Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 29(2): 2097 – 2107 (2025)
www.ejabf.journals.ekb.eg

Morphological and Molecular Survey of *Contracaecum* Larvae (Nematoda: Anisakidae); Endoparasites of Fish in Uzbekistan

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ARTICLE INFO
Article History:
Received: Feb. 2nd, 2025
Accepted: March 19, 2025
Online: April 10, 2025

Keywords:
Contracaecum larvae,
Nematode,
Prevalence,
Morphology,
RNA,
PCR

ABSTRACT

The range of the nematode of the genus *Contracaecum* (Anisakidae) covers almost the entire water area of the World Ocean from the Arctic to the Antarctic. Within the range, the larvae of this nematode at the 3rd stage of development are found in fish and invertebrates. The results of this research showed that the contracaecum 3rd stage larvae were detected in the body cavity of *Cyprinus carpio*, *Carassius gibelio*, and *Silurus glanis* fish distributed in the water bodies of the Jizzakh and Khorezm regions of Uzbekistan. The results of the helminthological study prevalence of *Contracaecum* sp. larvae with *Cyprinus carpio* fish was 30.8, 25% with *Carassius gibelio* fish, and 22.2% with *Silurus glanis* fish. Regarding morphological characterictics, it was found that these larvae are close to the species *Contracaecum quadripapillatum*. Molecular analysis confirmed that the ITS1-5.8S-ITS2 rDNA sequence exhibited 99.8% identity with *C. quadripapillatum* sequences available in GenBank based on BLAST comparison. To the best of our knowledge, the larvae of *C. quadripapillatum* has been recorded in Uzbekistan for the 1st time.

INTRODUCTION

Scientific research on the molecular taxonomy of various systematic groups of animals increases every year. In the identification of invertebrate and vertebrate species, in addition to traditional morphological methods, more and more attention is paid to modern molecular taxonomic methods. These methods are effective in identifying animal species, and they also allow for a comprehensive study and solution of some problems in the taxonomy, evolution, and phylogeny of animals **(Guo *et al.*, 2020; Kuchboev *et al.*, 2020a, 2021, 2023; Bazarbayeva *et al.*, 2024)**. Species belonging to the family Anisakidae (Nematoda: Ascaridida) are cosmopolitan in distribution **(Anderson, 2000; Murata, 2001; Shamsi *et al.*, 2020; Soatov *et al.*, 2023)** and cause zoonotic diseases **(Laffon, 2000; Ferrantelli *et al.*, 2015)**. Sometimes, humans are infected with nematodes of this family as a result of consuming raw or undercooked fish, leading to

impaired gastrointestinal function **(Nagasawa, 2012)**. The main characteristics considered by various authors for the morphological identification of representatives of this family include the presence of a cuticular tooth, ventral lips in the abdominal cavity, the shape of the excretory pore, pointed head shape and tail, as well as total body length, body diameter, esophagus length, and nerve ring length. Based on these morphological features, a total of 11 genera belonging to the Anisakidae family have been identified **(Moravec, 1997; Moravec, 1998; Pérez-i-García, 2015)**.

The genus *Contracaecum* Railliet & Henry, 1912, belonging to the family Anisakidae, has more than 50 species. Adults are parasitize fish-eating animals, such as mammals and birds, while eggs and various larval stages use invertebrates and fish as intermediate hosts. This parasitic nematode is the only one of the Anisakidae family that infects terrestrial, marine, and freshwater animals **(Koie & Fagerholm, 1995; Anderson, 2000; Valles-Vega, 2017)**. In Uzbekistan, *Contracaecum spiculigerum* Rudolphi, 1809 larvae were recorded in carp in the Syrdarya River (**Safarova, 2017**), in common rudd fish in the Shorkul reservoir **(Soatov *et al.*, 2023)**, and in the body cavity of carp from a fish farm in the Syrdarya region Uzbekistan **(Nomonov *et al.*, 2024)**.

In recent years, molecular taxonomy using ribosomal DNA and mitochondrial DNA markers has been proven to be an effective method for identifying new and problematic nematode species and for studying their phylogenetic relationships **(Shamsi *et al.*, 2018; Kuchboev *et al.*, 2020b, 2022; Madumarov *et al.*, 2021; Turgunov *et al.*, 2024)**. Identifying certain *Contracaecum* species based on morphological features remains challenging. Currently, species belonging to this genus are identified using the nucleotide sequence of the internal transcribed spacer (*its-1* and *its-2*) region of ribosomal DNA **(Nadler, 2000; Mattiucci, 2010; Davidovich *et al.*, 2023)**. The aim of this research work was to evaluate the morphological and molecular-genetic characteristics and identification of, at the species level, *Contracaecum* sp. larvae found in fish from reservoirs of the Jizzakh and Khorezm regions of Uzbekistan.

