGTCGTGATTACCCGCTGAATTTAAGCATATAATTAAGCGG
C.rudolphii-ITS2-C.regium       CCTCAGCTCAGTCGTGATTACCCGCTGAATTTAAGCATATAATTAAGCGG
C.rudolphii-ITS2-C.luteus       CCTCAGCTCAGTCGTGATTACCCGCTGAATTTAAGCATATAATTAAGCGG
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Fig. 5. Multiple sequence alignment for ITS-2 in *C. rudolphii* B in the five different fish species.

Fig. 6. Alignment of the ITS-1 and ITS-2 sequences representing genotype 1 (*C. rudolphii* B) from the present study and genotype 2 (*C. rudolphii* A) sequences have been deposited in GenBank under the accession numbers AJ634 782 and AJ634 785. Nucleotide differences between the aligned equences are indecated by having no asterisks.

in central Italy, which was examined previously and deposed in GenBank (AJ634786) (Li *et al*., 2005), and also matched 100 % to the previously reported reference gene sequences for the ITS-2 in *Contracaecum rudolphii* B from the same host from from the Guangzhou Zoo in Guangdong in China, which was examined previously and deposed in GenBank (FJ467618) (Zhang *et al*., 2009). The COX-2 sequences obtained from larvae-infected *A. marmid* matched 98.52 % to the previously reported reference gene sequences for the COX-2 in *Contracaecum rudolphii* B (Mattiucci *et al*., 2008) from the great cormorant *Phalacrocorax carbo sinensis* from Italy, which was examined previously and deposed in GenBank (EF122203) (Mattiucci *et al*., 2008). The COX-2 sequences obtained from larvae-infected *A. grypus*, *C. trutta* and *C. regium* matched 100 %, 99.19 % and 100 % respectively to the *Contracaecum rudolphii* B from *Phalacrocorax carbo sinensis* which was examined previously and deposed in GenBank (EF513509). While, the COX-2 sequences obtained from larvae-infected *C. luteus* matched 99.79 % to the *Contracaecum rudolphii* B from the same host which was examined previously and deposed in GenBank (EF558894) (Mattiucci *et al*., 2008).

The genetic characterisation of the parasite in the present study is available in the GenBank database; the ITS-1, ITS-2 and COX-2 sequences obtained were deposited in GenBank, and their accession numbers are stated in Table 3. The ITS-1, ITS-2 and COX-2

Table 4. *Contracaecum* nematodes and specimens/accession number (ITS) of taxa used to build phylogenetic trees and the nucleotide (bp) differences with the *Contracaecum* larvae collected from the present study.

