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Morphology and Molecular Studies of *Contracaecum* Larvae (Nematoda: Anisakidae) in Some Fish species from Sulaimani Province, Kurdistan Region, Iraq

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Abstract: *Contracaecum* is a genus of nematodes belonging to the family Anisakidae, that parasitise many fishes which act as an intermediate or paratenic hosts, while the piscivorous birds and mammals are definitive hosts. A total of 44 third larval stage of *Contracaecum* were collected from 13 infected freshwater fishes belonging to five different species in different water bodies in Sulaimani Province, Kurdistan Region, Iraq from January to the end of December 2018. In this investigation, 966 fishes were collected including six species of Nemacheilidae, five species of Cyprinidae, three species of Leuciscidae, two species of Xenocyprididae, one species of each of Bagridae, Heteropneustidae, Mastacembelidae, Mugilidae, Siluridae and Sisoridae. This study revealed that five fish species (*Cyprinus carpio*, *Luciobarbus barbulus*, *L. esocinus*, *L. xanthopterus* and *Mastacembelus mastacembelus*) were infected with *Contracaecum* larvae with the prevalence of 2.05%, 0.92%, 1.92%, 19.35% and 1.06%, respectively. The *Contracaecum* larvae were morphologically studied by compound light microscope and the molecular analyses was done by amplification, sequencing and comparing different gene loci (ITS1, ITS2 and COX2) of isolated third larval stage of *Contracaecum.* The ITS1, ITS2 and COX2 were amplified by polymerase chain reaction (PCR) and sequenced. The sequences of ITS1, ITS2 and COX2 reveal that all *Contracaecum* larvae from all infected fishes represented exactly one species (*Contracaecum rudolphii* B) based on compering and identity percentage in Gene Bank database. Phylogenetic analysis of the genotype (for ITS1) was described. The genetic characterization of the *Contracaecum* larvae in the present study is available in the GenBank database and they were deposited in GenBank and their accession numbers were demonstrated.

Keywords: *Contracaecum* larva, Nematoda, Freshwater fishes, Sequence analysis.

Introduction

Contracaecum is the most specious and diverse genus of parasitic nematodes of the family Anisakidae and they are cosmopolitan in their distribution (Szostakowska & Fagerholm, 2007). The larval stage of *Contracaecum* usually infect invertebrate

crustaceans and a wide range of fish species (Anderson, 2000). The adult stage can infect both terrestrial and aquatic animals (Shamsi, 2019). They also have a zoonotic significance (Bezerra *et al.*, 2019; Pekmezci and Yardimci, 2019). Anisakid larvae (L3) may accidentally infect human through eating raw, smoked, or undercooked fish and leading to a sever disease known as anisakidosis (anisakiasis), a zoonotic disease characterized by stomach pains, fever, diarrhea and vomiting, particularly the species belonging to *Anisakis*, *Contracaecum* and *Pseudoterranova* (Oshima, 1987; Arslan *et al*., 1995; Yagi *et al*., 1996; Audicana *et al.*, 2002; Shamsi & Butcher, 2011). This disease has been reported worldwide, and it is endemic in Southeast Asia (Audicana & Kennedy, 2008; Mattiucci & Nascetti, 2008).

Species of the genus *Contracaecum* differ from all other Anisakidae by having two oppositely-directed caecae as part of their digestive system, and their excretory pore is located at the anterior end of the parasite (Køie & Fagerholm, 1995). Specific identification of *Contracaecum* larvae in fish hosts to the species level, based on morphological characters is impossible. The scientists in the world investigate the genetic characterization for specific identification of *Contracaecum* larvae by using different genetic markers such as 28S rDNA, 18S rDNA, ITS1, ITS2, mtDNA COX2 (Garbin *et al*. 2013; Mattiucci *et al*. 2015; Younis *et al*., 2017; Malviya *et al*., 2018; Zuo *et al*. 2018). The first record of *Contracaecum* larva in fish from Iraq was by Herzog (1969). After that record, these larvae were recorded in many freshwater fishes in different Iraqi water bodies by many researchers as shown in table (1). Furthermore, there are few publications on the specific identification of *Contracaecum* larva in fishes from the world (Szostakowska & Fagerholm, 2007; Shamsi & Aghazadeh-Meshgi, 2011; Shamsi *et al.*, 2017; Molnár *et al.*, 2019; Pekmezci & Yardimci, 2019). The present investigation identifies the third larval stage of *Contracaecum* by using molecular genetic approach. The previous studies reveal that the molecular approach is useful for accurate identification of *Contracaecum* larva to species level (Shamsi *et al.*, 2011).

Materials & Methods:

Description of Study Area:

Sulaimani Province is situated in the northeast of Iraq, between the latitudes of 35^0 05' and 36^0 30' and between longitudes of '44^0 25' and 46^0 20'. It is located close to the Iraqi-Iranian border. This province is rich with many water bodies in addition to presence of two main rivers: Lesser Zab River and Sirwan River which pass through this Province (Fig. 1).

Collection and Preservation of the specimens

From January to the end of December 2018, a total of 966 fish specimens were collected and searched for infection with *Contracaecum* larvae.

These fishes belong to five species of Cyprinidae, three species of Leuciscidae, two species of Xenocyprididae, six species of Nemacheilidae and one species each of Bagridae, Mastacembelidae, Muglidae, Siluridae and Sisoridae (Table 2).