MATERIALS AND METHODS

Collection of helminthological material

For this study, *Cyprinus carpio* (13 samples), *Carassius gibelio* (12 specimens), and *Silurus glanis* (9 specimens) were collected from the Amu Darya River (from latitude 41°96'14.30″N, longitude 60°39'91.78″E to latitude 41°21'25.38″N, longitude 61°34'84.70″E) which flows through the Khazarasp, Khanka, Urgench, Yangibozor, and Gurlan districts of the Khorezm region and *Silurus glanis* fish from the Jizzakh reservoir basin (12 specimens) and from the "Erkin" fishing farm (6 specimens) of the Jizzakh region (latitude 40°27'28.2"N, longitude 67°38'57.8"E) and were examined using generally accepted helminthological dissection methods. Helminths were collected from the fish samples and were processed using helminthological and ichthyoparasitological methods **(Bykhovskaya-Pavlovskaya, 1985)**.

Morphological examination

The samples were placed on a slide and stained with the appropriate stains, examined under microscopes (Nexcope NE930-FL, Nexcope NSZ818) and photographed with a ToupCam camera. Morphometric analysis was performed according the method of **Anderson (2000)** and **Saad *et al.* (2018)** to determine the morphological and morphometric sizes of *Contracaecum* larvae collected from fish. Measures were given in millimeters.

Molecular indentification

Total DNA was extracted from the tissue of collected nematode samples using the DNeasy Blood and Tissue Kit for DNA Isolation (Qiagen Inc., November 2023). Nucleotide fragments of ribosomal DNA (rDNA) *its1-5.8s-its2* regions of nematodes were isolated using the forward primer TW81 (*ata tgc tta agt tca gcg ggt*) and the reverse primer AV28 (*gtt tcc gta ggt gaa cct gc*), which are commonly used in molecular taxonomy **(Curran *et al.*, 1994)**. Polymerase chain reaction (PCR) was performed according to the following temperature conditions by using a thermocycler ProFlexTM PCR System (TermofisherSientific, USA): Step 1, denaturation of DNA at 94°C for 5min. Step 2, denaturation of DNA at 95°C for 45sec. Step 3, annealing the primer at 55°C for 45sec. Step 4, elongation at 72°C for 1.0min and 40sec. Step 5, final elongation at 72°C for 5min. Steps 2 - 4 were repeated in a cycle up to 35 times **(Mirzaev *et al.*, 2024)**.

The presence of DNA was confirmed using electrophoresis in a 1.5% agarose gel at 80V. DNA was obtained from the agarose gel using reagent kits produced by Sileks M LLC (Moscow, Russia) in accordance with the manufacturer's instructions. DNA sequencing performed using the BigDe™ Terminator v. 3.1 reagent kit, and reaction DNA products were analyzed on an ABI PRISM 3100 Avant automated sequencer. Nucleotide sequence analysis was carried out using specialized bioinformatics software, including BioEdit, Clustal W, DNAstar™, and PAUP4.

RESULTS

The results of the helminthological study, larvae of the genus *Contracaecum* were extracted in the body cavity of 4 out of 13 *Cyprinus carpio* fish (30.8%), 3 out of 12 *Carassius gibelio* fish (25%), 2 out of 9 *Silurus glanis* fish (22.2%) from Amudariya Bassein of Khorezm, and 3 out of 12 *Silurus glanis* fish (25%) from the Jizzakh reservoir and 1 out of 8 *Silurus glanis* (12,5 %) from the "Erkin" fishing farm of Jizzakh region. The intensive infection of larvae was 5 – 35 larvae/fish.

The morphological studies resulted in the preparation of more than 20 permanent and temporary slides from the larvae of nematodes belonging to the genus *Contracaecum* found in *Cyprinus carpio*, *Carassius gibelio*, and *Silurus glanis*. The larvae were measured morphologically and morphometrically and original photographs were taken (Fig. 1). Third stage larvae of *Contraceacum* obtained from *Silurus glanis* (n=10). The

larvae were reddish-yellow in color and covered with a transparent cuticle. The length of the larvae was 14.2 – 28.6mm (18.2 ± 1.31), the width was 0.90 – 2.1mm (1.30 ± 0.5). The mouth was surrounded by three small lips with a protruding papilla, the length of the mouth was 0.02 – 0.04mm (0.03 ± 0.01) (Fig. 1). The esophagus was narrow and long and the length was 2.5 – 4.2mm (3.2 ± 0.7). The length of the ventricular process was 0.82 – 1.06mm (0.93 ± 0.3). The length of the cecum was 1.76 – 3.5mm (2.81 ± 0.4). The ratio of the ventricular process to the cecum of the intestine was 32.5 – 52%. The intestine opened ventrally with a slit-like anal opening, and the tail was conical in share the length about 0.1 – 0.15mm (0.12 ± 0.1) and 0.5 – 0.6% of the body length, with a conical process measuring 0.04 – 0.06mm (0.04 ± 0.01). The rectum was supplied with rectal cells. The length of the conical tail was 75.2 – 98.6mm (86 ± 15.3).