Parasite	GeneBank accession no. (ITS)	No. of nucleotide differences (bp)	Host	Source
C. rudolphii A	ITS1 (AJ634782)	9	*Phalacrocorax carbo sinensis*	Li *et al.* (2005)
	ITS2 (AJ634785)	14	*Phalacrocorax carbo sinensis*	Li *et al.* (2005)
C. rudolphii B	ITS1 (AJ634783)	1	*Phalacrocorax carbo sinensis*	Zhang *et al*., 2009
	ITS2 (AJ634786)	0	*Phalacrocorax carbosinensis*	Li *et al.* (2005)
C. rudolphii D	ITS1 (FM210251)	6	*Phalacrocorax varius*	Shamsi *et al.* (2009b)
	ITS2 (FM210268)	17	*Phalacrocorax carbo sinensis*	Shamsi *et al.* (2009b)
C. rudolphii E	ITS1 (FM210257)	6	*Phalacrocorax varius*	Shamsi *et al.* (2009b)
	ITS2 (FM210271)	15	*Phalacrocorax varius*	Shamsi *et al.* (2009b)
C. rudolphii F	ITS (JF424597)	21	*Pelecanus occidentalis*	D'Amelio et al. (2012)
		16	*Pelecanus occidentalis*	D'Amelio et al. (2012)
C. ogmorhini	ITS1 (AJ291468)	10	*Arctocephalus pusillus doriferus*	Zhu *et al.* (2001)
	ITS2 (AJ291471)	15	*Zalophus californianus*	Zhu *et al.* (2001)
C. eudyptulae	ITS1 (AJ007461)	8	-	Zhu *et al.*(unpublished)
	ITS2 (FM177565)	17	*Eudyptula minor*	Shamsi *et al.* (2009a)
C. chubutensis	ITS1 (HQ389546)	13	*Phalacrocorax brasilianus*	Garbin *et al.* (2011)
	ITS2 (HQ389548)	23	*Phalacrocorax atriceps*	Garbin *et al.* (2011)
C. variegatum	ITS1 (MK424804)	22	Bird	Hbaiel & Mohammad (unpublished)
	ITS2 (FM177537)	14	*Anhinga melanogaster*	Shamsi *et al.* (2009a)
C. microcephalum	ITS1 (FM177523)	50	*Phalacrocorax melanoleucos*	Shamsi *et al.* (2009a)
	ITS2 (FM177527)	72	*Phalacrocorax melanoleucos*	Shamsi *et al.* (2009a)
C. septentrionale	ITS1 (AJ634784)	24	*Phalacrocorax carbo sinensis*	Li *et al.* (2005)
	ITS2 (AJ634787)	35	*Alca torda*	Li *et al.* (2005)
C. bioccai	ITS (JF424598)	30	*Pelecanus occidentalis*	D'Amelio *et al.* (2012)
		40	*Pelecanus occidentalis*	D'Amelio *et al.* (2012)
C. radiatum	ITS (AY603529)	35	*Leptonechotes weddlli*	Kijewska *et al.* (2008)
		65	*Leptonechotes weddlli*	Kijewska *et al.* (2008)
C. osculatum	ITS (AB277825)	77	Arabesque greenling	Umehara *et al.* (2008)
		105	Arabesque greenling	Umehara *et al.* (2008)
C. multipapillatum	ITS1 (AM940056)	146	*Pelecanus conspicillatus*	Shamsi *et al.* (2008)
	ITS2 (AM940060)	132	*Pelecanus conspicillatus*	Shamsi *et al.* (2008)
C. pyripapillatum	ITS1 (AM940062)	141	*Pelecanus conspicillatus*	Shamsi *et al.* (2008)
	ITS2 (AM940066)	136	*Pelecanus conspicillatus*	Shamsi *et al.* (2008)
Contracaecum larva	ITS1 (MN557376)	-	*Acanthobrama marmid*	Present study
	ITS2 (MN526259)	-	*Acanthobrama marmid*	Present study
Contracaecum larva	ITS1 (MN557377)	-	*Arabibarbus grypus*	Present study
	ITS2 (MN563727)	-	*Arabibarbus grypus*	Present study
Contracaecum larva	ITS1 (MN557378)	-	*Capoeta trutta*	Present study
	ITS2 (MN563728)	-	*Capoeta trutta*	Present study
Contracaecum larva	ITS1 (MN557379)	-	*Carasobarbus luteus*	Present study
	ITS2 (MN563729)	-	*Carasobarbus luteus*	Present study
Contracaecum larva	ITS1 (MN557380)	-	*Chondrostoma regium*	Present study
	ITS2 (MN563730)	-	*Chondrostoma regium*	Present study
Ascaris suum	ITS1 (AB110023)	-	Pig	Ishiwata *et al*. (2004)
	ITS2 (FJ418786)	-	Pig	Wickramasinghe *et al*. (2009)

Table 5. *Contracaecum* nematodes and specimens/accession number (COX-2) of taxa used to build phylogenetic trees and the nucleotide (bp) differences with the *Contracaecum* larvae collected from the present study.