Table (1): Some previously record of *Contracaecum* larva in different fish species in Iraq.

Hosts	Locality	Sources
A. grypus, *C. luteus*, *H. fosilis*, *L. vorax*, *L. esocinus*, *L. xanthopterus*, *M. sharpeyi*, *M. pelusius*, *P. abu* and *S. triostegus*	Different inland water	Herzog, 1969
S. triostegus and *P. abu*	Local fish market	Shamsuddin *et al.*, 1971
S. triostegus	Tigris River, Baghdad	Khalifa *et al.*, 1978
C. luteus, *L. vorax* and *L. abu*	Shatt Al-Arab River, Basrah	Mhaisen *et al*., 1986
A. centisquama, *C. macrostomum*, *H. fossilis* and *L. cephalus*	Tigris River, Baghdad	Ali *et al*., 1987
P. abu	Babylon fish farm	Ali *et al*., 1989
P. abu and *H. fossilis*	Tigris River, Baghdad	Balasem *et al.*, 1993
A. vorax, A. grypus, *C. luteus*, *H. fosilis*, *L. clussumieri*, *L. xanthopterus*, *M. sharpeyi*, *M. pelusius*, *P. abu* and *S. triostegus*	Basrah	Mhaisen *et al*., 1993
P. abu	Al-Habbaniyah Lake	Mhaisen *et al*., 1999
C. carpio	Man-made lake, Baghdad	Al-Nasiri et al., 2002
P. abu	Al-Diwanyah	Al-Jadoa, 2008
A. dispar, *A. grypus*, *G. affinis* and *P. abu*	Al-Najaf	Al-Awadi & Mhaisen, 2010
C. carpio, *L. xanthopterus* and *L. vorax*	Euphrates, Al-Anbar	Al-Alusi, 2011
C. carpio	Basrah	Eassa *et al*., 2014
T. zilli	Al-Diwanyah, Thi-Qar	Mohammad, 2016
S. triostegus	Tigris River, Baghdad	Al-Moussawi *et al*., 2018

The fishes were caught by gillnetting and pulsed DC electro-shock device SAMUS 1000 (made in Poland). The collected fishes were identified based on their morphometric and meristic characters (Coad, 2010; Kamangar *et al*., 2014; Freyhof *et al*., 2016; Freyhof & Abdullah, 2017; Freyhof & Geiger, 2017). The fishes were transported to parasitological laboratory for parasitological examinations. The fishes were dissected; the body cavity, heart, liver, spleen, kidneys, swim bladder, gonads and muscles were all examined for *Contracaecum* cysts. The gastrointestinal tracts were dissected out from the rectum to the esophagus, opened and examined carefully (Amlacher, 1970). The cysts were removed and washed with normal saline (0.9%) in disposable plastic Petri dishes. Under dissecting microscope, the cysts were teared down with the aid of a fine needle to release the *Contracaecum* larvae, washed with saline solution and then preserved in

ethanol (70%). Prevalence and intensity of infection were calculated for each fish species based on the terminology of Bush *et al.* (1997).

Morphological study

The *Contracaecum* larvae were collected and washed with saline solution (0.9%), fixed in hot (60 °C) formaldehyde solution (4%) in order to relax their bodies and then preserved in ethanol (70%). A small piece of the larval mid-body was excised for molecular study and preserved directly in absolute ethanol. The *Contracaecum* larvae were cleared in glycerin (Moravec, 2009; Moravec & Yooyen, 2011).

The parasitic measurements were made with an ocular micrometer (Olympus) and given in millimeters. The photos were taken with Digital camera model DSC-W570, 16.1 mega pixels (Sony). The parasitic larvae were identified at a genus level according to their morphology and key features and descriptions of Anderson (2000).

Table (2): List of fishes collected from different water bodies in Sulaimani Province with their numbers.

Family and Scientific Names	**Number**
Family: Cyprinidae Rafinesque, 1815 *Cyprinus carpio* Linnaeus, 1758*	195
Garra rufa (Heckel, 1843)	57
Luciobarbus barbulus (Heckel, 1849)	108
Luciobarbus esocinus Heckel, 1843	52
Luciobarbus xanthopterus Heckel, 1843	31
Family: Leuciscidae Bonaparte 1835 *Leuciscus vorax* (Heckel, 1843)	1
Squalius cephalus (Linnaeus, 1758)	37
Squalius lepidus Heckel, 1843	62
Family: Xenocyprididae Günther 1868 *Hemiculter leucisculus* (Basilewsky, 1855) *	121
Hypophthalmichthys molitrix (Valenciennes, 1844) *	2
Family: Bagridae Bleeker, 1858 *Mystus pelusius* (Solander, 1794)	8
Family: Heteropneustidae Hora, 1936a *Heteropneustes fossilis* (Bloch, 1794)*	8
Family: Mastacembelidae Swainson, 1839 *Mastacembelus mastacembelus* (Banks & Solander, 1794)	94
Family: Mugilidae Cuvier, 1829 *Planiliza abu* (Heckel, 1843)	76
Family: Nemacheilidae Regan, 1911 *Eidinemacheilus proudlovei* Freyhof, Abdullah, Ararat, Hamad & Geiger, 2016	40
Oxynoemacheilus gyndes Freyhof & Abdullah, 2017	14
Oxynoemacheilus hanae Freyhof & Abdullah, 2017	5
Oxynoemacheilus kurdistanicus Kamangar, Prokofiev, Ghaderi & Nalbant,	12

2014	
Oxynoemacheilus zarzianus Freyhof & Geiger, 2017	2
Turcinoemacheilus kosswigi Bănărescu & Nalbant, 1964	2
Family: Siluridae Cuvier, 1816 *Silurus triostegus* Heckel, 1843	20
Family: Sisoridae Bleeker, 1858 *Glyptothorax kurdistanicus* (Berg, 1931)	19
Total	966

***:** Exotic fish;

Fig. (1): Map of Iraq showing the study area.