The morphometric measurements of *Contracaecum* larvae were compared with the data provided in **Saad *et al*. (2018)** and **Davidovich *et al*. (2023)** and confirming that the species is likely *Contracaecum quadripapillatum*.

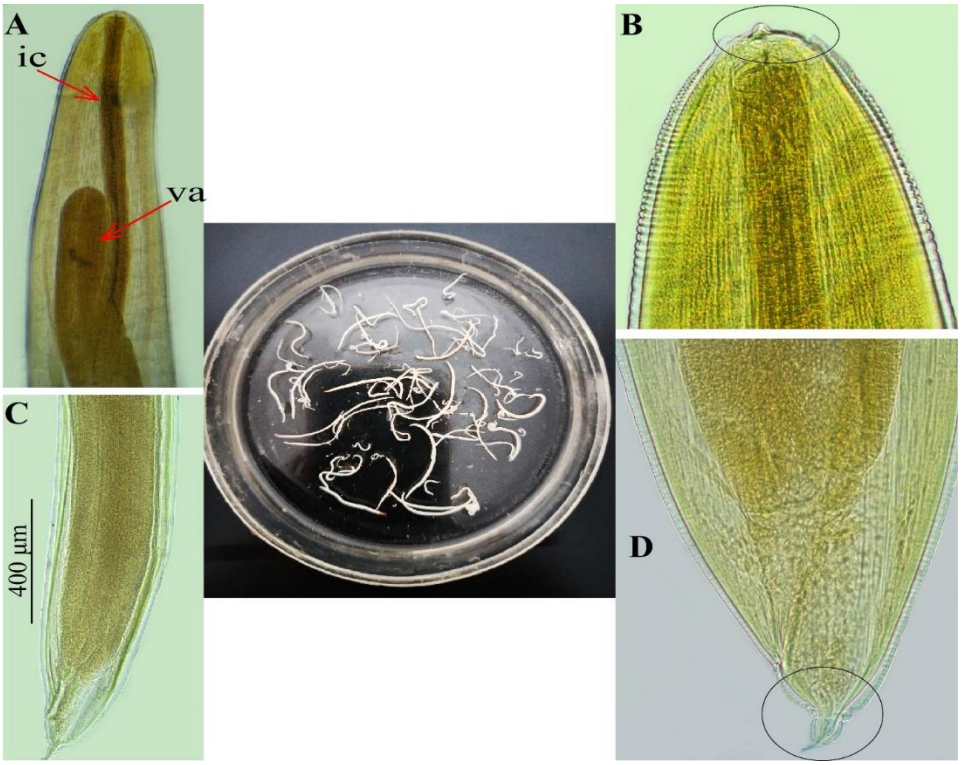

Fig. 1. *Contracecum* third stage larvae found in *Silurus glanis*. **A.** Cephalic region with distal part of intestinal caecum (ic), ventricular appendix (va). **B.** *Contracecum* larva anterior end. **C.** *Contracecum* larva tail view. **D.** *Contracecum* larva posterior end

Molecular analysis was performed on specimens of *Contracaecum* sp. collected from *Cyprinus carpio*, *Carassius gibelio*, and *Silurus glanis*. Sequencing of the ribosomal DNA (rDNA) ITS1-5.8S-ITS2 region yielded nucleotide sequences exceeding 814 base

pairs in length. These sequences were analyzed using bioinformatics software and compared with sequences from the National Center for Biotechnology Information (NCBI) GenBank database, specifically *C. quadripapillatum* (accession no. ON714982) and *C. aff. multipapillatum* (accession no. ON723788) (Fig. 2).