Parasite	GeneBank accession no. (COX-2)	No. of nucleotide differences (bp)	Host	Source
C. rudolphii A	EF122201	34	*Phalacrocorax carbo sinensis*	Mattiucci *et al*. (2008)
C. rudolphii B	EF558894	1	*Phalacrocorax carbo sinensis*	Mattiucci *et al*. (2008)
C. rudolphii C	EF014283	272	*Phalacrocorax auritus*	D'Amelio *et al*. (2007)
C. rudolphii F	JF727879	40	*Pelecanus occidentalis*	D'Amelio *et al*. (2012)
C. ogmorhini	MN624184	32	*Zalophus californianus*	Mladineo *et al.* (under press)
C. chubutensis	HQ328504	46	*Phalacrocorax atriceps*	Garbin *et al.* (2011)
C. microcephalum	EF122208	71	*Phalacrocorax pygmaeus*	Mattiucci *et al*. (2008)
C. septentrionale	EF558898	60	*Phalacrocorax carbo carbo*	Mattiucci *et al*. (2008)
C. bioccai	EF558899	50	*Pelecanus occidentalis*	Mattiucci *et al*. (2008)
C. osculatum	KC412224	58	*Chionodraco hamatus*	Santoro *et al.* (2013)
C. multipapillatum	AF179910	72	-	Nadler & Hudspeth (2000)
C. micropapillatum	EU852350	70	*Pelecanus onocrotalus*	Mattiucci *et al*. (2010)
C. austral	GQ847539	55	*Phalacrocorax brasilianus*	Garbin *et al.* (2011)
C. pelagicum	EF122210	60	*Spheniscus magellanicus*	Mattiucci *et al*. (2008)
Contracaecum larva	MN589997	-	*Acanthobrama marmid*	Present study
Contracaecum larva	MN589998	-	*Arabibarbus grypus*	Present study
Contracaecum larva	MN589999	-	*Capoeta trutta*	Present study
Contracaecum larva	MN590000	-	*Carasobarbus luteus*	Present study
Contracaecum larva	MN590001	-	*Chondrostoma regium*	Present study
Ascaris suum	HQ704901	-	swine	Liu *et al*. (2012)

sequence analysis confirmed that third larval stage of *Contracaecum* (L3) parasitizing the Cyprinid fish (*A. marmid*, *A. grypus*, *C. trutta*, *C. luteus* and *C. regium*) from the present study belong to species *C. rudolphii* type-B, a parasite at the adult stage of the great cormorant *Phalacrocorax carbo sinensis* mainly from Italy water (Li *et al.*, 2005; Mattiucci *et al*., 2008). ITS-1, ITS-2 and COX-2 markers may provide reliable evidence for specific species identification of *Contracaecum* larvae occurring in fish (Mattiucci *et al*., 2010). Therefore, the occurrence of *C. rudolphii* B larvae from Iraqi waters was also proved by molecular evidence inferred from the ITS-1, ITS-2 and COX-2 markers used in the present study. Furthermore, the sequences of ITS-1 and ITS-2 obtained from the collected larvae in different fish species were aligned with each other (the same gene). Pairwise comparisons of all nucleotides sequence among the *Contracaecum* larvae collected in the five different fish hosts revealed that only one nucleotide variation (0.2 %) in alignment position 161 for ITS-1 (Fig. 4) and there was no nucleotide variations for ITS-2 (Fig. 5).

Contracaecum rudolphii Hartwich, 1964 is a species complex it consist of several sibling species. *C. rudolphii* sensu lato (s.l.) named *C. rudolphii* A and *C. rudolphii* B (D'Amelio *et al*. 1990), they could also be differentiated from each other based on the ITS-1 and ITS-2 sequence data (Li *et al*. 2005). D'Amelio *et al*. (2007) indicated the existence of a third cryptic species of *C. rudolphii* complex (*C. rudolphii* C) in double-crested cormorants from west-central Florida based on PCR-RFLP and sequencing of the rrnS mitochondrial gene and nuclear ribosomal spacers. Shamsi *et al*. (2009) described two new sibling species of the *C. rudolphii* complex, *C. rudolphii* D from *Phalacrocorax carbo* and *Contracaecum rudolphii* E from *Phalacrocorax varius* in Australia, based on the ITS-1 and ITS-2 sequence data. Recently, D'Amelio *et al*. (2012) recorded new isolate of *C. rudolphi* complex (*C. rudolphii* F) from brown pelican *Pelecanus occidentalis* in the northern Gulf of Mexico.