Molecular study

A- DNA extraction

Before the molecular studies, each *Contracaecum* larva was identified based on morphology under optical microscope.

Genomic DNA was isolated from mid piece of individual larvae by using QIAamp® DNA Mini Kit with a bit modification. In brief, the mid piece of individual *Contracaecum* larval parasites were cut into small pieces, digested with proteinase K in ATL buffer for 3 hours at 56 °C and then the obtained DNA were

eluted into 50 µl of AE buffer (QIAamp® DNA Mini Kit).

B- DNA amplification

Polymerase chain reaction (PCR) was used to amplify three gene loci (ITS1, ITS2 and COX2). The specific sets of primer SS1F/NC13R, SS2F/NC2R (Shamsi *et al.*, 2008) and 210F/211R (Nadler & Hudspeth, 2000) were used to amplify two nuclear ribosomal markers (ITS1 and ITS2) and mitochondrial cytochrome oxidase II (COX2), respectively.

PCR was performed (in a volume of 30 µl) in 25 mM Tris-HCl, pH 9.0, at 25 °C, 2 mM $MgCl_2$, 50 mM KCl, 0.1 mg/ml gelatin, 200 µM de dATP, dGTP, dTTP, 100 µM [α32-P] de CTP (0.05 µCi/nmol) and 12.5 µg salmon sperm DNA (activated) and 10 pmol of each primer and 1.5 U *Taq* polymerase (Canvax Biotech, S.L.). The PCR reactions took place a thermocycler (Applied Biosystems 2720, USA) using this cycling instructions: initial denaturation at 94°C for 5 min, denaturation at 94°C, 30 sec for 35 cycles, annealing at 55°C, 30 sec, extension at 72°C, 30 sec and the final extension at 72°C for 7 min, 4°C ∞. 2 µl of genomic DNA (20-40 ng). Deionized distilled water was added to each PCR reaction. Specimen with fish genomic DNA that extracted from muscle was included in the PCR as negative control from these specimens, no amplicons were produced. Five µl of each PCR product was examined on a 1.5% w/v agarose gel, stained with DNA stain (Good View™ SBS Genetech Beijing, China), 1000 bp DNA ladder (Vivantis, Malaysia) was used and photographed by using a gel documentation system. The expected size of the PCR amplicon was ~530bp for ITS1, ~430bp for ITS2 and ~629bp for COX2. After that, the amplicon were purified using EasyPure® Quick Gel Extraction Kit (TRANSGEN BIOTECH), according to the manufacturer's protocols. The purified products were sent to the Macrogen Company (South Korea) for nucleotide sequence analyses by a dideoxy termination method using Genetic analyzer 3500, an applied Biosystems (USA) DNA Sequencer in the two directions (forward and reverse) with the same primer that used in the PCR.

C- Computer based sequence analysis

The resulted ITS1, ITS2 and COX2 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 program CLUSTALW (genome.jp/tools-bin/clustalw) to get the most homologous sequences (one sequence). Subsequently, the obtained sequences were put into the NCBI Blast program for homology search (http://www.ncbi.nlm.nih.gov/). In addition, the multiple sequence alignment were done for each obtained sequences from each gene (ITS1, ITS2 and COX2) in all *Contracaecum* larvae collected from five different fish host species by using the online software program CLUSTALW (genome.jp/tools-bin/clustalw), in order to obtain nucleotide variation among *Contracaecum* larvae in different fish host species.

The sequence data of ITS1 fragments obtained from *Contracaecum* larvae collected from five different fish host species in the present study 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 bp length of

ITS1 segments 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).

Results & Discussion

Prevalence of infection

In the present investigation, 966 fish specimens were examined for the presence of *Contracaecum* larvae. The larvae (n=44) were found in the mesentery, liver and on the intestinal wall of 13 fishes belonging to five different species. The total prevalence of infection was 1.34% (13\966) as shown in Table (3).

The highest prevalence of *Contracaecum* larva was recorded in *L. xanthopterus* (19.35%), while the lowest was occurred in *L. barbulus* (0.92%). These results agree with Abdullah & Rasheed (2004) who recorded *Contracaecum* larva in *L. xanthopterus* (reported as *Barbus xanthopterus*) and *L. barbulus* (reported as *Barbus barbulus*) with prevalences of 44.4% and 7.1%, respectively among 11 fish species in Dukan Lake. These variations in the prevalence may be due to water level, temperature, intensity of both intermediated host and piscivorous bird (final host), and types of food and feeding habits of the fishes (Younis *et al*., 2017).

