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Surface Ultrastructure and Molecular Studies of *Clinostomum complanatum* (Rudolphi, 1814) Braun, 1899 (Trematoda: Clinostomidae) Metacercariae in some Freshwater Fishes from Sulaimani Province, Iraq

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Abstract: *Clinostomum* is a trematode genus in the family Clinostomidae. The mentioned trematode species is parasitizing many fish species as intermediate hosts, while piscivorous birds and mammals are the main definitive hosts. Total of 58 metacercariae of *Clinostomum* larvae were dissected out from 25 infected fish specimens from eight different species from a number of water bodies of Sulaimani Province, Kurdistan Region, Iraq. In this investigation, 959 fish specimens were collected. These includes five cyprinid species: *Capoeta trutta* (Heckel), *C. umbla* (Heckel), *Carasobarbus kosswigi* (Ladiges), *Cyprinion macrostomum* Heckel and *Garra rufa* (Heckel), two leuciscid species: *Alburnus sellal* Heckel and *Squalius lepidus* Heckel and the mugilid fish *Planiliza abu* (Heckel). The prevalence of infection for each of these species was 1.8%, 5%, 20%, 1.5%, 1.7%, 4.9%, 4.9% and 1.3%, respectively. The morphology of the *Clinostomum* metacercariae was studied by using a compound microscope; their ultra-morphology was evaluated with a scanning electron microscopy (SEM); and the molecular analysis were performed by amplifying, sequencing and comparing the ITS1-5.8S-ITS2 and 28S rDNA gene loci from isolated *Clinostomum* metacercariae. The obtained sequences confirmed that all metacercariae of the *Clinostomum*, collected during the present study, represented with *C. complanatum* (Rudolphi, 1814) based on percent identity with sequences in the GenBank subject database. The molecular characterisations of the *C. complanatum* metacercariae in the present study were deposited in GenBank NCBI.

Keywords: Digenea, Freshwater fishes, Genetics, Iraq, Metacercaria, Yellow grub disease.

Introduction

The trematode *Clinostomum complanatum* (Rudolphi, 1814), is the most important species of the genus *Clinostomum* (Leidy, 1856) in the family Clinostomidae (Lühe, 1901) and has a cosmopolitan distribution (Lo *et al.*, 1981). The adult stage parasitises the oral cavity, pharynx or oesophagus of piscivorous birds, reptiles and occasionally mammals, including humans (Kanev *et al.*, 2002; Gustinelli *et al.*, 2010). Species of

Clinostomum has a complex life cycle. Snails are the first intermediate host, many fish species and amphibians as second intermediate hosts (Kanev *et al.*, 2002; Pinto *et al.*, 2015). The life cycle of clinostomids, as exemplified by *C. complanatum*, consists of adults laying eggs from which miracidia hatch and infect snails (the first intermediate hosts); then, miracidia develop into next stages (sporocysts followed by rediae, then producing brevifurcate cercariae). Cercariae penetrates the second intermediate host (fish) developing into metacercariae, which infect definitive hosts (Olsen, 1974; Smyth, 1994).

C. complanatum is economically important, as it is the causative agent of yellow grub disease in both wild and farmed fishes. This disease makes them unsuitable food for human consumption due to considerable damage to the skin, fins, and muscles (Lane & Morris, 2000; Yooyen *et al.*, 2006; Bonett *et al.*, 2011). The parasite also has zoonotic significance, as accidental human infection causing pharyngitis or laryngitis through consumption of infected raw or/and improperly cooked freshwater fishes haves frequently been reported from various geographical regions (Park *et al.*, 2009; Hara *et al.*, 2014; Lee *et al.,* 2017).

In the past, *Clinostomum* has been subjected to several taxonomic revisions due to the high degree of morphological variability within the same species (Gustinelli *et al.*, 2010). Scientists have used genetic characterisation in specific identification of digeneans metacercariae by using different loci as markers, such as 18S rDNA, ITS1, ITS2, 28S rDNA and mtDNA COI (Nolan & Cribb, 2005; Locke *et al.*, 2010; Caffara *et al.*, 2011). It was demonstrated that combining a molecular approach with morphological analyses provides accurate identification of *Clinostomum* species (Simsek *et al.*, 2018).

C. complanatum metacercariae were recorded for first time in Iraq by Mhaisen *et al.* (1986), who identified this parasite in both *Carasobarbus luteus* (reported as *Barbus luteus*) and *Leuciscus vorax* (reported as *Aspius vorax*) in Mehaijeran Creek in Basrah Province in south of Iraq. After that, these metacercariae were recorded continuously in many freshwater fishes from Basrah Province (Mhaisen *et al.*, 2013), Al-Najaf Al-Ashraf Province (Al-Joborae *et al.*, 2010), Kurdistan (Mhaisen & Abdullah, 2017) and Thi Qar Province (Mhaisen, 2019). To date, a total of 27 fish species have been found to be hosts for *C. complanatum* metacercariae in Iraq (Mhaisen, 2023).

Many publications revealed the specific identification of *Clinostomum* metacercariae in fish hosts, based on molecular studies, conducted around the world (Gustinelli *et al.*, 2010; Bonett *et al.*, 2011; Acosta *et al.*, 2016; Caffara *et al.,* 2017; Simsek *et al.*, 2018; Caffara *et al.*, 2019; Caffara *et al.*, 2020). However, to our knowledge, there has not yet been any study on *C. complanatum* metacercarial ultrastructure and molecular characterisation from freshwater fishes in Iraq. Thus, the present study aimed to evaluate variations in the prevalence of *C. complanatum* metacercariae among edible freshwater fishes in Sulaimani Province, Kurdistan region, Iraq. In addition, performing ultra-morphological study and molecular characterisation for the first time based on ITS1-5.8S-ITS-2 and 28S gene regions will further confirm the accurate diagnosis of this parasite.

Materials & Methods

A-Description of collection area

Sulaimani city is situated in the north eastern of Iraq, and regards as a second city of the Kurdistan Region, on the border with Iran.