Various studies demonstrated that internal transcribed spacers (ITS-1 and ITS-2) of the nuclear ribosomal DNA (rDNA) provide genetic markers for the accurate identification of a range of species of Ascaridoids. In addition, more studies indicated that sibling species can be differentiated based on the ITS sequences (Jacobs *et al*., 1997; Zhu *et al*., 2000; 2001; 2002). The ITS-1 sequences of the obtained larvae (*C. rudolphii* B) in the present study show 11 (2.46 %) nucleotide differences with the previously reported reference gene sequence for the ITS-1 in *C. rudolphii* A which was examined and deposed in GenBank (Accession number: AJ634782). While, ITS-2 shows 14 (5.22 %) nucleotide differences with the *C. rudolphii* A which previously reported reference gene sequence for the ITS-2 in *C. rudolphii* A which was examined and deposed in GenBank (Accession number: AJ634785) (Li *et al*., 2005) (Fig. 6). This clear genetic differentiation support previous sequence analyses (Li *et al*., 2005) that there are sequence differences (1.8 %) in the ITS-1 and (5.1 %) in ITS-2 between the sibling species of *C. rudolphii* A and B (Li *et al*., 2005). Extending these studies, we investigated that there is no significant sequence variation in the ITS-1 and ITS-2 within and among the larvae collected from dif-