Morphological identification

Morphological study and measurements of the larvae were done by optical microscope and showed that the larvae of the present study were the third larval stage (L3) of *Contracaecum* as described by Moravec (2009). As well as there were no any significant morphological differences among the larvae which were recorded in different fish species.

The collected *Contracaecum* larvae were light brownish-yellow. They have cylindrical body, anterior end was provided with a distinct boring tooth, tail was short with rounded tip. Their bodies were covered with fine, dense transverse striation of cuticle. The encapsulated larvae were with slender body, posterior ventricular appendix and anterior intestinal caecum. Excretory pore is located at the level of base of lips, cuticular striations were observed throughout the all length of the body. Esophagus is composed of a long muscular part and a short glandular ventriculus. Small numerous brownish granules filled the intestine. Reproductive system (gonads and other accessory organ) were not developed (Fig. 2).

Total length of the larva is 3.3-5.20 mm, width 0.17-0.75 mm. Esophagus length 0.07-0.75 mm. Intestinal caecum length 0.04-0.40 mm. esophageal caecum length 0.07-0.52 mm (Table 4). This parasite, in the third larval stage (L3), lacks the gonads and other parts of the reproduction system. It is difficult to determine the exact classification status at the species level based on morphological study only. The present *Contracaecum* larvae are in a close resemblance to the third larval stage of *Contracaecum* that studied by Moravec (2009) in *C. carpio* from Czech Republic in both measurements and characters. There were no significant morphological variations among the *Contracaecum* larvae which they were recorded in the present study in the different fish species. Photomicrographs of the third larval stage (L3) of *Contracaecum* in *L. xanthopterus* were demonstrated in fig. (2).

Polymer chain reaction (PCR)

The PCR were done by amplifying ITS1, ITS2 and COX2 regions from individual larvae. The agarose gels analyses revealed the same size for each ITS1, ITS2 and COX2 regions. The amplicons were ~530 bp, ~430 bp and ~630 bp for the ITS1, ITS2, and COX2 (not shown), respectively, which confirm that all the obtained sequences were from the same genus.

Sequence and phylogenic analysis

The alignment of resulted sequences demonstrated that there was no significant variation of each ITS1, ITS2 and COX2 regions, which indicates the presence of only one type of larvae.

Based on percentage identities of nucleotide sequences from GenBank, the online BLAST tool showed the ITS1 sequences obtained from larvae from *C. carpio*, *L. barbulus*, *L. esocinus*, *L. xanthopterus* and *M. mastacembelus* matched 100%, 100%, 99.55%, 100% and 100%, respectively to the previously reported reference gene sequences for the ITS-1 in *Contracaecum rudolphii* type-B (Zhang *et al*., 2009) isolated from the intestinal canal of great cormorant *Phalacrocorax carbo sinensis* from the Guangzhou Zoo in Guangdong in China, which was previously examined and deposited in GenBank under accession number FJ467618 (Zhang *et al*., 2009).

The ITS2 sequences obtained from larvae infecting *C. carpio*, *L. barbulus*, *L. esocinus*, *L. xanthopterus* and *M. mastacembelus* matched 100% to the previously reported reference gene sequences for the ITS-2 in *Contracaecum rudolphii* type-B (Zhang *et al*., 2009) isolated from the intestine of great cormorant *Phalacrocorax carbo sinensis* from the Guangzhou Zoo in Guangdong in China, which was previously examined and deposited in GenBank under accession number FJ467620 (Zhang *et al*., 2009).

Table (3): Prevalence of *Contracaecum* larva and mean of intensity among fish species.

Hosts	Fishes		Prevalence %	Mean intensity	Site of infection
	Examined	Infected			
C. carpio	195	4	2.05%	2.75	Intestinal wall
L. barbulus	108	1	0.92	6	Intestine wall
L. esocinus	52	1	1.92	6	Intestine wall
L. xanthopterus	31	6	19.35	2.66	Intestine wall
M. mastacembelus	94	1	1.06	5	Mesentery, liver