Many bodies of water are found in Sulaimani Province, including two rivers (Lesser Zab and Sirwan Rivers) that passing the province. Lesser Zab River comprises 20-30%, while Sirwan River 12-15% of total Tigris River water flows (Abdulla & Al-Badranih, 2000).

B-Collection and preservation of specimens

A total of 959 freshwater fishes were collected from different water bodies and searched for infection with *Clinostomum* metacercariae. The fishes were five cyprinid species: *Capoeta trutta* (n= 217), *Capoeta umbla* (n= 160), *Carasobarbus kosswigi* (n= 5), *Cyprinion macrostomum* (n=324) and *Garra rufa* (n=58), two luciscid species: *Alburnus sellal* (n= 61) and *Squalius lepidus* (n= 61); and one mugilid species: *Planiliza abu* (n= 73).

The fishes were caught by local fishermen by using gillnetting. Morphometric and meristic characters were used to identify the collected fishes (Coad, 2010), and the scientific names were updated according to Fricke *et al*. (2023), while Mohammadian-Kalat *et al*. (2017) for validity of *A. sellal*. Fishes were transported as alive to the parasitological laboratory immediately for examination. The fishes were examined externally and internally for *Clinostomum* metacercarial cysts, which were removed from the gill arch, skin, muscles and viscera, then washed with normal saline (0.9% NaCl) in separate petri dishes. The cysts were disrupted under a dissecting microscope with the aid of two fine needles to release the metacercaria, then washed with normal saline. The metacercariae were fixed in 5% hot formalin (60°C) and then preserved in 70% ethanol (Scholz, 1989). Margolis *et al*. (1982) was followed for the ecological terms of Prevalence and mean of intensity.

Morphological investigation

A- Light microscopy

The preserved Clinostomum metacercariae were stained with aceto-carmine, cleared in xylene and mounted in Canada balsam to prepare a permanent slide (Scholz 1989). Before the fixation process, some larvae specimen pieces were fixed and preserved directly in absolute ethanol for molecular study.

Morphological characteristics and morphometric measurements of the metacercariae were revealed by using an optical microscope with ocular micrometre (Olympus, Japan) and are presented in millimetres. Photomicrographs shots were achieved with optical steady digital camera (Sony Cyber-shot DSC-W570 16.1 MP, Japan). The metacercariae were identified according to morphological keys and descriptions given by Yamaguti (1958) and Gibson *et al*. (2002).

B- Scanning electron microscopy

For the scanning electron microscopy, the preserved metacercariae were post-fixed in 1% osmium tetroxide. The specimens were dehydrated, dried and mounted on aluminium stubs, followed by coating with gold (Chai *et al*., 2002). The specimens were observed with a scanning electron microscope (FEI Quanta 400, USA) with accelerating voltage of 25 kV.

Molecular study

A-DNA extraction

A small piece of each individual metacercariae were used for total genomic DNA extraction by using the QIAamp® DNA Mini Kit (Germany). In brief, the small piece of individual metacercaria was cut to smaller pieces, digestion was performed by proteinase K in ATL buffer for 1–3 hours at 56°C. The

obtained DNA was eluted into 100µl of AE buffer (QIAamp® DNA Mini Kit).

B- Amplification on extracted DNA

Two gene loci were amplified with Polymerase Chain Reaction (PCR), the ITS1-5.8S-ITS2 gene cluster and the 28S rDNA gene. The specific sets of primers used for the amplification of the ITS1-5.8S-ITS2 gene cluster were D1F (5′-AGG AAT TCC TGG TAA GTG CAA G-3′) and D2R (5′-CGT TAC TGA GGG AAT CCT GGT-3′) (Galazzo et al., 2002), and those used for the 28S rDNA gene were 28F1 (5′-ACG TGA TTA CCC GCT GAA CT-3′) and 28R600 (5´CTG AGA AAG TGC ACT GAC AAG-3´) (Marcilla *et al*., 2002).

PCR was performed in a volume of 40 µl by using the following cycling protocol: initial denaturation at 94°C (5 min); denaturation at 94°C (35 cycles for 30 sec), annealing at 55°C (30 sec) and extension at 72°C (30 sec); and final extension at 72°C (7 min), with a 4°C hold. Fish genomic DNA (fish muscle extracted) was included as a negative control; no amplicons were seen from this DNA. The PCR product was run and visualised on a 1% agarose gel, DNA stain (GoodViewTM, SBS Genetech, Beijing, China) used for staining and imaged using a gel documentation system (SmartDoc Imaging Enclosure, ACCURISTM, USA). The amplicons purification done with EasyPure® Quick Gel Extraction Kit (TransGen Biotech, Beijing, China) following the manufacturer's protocols. The purified products were sequenced in forward and reverse directions of the same primers used for PCR.

C- Computer-based sequence analysis

The resulting ITS1-5.8S-ITS2 and 28S rDNA sequences (forward) were compared with their complements (reverse) and adjusted by using online software tool (bioinformatics.org\ sms\ rev_comp.html). The reverse sequences were reverse complemented and compared to the forward sequences. Sequences were then aligned by using a multiple sequence alignment program (the online software program CLUSTALW [genome.jp/tools-bin/clustalw]) for checking the sequences quality. The obtained sequence blasted into the online NCBI BLAST program to conduct a homology search (http://www.ncbi.nlm.nih.gov/). In addition, multiple sequence alignment was performed for all obtained sequence from each gene (ITS1-5.8S-ITS2 and 28S rDNA) in all *Clinostomum* metacercariae collected in all the infected fishes by using an online software program (CLUSTALW) (genome.jp/tools-bin/clustalw) to determine whether the nucleotide sequences varied among *Clinostomum* metacercariae from studied fish hosts.