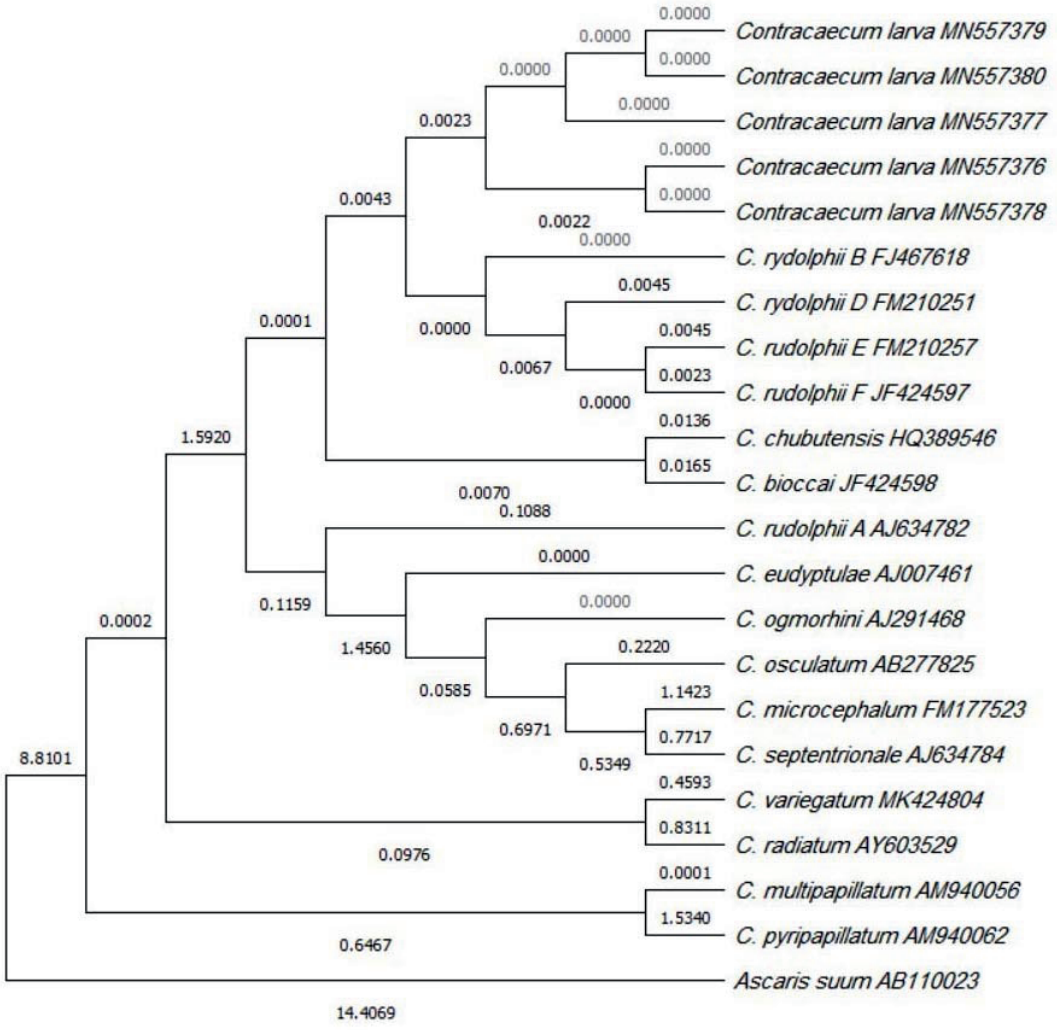

Fig. 7. Phylogenetic relationships between *Contracaecum* larvae from the present study and other *Contracaecum* species as inferred by maximum likelihood obtained from ITSI. *Ascaris suum* was used as outgroup.

ferent fish host in the present investigation. The molecular finding of the present investigation support that the present finding larvae belong to *C. rudolphii* type-B.

In the phylogenetic analysis, the sequence data aligned with the data sequences of ITS-1, ITS-2 and COX-2 form other different *Contracaecum* species (different genotypes) and *Ascaris suum* used as out group detected in GenBank (Table 4, 5). Phylogenetic analyses were conducted in MEGA X (Kumar *et al*., 2018). The evolutionary histories were inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura & Nei, 1993). The tree with the highest log likelihood (-6713.31, -3848.19 and -385797 for ITS-1, ITS-2 and COX-2 respectively) are shown (Fig. 7, 8, 9). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The *Contracaecum* larvae from all different fish hosts were clustered in the same clade of *Contracaecum rudolphii* B. Moreover, the concatenated phylogenetic tree of the ITS-1, ITS-2 and COX-2 sequences using ML analyses indicated that *Contracaecum* larvae clades were distinct species by high bootstrap values (Fig. 7, 8, 9).

The first information on *Contracaecum* larvae from Iraqi freshwater fishes was provided by Herzog (1969) from ten fish species collected from different inland water bodies in Iraq. In the Kurdistan Region, larvae were recorded from Dukan Lake in Sulaimani Province by Abdullah and Rasheed (2004) in *Arabibarbus grypus*, *Carasobarbus luteus*, *Chondrostoma regium*, *Cyprinion macrostomum*, *Cyprinus carpio*, *Luciobarbus barbulus*, *L. esocinus, L. kersin*, *L. subquincunciatus*, *L. xanthopterus*, and *Squalius lepidus*. Thus far,