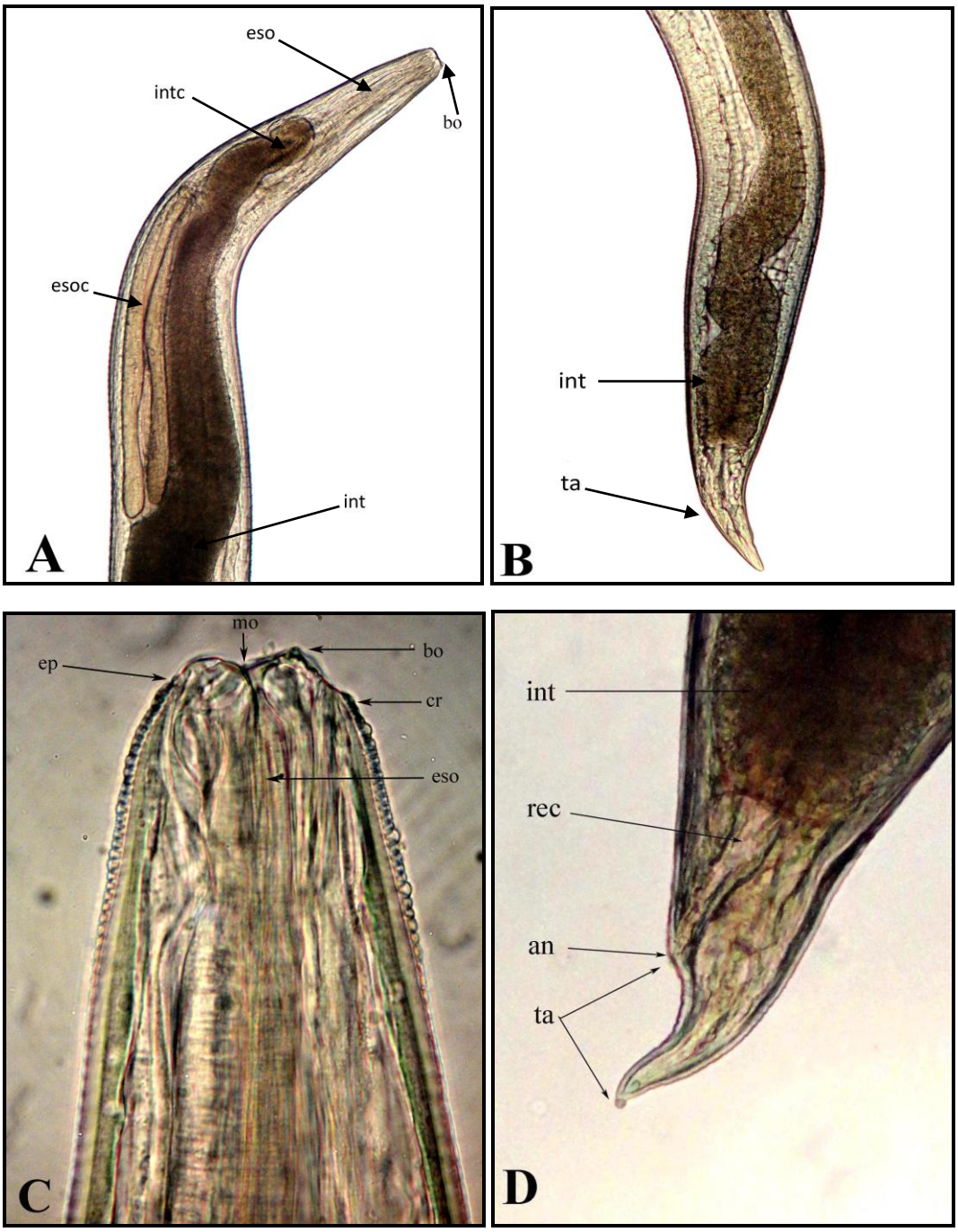

Fig. (2): Photomicrographs of *Contracaecum* larva in *L. xanthopterus*

A- Anterior part of the larva (100X).
B- Posterior part of the larva (100X).
C- Mouth region of the larva (400X).
D- Tail region of the larva (400X).

an= anus; **bo**= boring tooth; **cr**= cuticle ridges; **ep=** excretory pore; **eso**= esophagus; **esoc**= esophageal caecum; **int**= intestine; **intc**= intestinal caecum; **mo**= mouth opening; **rec**= rectum; **ta**= tail.

Table (4): Comparison of measurements of systematically important features in *Contracaecum* larvae in different fish species in the present study (in millimeter).

Host / Measurements	*C. carpio*	*L. barbulus*	*L. esocinus*	*L. xanthopterus*	*M. mastacembelus*
Total length	3.20-3.80 (3.50)	3.10-3.90 (3.50)	4.30-5.70 (5.00)	3.00-3.70 (3.35)	4.80-5.60 (5.20)
Maximum width	0.22-0.28 (0.25)	0.17-0.23 (0.20)	0.20-0.30 (0.25)	0.15-0.19 (0.17)	0.72-0.78 (0.75)
Tail length	0.08-0.09 (0.085)	0.078-0.082 (0.08)	0.078-0.082 (0.08)	0.08-0.10 (0.09)	0.035-0.045 (0.04)
Rectum length	0.06-0.08 (0.07)	0.064-0.086 (0.07)	0.06-0.08 (0.07)	0.06-0.08 (0.07)	0.029-0.031 (0.03)
Boring tooth length	0.004-0.006 (0.005)	0.004-0.006 (0.005)	0.004-0.006 0.005	0.004-0.006 (0.005)	0.0074-0.0076 (0.0075)
Esophagus length	0.60-0.80 (0.70)	0.66-0.70 (0.68)	0.72-0.78 (0.75)	0.65-0.71 (0.68)	0.65-0.75 (0.70)
Esophageal caeca length	0.68-0.72 (0.70)	0.43-0.57 (0.50)	0.48-0.52 (0.52)	0.44-0.56 (0.50)	0.47-0.53 (0.50)
Intestinal caeca length	0.46-0.50 (0.48)	0.28-0.32 (0.30)	0.38-0.42 (0.40)	0.28-0.32 (0.30)	0.35-0.39 (0.37)
% tail to body length	2.50%-2.36% (2.42%)	2.51%-2.10% (2.28%)	1.81%-1.43% (1.60%)	2.66%-2.70% (2.68%)	0.72%-0.80% (0.76%)
Ratio of intestinal caecum to esophageal caecum	1:0.14	1:1.66	1:1.30	1:1.66	1:1.35

Furthermore, they matched 100% to the previously reported reference gene sequences for the ITS2 in *Contracaecum rudolphii* type-B (Li *et al*., 2005) from the same host from the Venice lagoon in northeastern Italy and from Monaci Lake in central Italy, which was previously examined and deposited in GenBank under accession number AJ634786 (Li *et al*., 2005).