Results & Discussion

Morphological identification

Many whitish-yellow encysted metacercariae were dissected out from the pharynx, skin, muscle and viscera in different fish species. The cysts were a round to oval in shape and visible to the naked eye, and each cyst contained only one metacercaria (Fig. 1). After their cysts were ruptured, all parasites were determined to be *Clinostomum* spp. metacercariae, as described by Gibson *et al.* (2002). In addition, no any distinct morphological variances among the studied metacercariae were obtained from infected fish species.

The isolated *Clinostomum* spp. metacercariae in the different infected fishes were ligulae shaped. They had subterminal oral sucker with a mouth located in the centre of the sucker. The ventral sucker, located in the

first third of the body, was larger than the oral sucker. The oesophagus was extremely short, and it had two thick, wrinkled, unbranched and blind intestinal caeca. The testes and ovary were not well developed (Fig. 2). The body length was 3.5–6.0 mm and maximum width was 1.0–1.5 mm at starting of the third body part. The diameter of oral sucker was 0.25–0.35 mm and that of the ventral sucker was 0.75–0.85 mm.

The *Clinostomum* metacercariae in the present study closely resemble the *C. complanatum* metacercariae reported by Al-Maliki *et al.* (2018) in *Alburnus mossulensis* (= *A. sellal*), *Capoeta damascina*, *Garra rufa* and *Squalius cephalus* from Swarian station, Gheshlagh River in Kurdistan Province, Iran; and Simsek *et al.* (2018) in *Squalius cephalus* from the Central Anatolia Region in Turkey. There were no significant morphological variations among the metacercariae of this parasite obtained from the different fish species hosts of the present study. Photomicrographs of the metacercariae of *C. complanatum* in *S. lepidus* included as an example (Fig. 2).

This present species was first noticed in Iraq from both *Carassobarbus luteus* (reported as *Barbus luteus*) and *Leuciscus vorax* (reported as *Aspius vorax*) in Mehaijeran Creek in Basrah Province in the south of Iraq (Mhaisen *et. al.*, 1983). Subsequently, it was reported from 27 fish host species in Iraq (*Acanthobrama marmid*, *Alburnus caeruleus*, *A. sellal*, *Aphanius stoliczkanus*, *Arabibarbus grypus*, *Capoeta umbla*, *Carasobarbus luteus*, *Carassius auratus*, *Chondrostoma regium*, *Coptodon zillii*, *Cyprinion kais*, *C. macrostomum*, *Cyprinus carpio*, *Gambusia holbrooki*, *Garra rufa*, *Glyptothorax kurdistanicus*, *Heteropneustes fossilis*, *Leuciscus vorax*, *Luciobarbus esocinus*, *L. xanthopterus*, *Mastacembelus mastacembelus*, *Mystus pelusius*, *Planiliza abu*, *P. subviridis*, *Poecilia latipinna*, *Silurus triostegus* and *Squalius lepidus*). To our knowledge, no additional host species have been reported (Mhaisen, 2023). Thus, *C. kosswigi* and *C. trutta* in the present study could be regarded as new hosts for this parasite in Iraq.

Prevalence of infection

A total of 959 specimens of freshwater fishes collected from Sulaimani Province, Iraq, were examined for the presence of yellow grub disease. The encysted metacercaria of *Clinostomum* (n= 58) were found in the branchial cavity, viscera and muscle of 25 infected fishes belonging to eight different species. The prevalence and mean intensity for each fish species were shown in table (1). The highest prevalence was in *C. kosswigi* (20%), followed by *C. umbla* (5%), while the lowest was observed in *P. abu* (1.3%). These results agree with those of Gholami *et al.* (2011), who recorded *C. complanatum* metacercariae in *A. dispar* caught from the Mehran River in the southern of Iran, with a prevalence of 4.12%. Malek & Mobedi (2001) recorded this metacercaria in *C. capoeta gracilis*, collected from Shiroud River in Iran with a prevalence of 47.3%. Ahammed-Shareef & Abidi (2012) recorded *C. complanatum* metacercaria in *Channa punctatus* from India with a prevalence of 24.7%. The branchial cavity of spiny eel *Mastacembelus mastacembelus* caught in Greater Zab River in Erbil Province was seen infected by these metacercariae with a prevalence of 0.78% (Bashě & Abdullah, 2010). In addition, *C. complanatum* metacercariae were reported in *Tilapia zillii* (=*Coptodon zillii*) collected from Qurna in Basrah Province at a prevalence of 26.1% (Al-Maliki *et al.*, 2015). The variations in the present prevalence of different fish hosts may be due to water level, temperature and/or

density of both the intermediate and final hosts.

The metacercariae of *C. complanatum* are known to cause considerable damage to the viscera and the musculature of fish species (Ahammed-Shareef & Abidi, 2012; Wang *et al.*, 2017). In addition, the metacercaria of *Clinostomum* can infect many amphibians, such as frogs, toads, salamanders and tritons (Goldberg *et al.*, 1998). Previous studies showed that *C. complanatum* had low host specificity and is found in many freshwater fishes (Chung *et al.*, 1995; Gholami *et al.*, 2011). Chai *et al.* (2005) reported *Clinostomum* metacercaria as a fish born trematode of serious zoonotic concern. Many cases of human infection with *C. complanatum* metacercaria have been reported from different parts of the world. Thus, there is a possibility of zoonotic infection in this region.

Scanning electron microscopy

The SEM study showed that the metacercariae are linguiform and dorsoventrally flattened, and that the anterior and posterior ends of the body are rounded and blunt.

The body becomes gradually wider when extended posteriorly (the posterior part is slightly broader than the anterior part) and is ventrally concave (Fig. 3). They have oral and ventral suckers. Tegument lacking spines, body surface is covered with numerous tegumental sensory papillae. The oral sucker is smaller in size from the ventral sucker that located at the anterior end, surrounded by a thick collar-like rim and margined densely with sensory papillae that are arranged radially around the mouth (Fig. 4). The ventral sucker is large, sub-median and anteriorly located. Some smooth dome-shaped papillae are presented around the ventral sucker. The area between the oral and ventral suckers has a corrugated surface with some sensory papillae (Fig. 5).