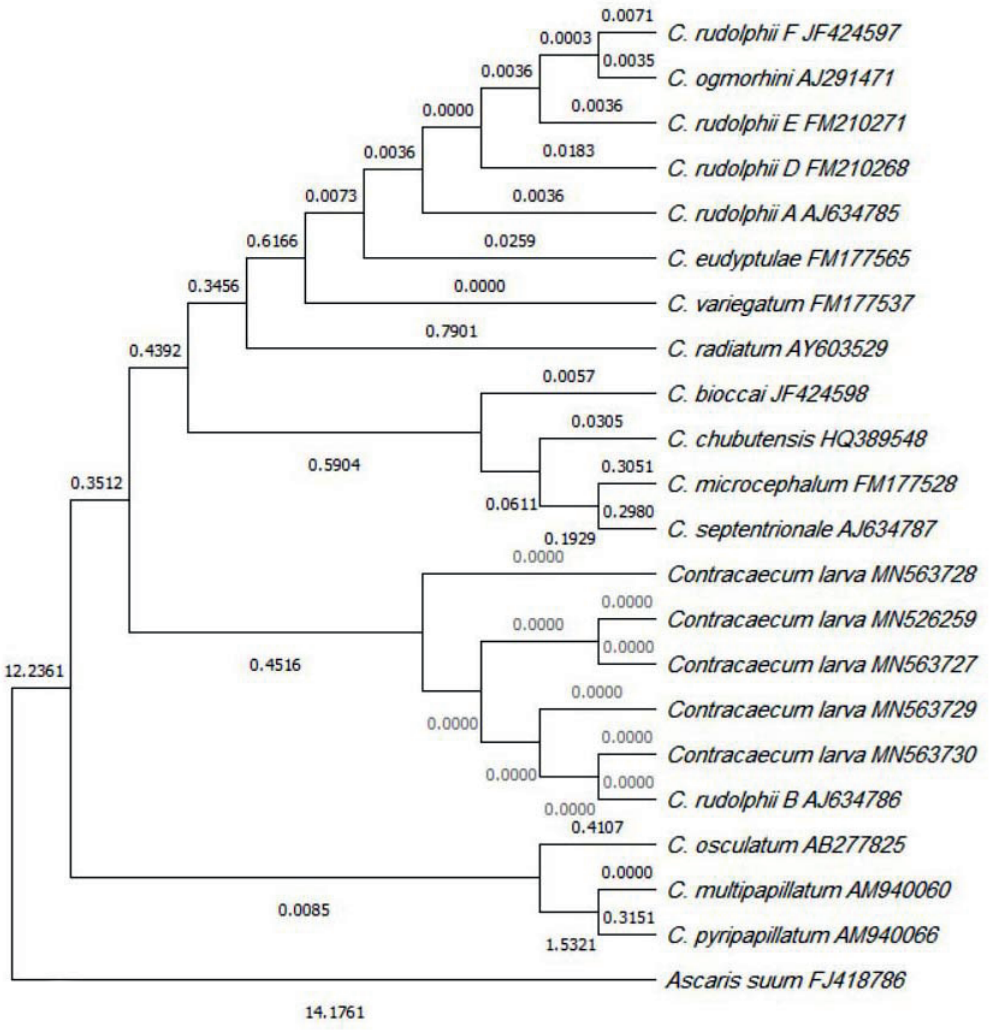

Fig. 8. Phylogenetic relationships between *Contracaecum* larvae from the present study and other *Contracaecum* species as inferred by maximum likelihood obtained from ITS2. *Ascaris suum* was used as outgroup.

a total of 21 host fish species are known for *Contracaecum* larvae in the Kurdistan Region of Iraq (Mhaisen & Abdullah, 2017). In addition, molecular identification of *Contracaecum* larvae in fish species have also not been studied, and there is still no specific identification of the *Contracaecum* species in fresh and marine water fish species in Iraq. Till now, morphological identification based on only optical microscopy has been used to identify larval stage of *Contracaecum* only at genus level in Iraq. Recently, 42 different fish species were known as hosts for *Contracaecum* sp. larvae in Iraq from north to south including marine water fish (Mhaisen, 2019). Therefore, the authors provide the first molecular evidence of *Contracaecum* infection in fish from Iraqi waters in the present study. Notably, the *Contracaecum* larvae can infect many types of fish in Iraq. This nonspecific infection ability towards a variety of different organs and fish species may lead to the infection of a variety of piscivorous birds and mammals in the region. Anisakidosis is a disease caused by the accidental ingestion of larval anisakid nematodes in raw fish. All fishes which infected with *Contracaecum* larvae in the present study are edible, particularly *A. marmid* (prevalence 35 %) in Sulaimani Province. This may affect human health in this region, because this fish is used by local people and other consumers as a food source.