The COX2 sequences obtained from larvae infecting *C. carpio* matched 99.79% to the previously reported reference gene sequences for the COX2 in *Contracaecum rudolphii* type-B isolated from the intestine of great cormorant *Phalacrocorax carbo sinensis* from Italy, which was previously examined and deposed in GenBank under accession number EF558894 (Mattiucci *et al*., 2008). COX2 sequences obtained from larvae infecting *L. barbulus*, *L. esocinus*, *L. xanthopterus* and *M. mastacembelus* matched 100%, 99.37%, 100% and 99.58%, respectively to the previously reported reference gene sequences for the COX2 in *Contracaecum rudolphii* type-B (Mattiucci *et al*., 2008) isolated from the great cormorant *Phalacrocorax carbo sinensis* from Italy, which previously was examined and deposited in GenBank under accession number EF513509 (Mattiucci *et al*., 2008).

The genetic characterization of the *Contracaecum* parasite in the present investigation is available in the GenBank. In addition, the ITS1, ITS2 and COX2 sequences obtained were deposited in GenBank and their accession numbers were demonstrated in Table (5). The obtained sequences (ITS1, ITS2 and COX2) from individual *Contracaecum* larva in the different hosts were aligned with the aid of

the online computer program CLASTALAW (https://www.genome.jp/tools-bin/clustalw) then adjusted manually. The results as follow:

ITS1 appeared nucleotide variations in alignment position 161, and the ITS2 showed no nucleotide variations in alignment while, COX2 showed nucleotide variations in alignment positions 27, 33, 36, 54, 57, 78, 117, 168, 177, 282, 318, 336, 474 and 480 (not shown).

For the evolutionary study, the obtained sequence data of ITS1 from collected *Contracaecum* larvae in the present study were subjected to phylogenetic analysis. The sequence data aligned with the data sequence of ITS1 form other 12 different species (16 different genotypes) of *Contracaecum* detected in GenBank (Accession numbers: AJ634782 *Contracaecum rudolphii* A, AJ634783 *C. rudolphii* B, FM210251 *C. rudolphii* D, FM210257 *C. rudolphii* E, JF424597 *C. rudolphii* F, AJ291468 *C. ogmorhini*, AJ007461 *C. eudyptulae*, HQ389546 *C. chubutensis*, MK424804 *C. variegatum*, FM177523 C. *microcephalum*, AJ634784 *C. septentrionale*, JF424598 *C. bioccai*, AY603529 *C. radiatum*, AB277825 *C. osculatum*, AM940056 *C. multipapillatum*, AM940062 *C. pyripapillatum*) and *Ascaris sum* (AB110023) as outgroup. So, in this analysis, 22 nucleotide sequences were involved. Evolutionary analyses were conducted in MEGA X (Kumar *et al*., 2018). The phylogenetic analysis was done by using the maximum likelihood (ML) method (Fig. 3). The evolutionary history was inferred by using the Maximum Likelihood (-6699.73) is shown. Intial tree for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distance 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).

Table (5): Accession numbers of different *Contracaecum* spp. provided by NCBI and used for building the phylogenetic tree.

Host of *Contracaecum rudolphii*	DNA region	Accession numbers	References
Arabesque greenling	ITS	AB277825	Umehara *et al.* (2008)
Arctocephalus pusillus doriferus	ITS1	AJ291468	Zhu *et al.* (2001)
Bird	ITS1	MK424804	Hbaiel & Mohammad (unpublished)
Leptonechotes weddlli	ITS	AY603529	Kijewska *et al.* (2008)
Pelecanus conspicillatus	ITS1	AM940056	Shamsi *et al.* (2008)
	ITS1	AM940062	Shamsi *et al.* (2008)
Pelecanus occidentalis	ITS	JF424597	D'Amelio *et al.* (2012)
	ITS	JF424598	D'Amelio *et al.* (2012)
Phalacrocorax brasilianus	ITS1	HQ389546	Garbin *et al.* (2011)
Phalacrocorax carbo sinensis	ITS1	AJ634782	Li *et al.* (2005)
	ITS1	AJ634783	Li *et al.* (2005)
	ITS1	AJ634784	Li *et al.* (2005)
Phalacrocorax melanoleucos	ITS1	FM177523	Shamsi *et al.* (2009a)
Phalacrocorax varius	ITS1	FM210251	Shamsi *et al.* (2009b)
	ITS1	FM210257	Shamsi *et al.* (2009b)
Unknown	ITS1	AJ007461	Zhu *et al.* (unpublished)
	ITS1	MN557381	Present study

C. carpio	ITS2	MN563731	Present study
	Cox2	MN590002	Present study
L. barbulus	ITS1	MN557382	Present study
	ITS2	MN563732	Present study
	Cox2	MN590003	Present study
L. esocinus	ITS1	MN557383	Present study
	ITS2	MN563733	Present study
	Cox2	MN590004	Present study
L. xanthopterus	ITS1	MN557384	Present study
	ITS2	MN563734	Present study
	Cox2	MN590005	Present study
M. mastacembelus	ITS1	MN557385	Present study
	ITS2	MN563735	Present study
	Cox2	MN590006	Present study