Fig. (1): Photomicrograph of encysted *Clinostomum* metacercariae in caudal muscle of *Cyprinion macrostomum.*

Fig. (2): Photomicrograph of *Clinostomum complanatum* metacercariae. Left: Unstained; Right: stained with acetocarmine.

Table (1): Prevalence of *Clinostomum complanatum* metacercariae and mean of intensity among fish species in the present study

Host	Fish		Prevalence %	Mean intensity	Site of infection
	Examined	Infected			
Alburnus sellal	61	3	4.9	1.3	Muscles, Pharynx
Capoeta trutta	217	4	1.8	1.2	Muscles, Pharynx
Capoeta umbla	160	8	5	2.1	Muscles, Pharynx
Carasobarbus kosswigi	5	1	20	2	Pharynx
Cyprinion macrostomum	324	5	1.5	2.4	Muscles, Pharynx
Garra rufa	58	1	1.7	2	Muscles
Planiliza abu	73	1	1.3	2	Muscles
Squalius lepidus	61	3	4.9	5.3	Muscles, Mesentery

Several crescent-shaped slits are distributed marginally in the anterior region of the body. The ventral part of the body has a papillary tegument and includes a genital pore with some sensory papillae surrounding it. The mid ventral posterior tegument has a cobblestone-like structure (Fig. 6). The SEM study revealed no significant ultrastructural differences among the *C. complanatum* metacercariae from the different fish hosts of the present study. The surface ultrastructures of *C. complanatum* metacercariae generally showed great similarity to *C. complanatum* metacercarial flukes collected from *Trichogaster fasciata* (recorded as *Trichogaster fasciatus*) in India (Abidi *et al.*, 1988). However, some differences were recognised in the shape and distribution of the tegumental sensory papillae around the genital pore. Identification of *Clinostomum* species based on morphology (light and SEM) alone can lead to misidentification due to phenotypic variability within the same species (Feizullaev & Mirzoeva, 1983). Even among different species of *Clinostomum*, there are no reliable morphological characters, which has led to instability in their taxonomic status and frequent revision in the taxonomic position of

this genus. Moreover, the identification at the species level could not be revealed with morphological analysis alone (Gustinelli *et al.*, 2010; Acosta *et al.*, 2016). In contrast, molecular approaches have proven to be useful in the specific identification of parasites up to the species level (Nolan & Cribb, 2005; Ghatani *et al.*, 2012). Furthermore, molecular approaches can be used to differentiate between *C. complanatum* and *C. marginatum*, which are similar to each other (Caffara *et al.*, 2011).

Polymerase chain reaction

PCR was performed to amplify the ITS1-5.8S-ITS2 and 28S rDNA gene loci from extracted genomic DNA. The agarose gel analysis revealed that all ITS1-5.8S-ITS2 (Fig. 7) and 28S rDNA (Fig. 8) regions were of the same size, thus the obtained sequences all were to the same genus.

Previously referenced gene sequences of the ITS1-5.8S-ITS-2 in *C. complanatum* metacercariae isolated from *Scardinius erythropthalmus* and *Perca fluviatilis* collected in Romania near Tulcea City (from the Danube Delta) and Rosu Lake, respectively, which were examined previously; deposited in GenBank with accession number MK811210 (Locke *et al.*, 2019) (Fig. 9).

The 28S rDNA sequences extracted from *Clinostomum* metacercariae in all infected fishes in the present study matched 99.82% to the reported reference gene sequences for the 28S rDNA in *C. complanatum* metacercariae isolated from previous hosts and documented in GenBank with accession number MK811210 (Fig. 10) (Locke *et al.*, 2019); only one substitution was observed (T instead of C at position 542 from the 5′ end) and it could be a natural variation.

Fig. (3): Scanning Electron micrograph of *Clinostomum complanatum* metacercaria.

Fig. (4): Scanning Electron micrograph of the oral sucker of *Clinostomum complanatum* metacercaria.

The obtained sequences (ITS1-5.8S-ITS2 and 28S) from every single metacercaria specimen from different hosts were aligned by using the online computer program CLUSTALAW (https://www.genome.jp/tools-bin/clustalw) and adjusted manually. The present results showed that there are no nucleotide variations in alignment of both gene loci (Figs. 7 and 10).

The genetic characterisation (ITS1-5.8S-ITS2 and 28S rDNA) of *C. complanatum* metacercariae in the present study are available in the GenBank database with their accession numbers that provided in table (2).

In Kurdistan Region, Iraq, this metacercaria was reported from the gills of *Luciobarbus esocinus* (as *Barbus esocinus*) from the Greater Zab River (Ali, 1989), the gill cavity of *Squalius lepidus* (as *Leuciscus lepidus*) from Dokan Lake (Abdullah & Rasheed, 2004), the gill cavity of *Cyprinion macrostomum* from the Bahdinan River (Bilal & Abdullah, 2009), the gill cavity of *Capoeta umbla* (as *Varicorhinus umbla*) from the Greater Zab River (Abdullah & Mhaisen, 2010) and from Darbandikhan Lake (Abdullah & Abdullah, 2015) and the gill cavity of *Glyptothorax kurdistanicus* from some watersheds in the Sharbazher Area in Sulaimani Province (Abdullah *et al.*, 2018). Furthermore, Mhaisen & Abdullah (2017) listed six fish host species (*Capoeta umbla*, *Carasobarbus luteus*, *Cyprinion macrostomum*, *Luciobarbus esocinus*, *Mastacembelus mastacembelus* and *Squalius lepidus*) for *C. complanatum* metacercaria in the Kurdistan Region of Iraq.

Table (2): NCBI GenBank accession numbers for newly collected *Clinostomum complanatum* metacercariae from various fish hosts.