It is noteworthy that the adult *Contracaecum rudolphii* sensu lato was recorded previously for the first time in Iraq in the digestive tract of the great black cormorant *Phalacrocorax carbo* from Baghdad province in Iraq (Al-Moussawi & Mohammad, 2011). Furthermore, three other species of this genus (*Contracaecum*) were reported in birds (final host) in Iraq, namely, *C. microcephalum*, *C. multipapillatum* and *C. ovale* (Habish, 1977; Al-Hadithi & Abdullah, 1991).

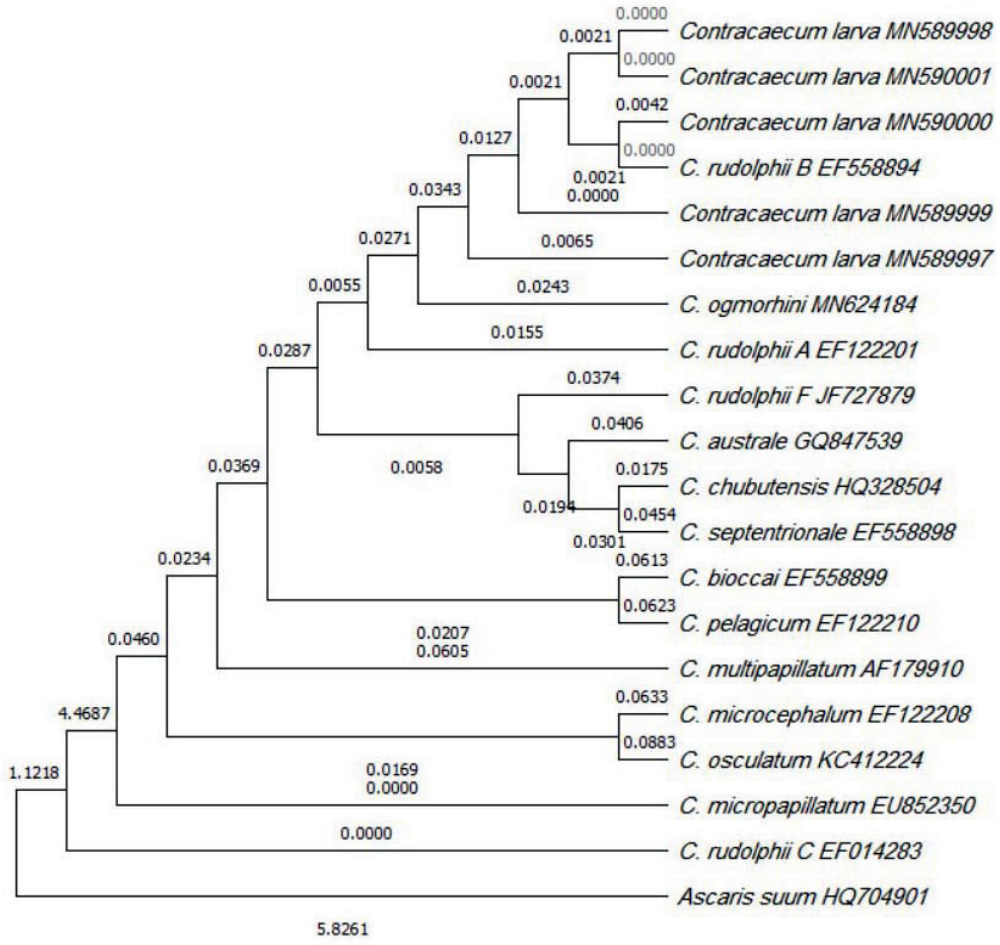

Fig. 9. Phylogenetic relationships between *Contracaecum* larvae from the present study and other *Contracaecum* species as inferred by maximum likelihood obtained from COX2. *Ascaris suum* was used as outgroup.

Conclusion

The *Contracaecum* larvae in these five fish species in Sulaimani Province were found to be *Contracaecum rudolphii* B. A study of the complete life cycle of these larvae in the laboratory is necessary to examination the morphology and ultrastructure of the adult stage (male and female).

Conflict of Interest:

The authors declare that they have no conflict of interest.

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