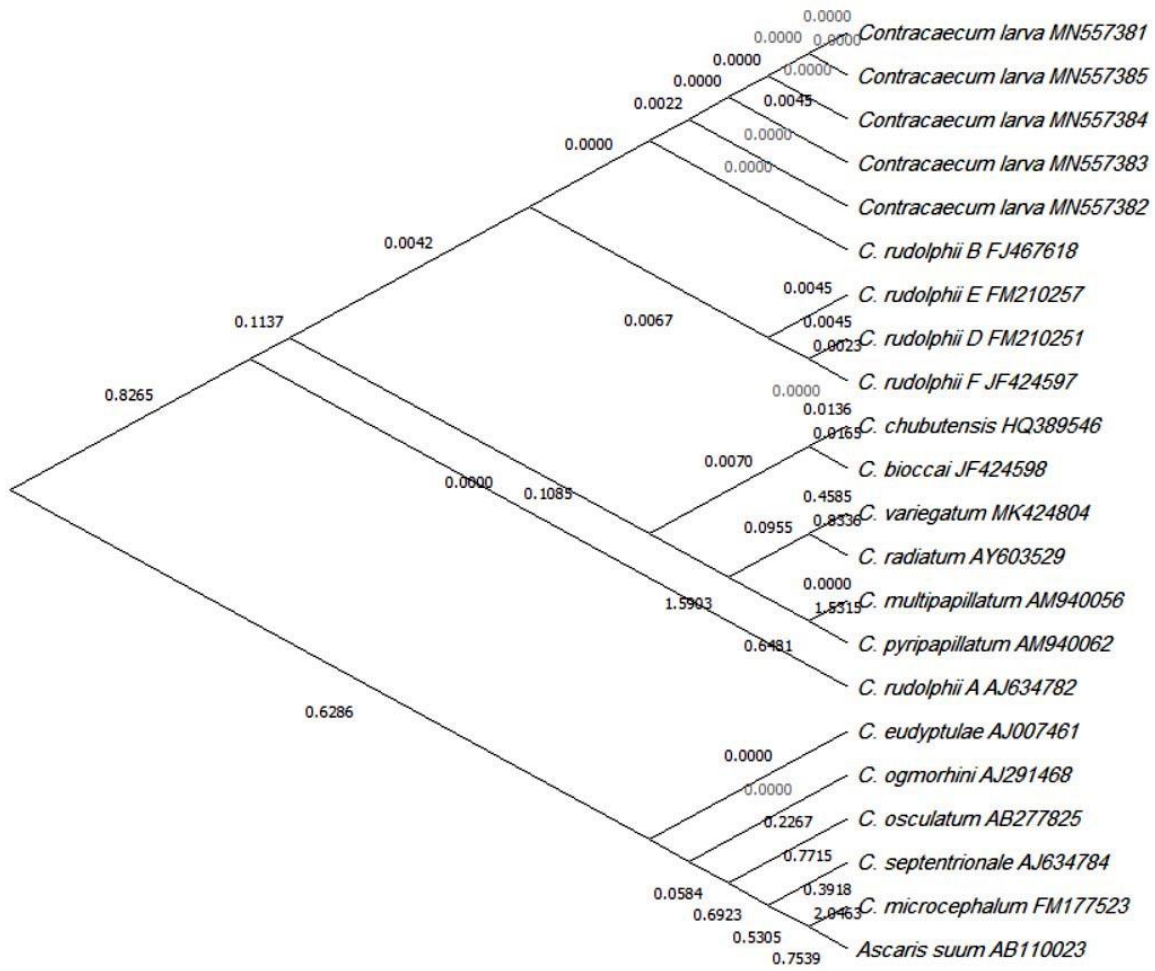

Fig. (3): Phylogenetic relationships between *Contracaecum* larvae from the present study and other *Contracaecum* species as inferred by maximum likelihood obtained from ITS1. *Ascaris suum* was used as outgroup.

The *Contracaecum* larvae (MN557381, MN557382, MN557383, MN557384 and MN557385) collected from *C. carpio, L. barbulus, L. esocinus, L. xanthopterus* and *M. mastacembelus* in Sulaimani Province, Iraq, and adult *C. rudolphii* B (FJ467618) from great cormorant *Phalacrocorax carbo sinensis* in Cuangdong, China were closely related to each other and clustered in the same clade with no (0.0000) sequence divergence, and they were very well supported in the ML tree (Fig. 3) inferred from the ITS1 sequence analysis. Moreover, the phylogenetic tree of the ITS1 sequences using ML analyses indicated that *Contracaecum* larvae in the present study were distinct species by high bootstrap values (Fig. 3).