Host	28S sequences	ITS1-5.8S-ITS2 sequences
A. sellal	OM001620	OM001708
Cyprinion macrostomum	OM001621	OM001709
Carasobarbus kosswigi	OM001622	OM001710
Capoeta trutta	OM001623	OM001711
Capoeta umbla	OM001624	OM001712
Garra rufa	OM001625	OM001713
Planiliza abu	OM001626	OM001714
Squalius lepidus	OM001627	OM001715

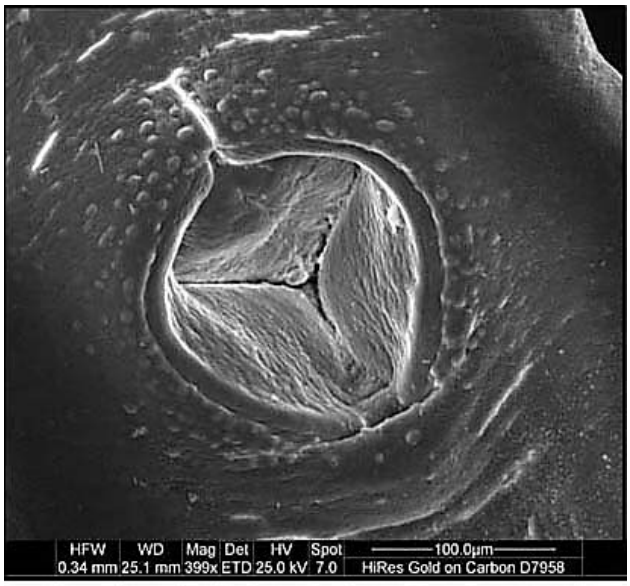


Fig. (5): Scanning Electron micrograph of the ventral sucker of *C. complanatum* metacercaria.

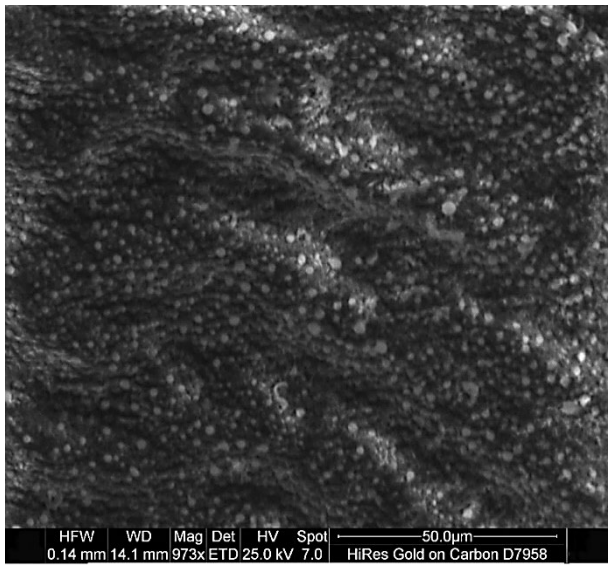


Fig. (6): Scanning Electron micrograph of the tegument of *C. complanatum* metacercaria.

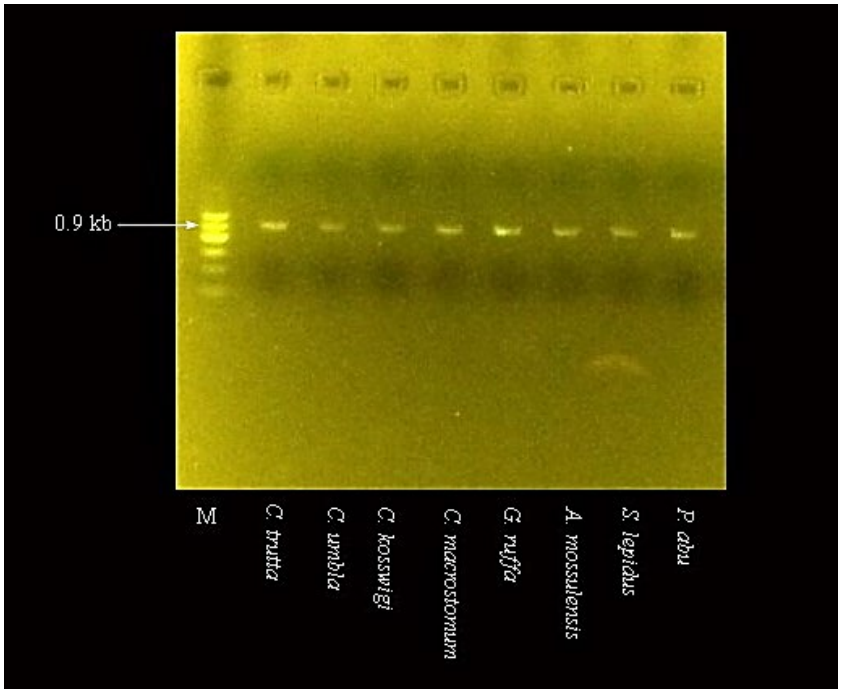


Fig. (7): Amplified bands on the agarose gel after Gel electrophoresis of *Clinostomum* ITS1-5.8S-ITS2 from different fish host species. M= Marker (Ladder).

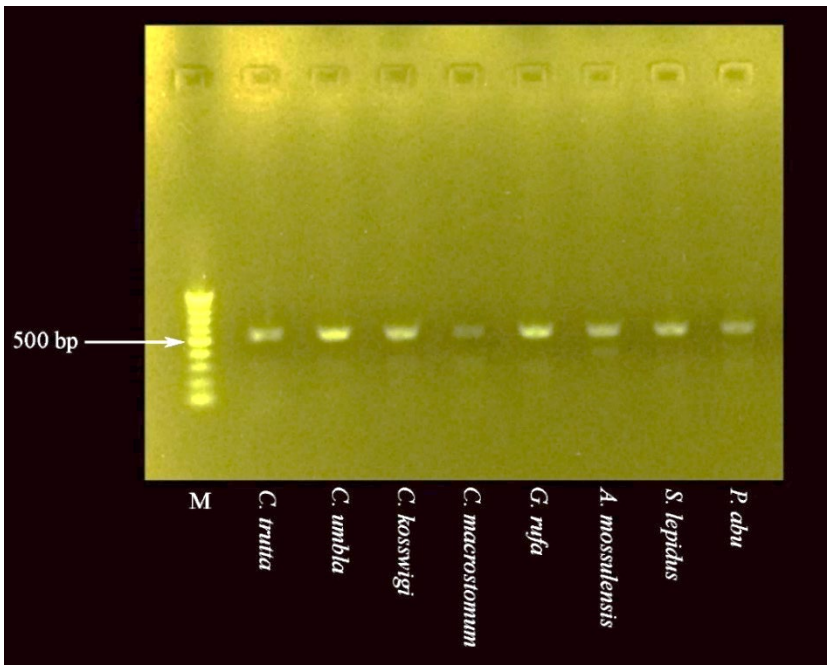
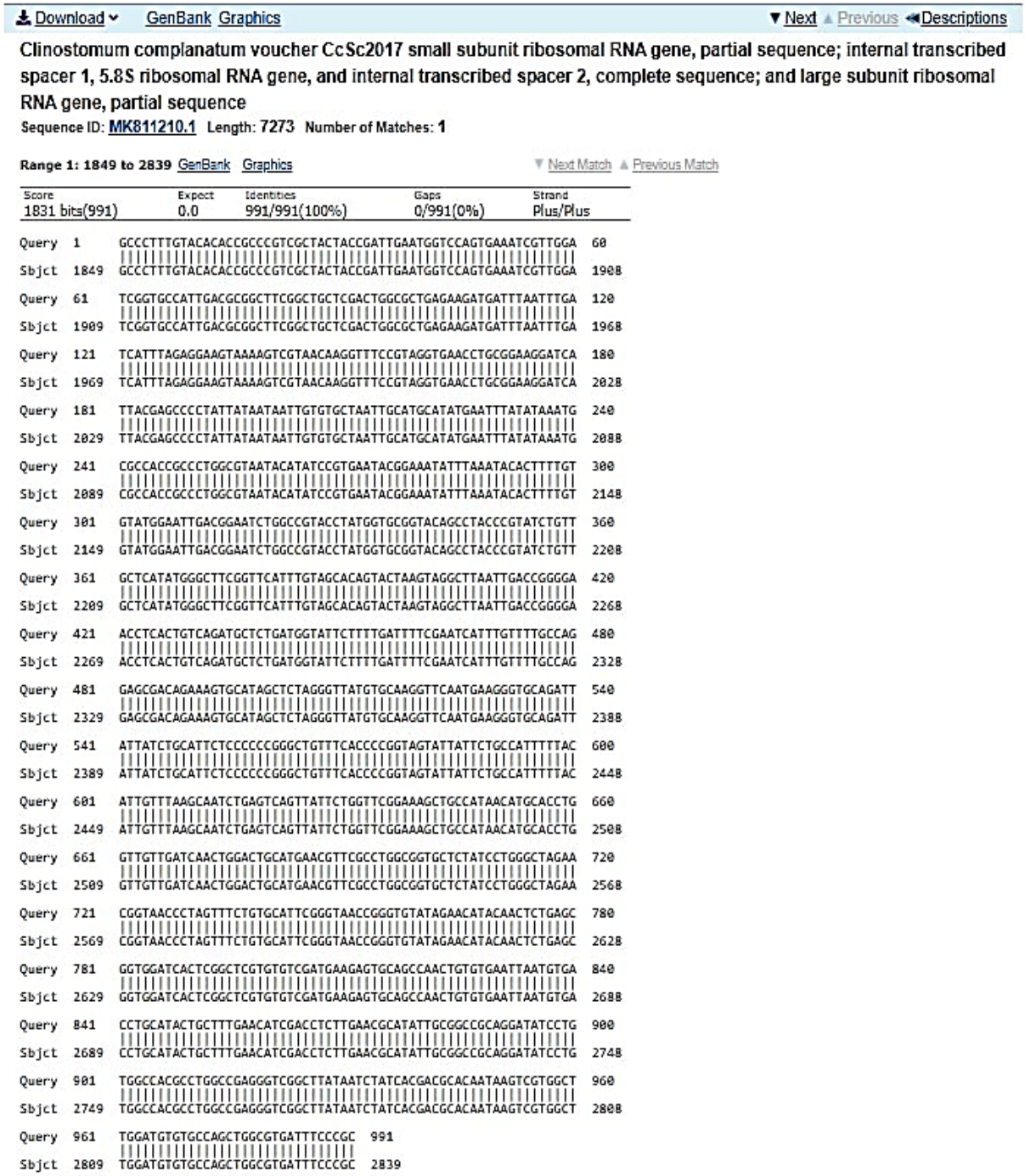


Fig. (8): Amplified bands on the agarose gel after Gel electrophoresis of *Clinostomum* 28S rDNA from different fish host species. M= Marker (Ladder).



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Clinostomum complanatum voucher CcSc2017 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MK811210.1 Length: 7273 Number of Matches: 1

Range 1: 1849 to 2839 GenBank Graphics ▼Next Match ▲Previous Match

Score	Expect	Identities	Gaps	Strand
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Query  121   TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  180
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Sbjct  1969  TCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCA  2028

Query  181   TTACGAGCCCCTATTATAATAATTGTGTGCTAATTGCATGCATATGAATTTATATAAATG  240
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Sbjct  2029  TTACGAGCCCCTATTATAATAATTGTGTGCTAATTGCATGCATATGAATTTATATAAATG  2088

Query  241   CGCCACCGCCCTGGCGTAATACATATCCGTGAATACGGAAATATTTAAATACACTTTTGT  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2089  CGCCACCGCCCTGGCGTAATACATATCCGTGAATACGGAAATATTTAAATACACTTTTGT  2148