The first record of *Contracaecum* larva in freshwater fishes of Iraq was done by Herzog (1969) from ten fish species from different water bodies of Iraq. In Kurdistan Region, this larva was recorded for the first time by Abdullah & Rasheed (2004) from Dukan Lake in Sulaimani Province in *Arabibarbus grypus* (reported as *Barbus grypus*), *Carasobarbus luteus* (reported as *B. luteus*), *Chondrostoma regium* (misspelled as *Chondrostoma regius*), *Cyprinion macrostomum* (misspelled as *Cyprinion macrostomus*), *Cyprinus carpio*, *Luciobarbus barbulus* (reported as *Barbus barbulus*), *L. esocinus* (reported as *B. esocinus*), *L. kersin* (reported as *B. kersin*), *L. subquincunciatus* (reported as *B. subquincunciqtus*), *L. xanthopterus* (reported as *B. xanthopterus*), and *Squalius lepidus* (reported as *Leuciscus lepidus*). According to Mhaisen & Abdullah (2017) a total of 21 fish host species are known for *Contracaecum* larva in Kurdistan Region of Iraq. Furthermore, there are 41 fish host species are known for *Contracaecum* larvae in Iraq (Mhaisen, 2020).

In the present study, the molecular approach is used toward characterization of larval anisakid nematodes (*Contracaecum*) in some different fish species in Iraq. On the view of molecular characters, all larvae which were collected from (*C. carpio*, *L. barbulus*, *L. esocinus*, *L. xanthopterus* and *M. mastacembelus*) in the present investigation are belonging to *Contracaecum rudolphii* type-B. It was cleared that the *Contracaecum* larva can infect more different fish species in Iraq. This larva has low host specificity and this may lead to infect a variety of piscivorous birds and mammals in the region.

Contracaecosis is a disease caused by the accidental ingestion of larval *Contracaecum* nematodes mainly in raw fish. *Contracaecum* larvae infected those fishes which mentioned above in Sulaimani Province in the present study, especially *L. xanthopterus* with prevalence of 19.35% and it may affect the human health in this region because this fish is used by local people consumers as one of the most delicious fishes.

Conclusion and recommendation

The *Contracaecum* larvae infect many different fish species in Sulaimani Province and they are belonging to *Contracaecum rudolphii* B. The study of larval stage (inside their cysts) resistance to salt, pH, temperature and freezing in the laboratory is necessary in order to know the weak point of the larva and prevent contracaecosis.

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Conflicts of interest

The authors declare that they have no conflict of interests.

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دراسة مظهرية وجزيئية ليرقة الديدان الخيطية *Contracaecum* في بعض أنواع الأسماك في محافظة السليمانية، أقليم كوردستان، العراق

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المستخلص: كونتراسيكام (*Contracaecum*) هو جنس من الديدان الخيطية ينتمي إلى عائلة Anisakidae، وهي طفيليات للعديد من الأسماك التي تعمل كمضيف وسطي، الطيور والثدييات الآكلة الأسماك هي مضايف نهائية. تم عزل 44 يرقة من الطور الثالث من *Contracaecum* من 13 سمكة مصابة تعود إلى خمسة أنواع مختلفة. جمعت الأسماك من المسطحات المائية المختلفة في محافظة السليمانية، إقليم كردستان العراق، خلال الفترة المحصورة بين شهر كانون الثاني وحتى نهاية كانون الأول 2018 خلال الدراسة الحالية، تم جمع 966 سمكة وهي ستة انواع من العائلة Nemacheilidae وخمسة انواع من عائلة الشبوطيات (Cyprinidae)، وثلاثة انواع من العائلة Leuciscidae، ونوعين من العائلة Xenocyprididae، ونوع واحد من كل من عائلة ابو الزمير (Bagridae)، والجري اللاسع (Heteropneustidae)، والمرمريج (Mastacembelidae)، واالبياح (Mugilidae)، ,والجري (Siluridae) والصقنقرور (Sisoriae). أظهرت الدراسة أن خمسة أنواع من الأسماك (الكارب الإعتيادي *Cyprinus carpio*, أبو براطم *Luciobarbus barbulus*, البزَ *L. esocinus*, الكطَان *L. xanthopterus* والمرمريج *Mastacembelus mastacembelus*) أصيبت بيرقات *Contracaecum* بنسبة انتشار 2.05٪ و 0.92٪ و 1.92٪ و 19.35٪ و 1.06٪. على التوالي. تمّت دراسة الشكل الخارجي لهذه اليرقات بواسطة المجهر الضوئي.كما أجريت دراسة التحليلات الجزيئية عن طريق التضخيم والتسلسل ومقارنة مواقع الجينات المختلفة (ITS1، ITS2 و COX2) لمختلف يرقات *Contracaecum* المعزولة. تمّ الحصول على 15 تسلسلًا لهذه اليرقة التي تمّ جمعها. تمّ تضخيم ITS1، ITS2 و COX2 عن طريق تفاعل سلسلة البلمرة Polymer chain reaction وتسلسلها. وكشفت أن عينات يرقات *Contracaecum* التي تمّ جمعها من خمسة أنواع من الأسماك تعود لنوع واحد وهو *Contracaecum rudolphii* B إستنادًا إلى نسبة الهوية في قاعدة بيانات بنك الجينات. وقد تمّ وصف التحليل الوراثي للنمط الوراثي. التوصيف الوراثي لهذه اليرقات في هذه الدراسة متاح في قاعدة بيانات بنك الجينات. تمّ إيداع تسلسلات ITS1، ITS2 و COX2 التي تمّ الحصول عليها في GenBank وأظهرت أرقام إنضمامها.

الكلمات المفتاحية: يرقة *Contracaecum* ، الديدان الخيطية ، أسماك المياه العذبة، دراسة سلسلة القواعد النتروجينية.