Query  301   GTATGGAATTGACGGAATCTGGCCGTACCTATGGTGCGGTACAGCCTACCCGTATCTGTT  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2149  GTATGGAATTGACGGAATCTGGCCGTACCTATGGTGCGGTACAGCCTACCCGTATCTGTT  2208

Query  361   GCTCATATGGGCTTCGGTTCATTTGTAGCACAGTACTAAGTAGGCTTAATTGACCGGGGA  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2209  GCTCATATGGGCTTCGGTTCATTTGTAGCACAGTACTAAGTAGGCTTAATTGACCGGGGA  2268

Query  421   ACCTCACTGTCAGATGCTCTGATGGTATTCTTTTGATTTTCGAATCATTTGTTTTGCCAG  480
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Sbjct  2269  ACCTCACTGTCAGATGCTCTGATGGTATTCTTTTGATTTTCGAATCATTTGTTTTGCCAG  2328

Query  481   GAGCGACAGAAAGTGCATAGCTCTAGGGTTATGTGCAAGGTTCAATGAAGGGTGCAGATT  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2329  GAGCGACAGAAAGTGCATAGCTCTAGGGTTATGTGCAAGGTTCAATGAAGGGTGCAGATT  2388

Query  541   ATTATCTGCATTCTCCCCCCGGGCTGTTTCACCCCGGTAGTATTATTCTGCCATTTTTAC  600
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2389  ATTATCTGCATTCTCCCCCCGGGCTGTTTCACCCCGGTAGTATTATTCTGCCATTTTTAC  2448

Query  601   ATTGTTTAAGCAATCTGAGTCAGTTATTCTGGTTCGGAAAGCTGCCATAACATGCACCTG  660
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2449  ATTGTTTAAGCAATCTGAGTCAGTTATTCTGGTTCGGAAAGCTGCCATAACATGCACCTG  2508

Query  661   GTTGTTGATCAACTGGACTGCATGAACGTTCGCCTGGCGGTGCTCTATCCTGGGCTAGAA  720
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2509  GTTGTTGATCAACTGGACTGCATGAACGTTCGCCTGGCGGTGCTCTATCCTGGGCTAGAA  2568

Query  721   CGGTAACCCTAGTTTCTGTGCATTCGGGTAACCGGGTGTATAGAACATACAACTCTGAGC  780
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2569  CGGTAACCCTAGTTTCTGTGCATTCGGGTAACCGGGTGTATAGAACATACAACTCTGAGC  2628

Query  781   GGTGGATCACTCGGCTCGTGTGTCGATGAAGAGTGCAGCCAACTGTGTGAATTAATGTGA  840
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2629  GGTGGATCACTCGGCTCGTGTGTCGATGAAGAGTGCAGCCAACTGTGTGAATTAATGTGA  2688

Query  841   CCTGCATACTGCTTTGAACATCGACCTCTTGAACGCATATTGCGGCCGCAGGATATCCTG  900
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Sbjct  2689  CCTGCATACTGCTTTGAACATCGACCTCTTGAACGCATATTGCGGCCGCAGGATATCCTG  2748

Query  901   TGGCCACGCCTGGCCGAGGGTCGGCTTATAATCTATCACGACGCACAATAAGTCGTGGCT  960
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2749  TGGCCACGCCTGGCCGAGGGTCGGCTTATAATCTATCACGACGCACAATAAGTCGTGGCT  2808

Query  961   TGGATGTGTGCCAGCTGGCGTGATTTCCCGC  991
             |||||||||||||||||||||||||||||||
Sbjct  2809  TGGATGTGTGCCAGCTGGCGTGATTTCCCGC  2839
```

Fig. (9): Pair wised alignment of ITS1-5.8S-ITS2 sequence of *Clinostomum complanatum* Query is the study or sample sequence and Sbjct is the GenBank sequence.


Download GenBank Graphics ▼ Next ▲ Previous ◀◀ Descriptions

Clinostomum complanatum voucher CcSc2017 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MK811210.1 **Length:** 7273 **Number of Matches:** 1

Range 1: 3119 to 3673 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1020 bits(552)	0.0	554/555(99%)	0/555(0%)	Plus/Plus

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Query  1     CAGGATTCCCCTAGTAACTGCGAGTGAAGAGGGATTAGCCCAGCACCGAAGCCTGCGGTC  60
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3119  CAGGATTCCCCTAGTAACTGCGAGTGAAGAGGGATTAGCCCAGCACCGAAGCCTGCGGTC  3178

Query  61    ATTTGACTGCTAGGCAATGTGGTGTTTAGGTTGGTTCTTGGCATTACTGCTCCACCCTAA  120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3179  ATTTGACTGCTAGGCAATGTGGTGTTTAGGTTGGTTCTTGGCATTACTGCTCCACCCTAA  3238

Query  121   GTCCAGCAATGAGTACGGCTTACTGGATTGGCCCATTGAGGGTGAAAGGCCCGTGTGGGT  180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3239  GTCCAGCAATGAGTACGGCTTACTGGATTGGCCCATTGAGGGTGAAAGGCCCGTGTGGGT  3298

Query  181   GGAGACTCAGATTATCAGTTTTGCCCTGAATTAACCTTGGAGTCGGGTTGTTTGTGAATG  240
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3299  GGAGACTCAGATTATCAGTTTTGCCCTGAATTAACCTTGGAGTCGGGTTGTTTGTGAATG  3358

Query  241   CAGCCCAAAGTGGGTGGTAAACTCCATCTAAGGCTAAATACTGGCACGAGTCCGATAGCG  300
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3359  CAGCCCAAAGTGGGTGGTAAACTCCATCTAAGGCTAAATACTGGCACGAGTCCGATAGCG  3418

Query  301   AACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAAGAGAGAGTAAACAGTTCGTGAA  360
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3419  AACAAGTACCGTGAGGGAAAGTTGAAAAGTACTTTGAAGAGAGAGTAAACAGTTCGTGAA  3478

Query  361   CCCGCTCAGAGGTAAACGGGTGGAGTTGAACTGGAAGCCCTGGGGATTCAGCTGATGAGT  420
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3479  CCCGCTCAGAGGTAAACGGGTGGAGTTGAACTGGAAGCCCTGGGGATTCAGCTGATGAGT  3538

Query  421   ATGGCATGAGCTTGGGCATATTGGTCGGCTTTCAGAGTCCGCTTAGCTGCGGGTCCTTGC  480
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3539  ATGGCATGAGCTTGGGCATATTGGTCGGCTTTCAGAGTCCGCTTAGCTGCGGGTCCTTGC  3598

Query  481   CCTAACGGGTTTGGATGTGCGTTACGCTCATTAGGCGTTGTGCGCTCTGTCTGTTCCGGG  540
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3599  CCTAACGGGTTTGGATGTGCGTTACGCTCATTAGGCGTTGTGCGCTCTGTCTGTTCCGGG  3658

Query  541   CCTGCTTGTCAGTGC  555
             ||||||  ||||||||
Sbjct  3659  CCTGCTCGTCAGTGC  3673
```

Fig. (10): Pair wised alignment of 28S rDNA sequence of *Clinostomum complanatum* Query is the study or sample sequence and Sbjct is the GenBank sequence.

Conclusion

Basing on recent knowledge, the present study is the first ultrastructural and molecular identification of *C. complanatum* metacercariae in different freshwater fish species in Iraq. Based on molecular characteristics, all metacercariae dissected out from the infected fishes (*A. sellal*, *C. trutta*, *C. umbla*, *C. kosswigi*, *C. macrostomum*, *G. rufa*, *P. abu* and *S. lepidus*) in the present investigation belong to the same species, *C. complanatum*. Thus, it was revealed that the *Clinostomum* metacercariae can infect additional fish hosts in Iraq. Low host specificity is a character of this species, which results in the infection of a various piscivorous birds as well as mammals in the region. Accidental ingestion of *Clinostomum* metacercariae results in clinostomiasis, a disease caused mainly by raw and/or undercooked fish. This metacercaria infected the fishes mentioned above, all of which are edible and consumed by local people; because these fishes serve as intermediate hosts for this parasite, human health in this region may be affected.

The ultrastructural study of all life cycle stages of this parasite in the laboratory is necessary to understand the ultrastructural changes that occur during transformation from egg to adult worm. Furthermore, *C.*

complanatum metacercariae infecting various fish species in Sulaimani Province; thus, it is important to control this infection to decrease the possibility of zoonosis.

Acknowledgement

Our thanks are due to Prof. Dr. Furhan T. Mhaisen for providing the information on the previous records of *C. complanatum* metacercariae from different fish species in Iraq based on the unpublished index catalogue of parasites and disease agents of fishes of Iraq (Mhaisen, 2023).

Contributions of authors

Y.S.A., Sample collections, fish hots identification, parasite identification, especially the SEM part, writing of the paper.

S.J.B., Fish host identification, molecular identification designing, performing, and writing of the paper.

T.A.H.S., Sample collection.

Y.O.M., Sample collection.

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Conflicts of interest

The authors declare that we have no conflicts of interest.

Ethical approval

All fish specimens were collected (dead) and bought from local fishermen. The fishes were hunted in the permitted season as directed by the local governorate.

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التراكيب الدقيقة السطحية والدراسات الجزيئية لليرقة البعدية *Clinostomum complanatum* (Rudolphi, 1819) Braun, 1899 (Trematoda: Clinostomidae) في بعض أسماك المياه العذبة من محافظة السليمانية، العراق

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المستخلص: *Clinostomum* هو جنس من المخرّمات التابعة لعائلة Clinostomidae. أنواع المخرّمات المذكورة تتطفل على العديد من أنواع الأسماك كمضيّفات وسطية، في حين أن الطيور والثدييات الآكلة للأسماك هي المضيّفات الرئيسية النهائية. تم تشريح ما مجموعه 58 متكيّسة من يرقات *Clinostomum* من 25 عينة سمكية مصابة تابعة لثمانية أنواع مختلفة من عدد من المسطحات المائية في محافظة السليمانية، إقليم كردستان، العراق. في هذا البحث، تم جمع 959 سمكة، تضمنت خمسة أنواع من العائلة الشبوطية Cyprinidae وهي: أسماك التيلة المرقطة *Capoeta trutta* وكناس دجلة *C. umbla* وسمكة أبو حنك *Carasobarbus kosswigi* والبنيني كبير الفم *Cyprinion macrostomum* والكركور الاحمر *Garra rufa* وعائلة Leuciscidae ومنها السمنان النحيف *Alburnus sellal* والبرعان *Squalius lepidus* وعائلة البياح Mugilidae الممثلة باسماك الخشني *Planiliza abu*. بلغ معدل نسبة الإصابة لكل من هذه الأنواع 1.8٪، 5٪، 20٪، 1.5٪، 1.7٪، 4.9٪، 4.9٪ و 1.3٪ على التوالي. تمت دراسة الخصائص المظهرية للسركاريا البعدية للمخرمة *Clinostomum* باستخدام المجهر الضوئي؛ تم تقييم الشكل الفائق باستخدام الفحص المجهري الإلكتروني؛ وتم إجراء التحليل الجزيئي عن طريق تضخيم وتسلسل ومقارنة مواقع الجين ITS1–5.8S–ITS2 و 28 S rDNA المعزولة من السركاريا البعدية للجنس *Clinostomum*. أكدت التسلسلات التي تم الحصول عليها أن جميع السركاريا البعدية التي تم جمعها خلال الدراسة الحالية ممثلة بنوع واحد هو *C. complanatum* (Rudolphi, 1814). تم ايداع الخصائص الجزيئية لليرقة البعدية *C. complanatum* في هذه الدراسة في البنك الجيني.

الكلمات المفتاحية: ثنائية المنشأ، المياه العذبة، الوراثة، العراق، المتكيسة البعدية، مرض الجرب الاصفر.