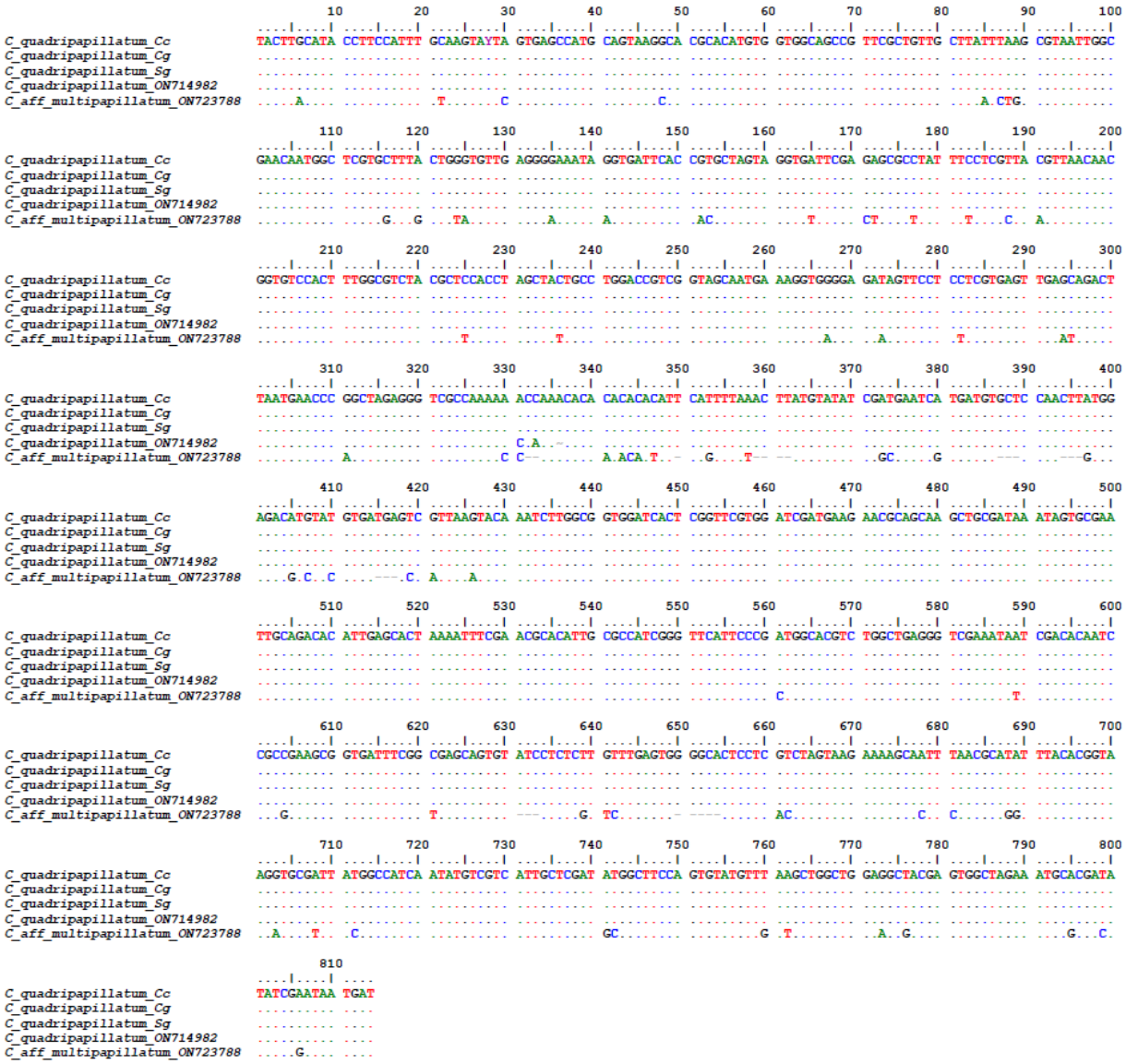

Fig. 2. Comparative comparison of nucleotide sequence of the *its* region of rDNA of *C. quadripapillatum* and other species of the genus *Contracaecum*

Note: *C_quadripapillatum_Cc* – from *C. carpio, C_quadripapillatum_Cg* – from *C. gibelio, C_quadripapillatum_Sg* – from *S. glanis.*

Bioinformatical analysis showed that the *C. quadripapillatum* nematodes found in *C. carpio, C. gibelio,* and *S. glanis* differ from the NCBI reference sequence *C. quadripapillatum* (ON714982) at two nucleotide positions (0.24 %): At position 331,

C_quadripapillatum_Cc, *C_quadripapillatum_Cg*, and *C_quadripapillatum_Sg* contained A (adenine), while *C. quadripapillatum* (ON714982) contained S (cytosine). At position 333, *C_quadripapillatum_Cc*, *C_quadripapillatum_Cg*, and *C_quadripapillatum_Sg* contained S (cytosine), while *C. quadripapillatum* (ON714982) contained A (adenine) (Fig. 2). These differences in the nucleotide sequence may be attributed to the ecological conditions of the sampling locations and the type of host species. The comparison between *C_quadripapillatum_Cc*, *C_quadripapillatum_Cg*, and *C_quadripapillatum_Sg* with *C. aff. multipapillatum* (ON723788) revealed 75 nucleotide differences, corresponding to an overall sequence divergence of 9.2%. The ITS1-5.8S-ITS2 rDNA sequences were compared using BLAST with available sequences in the GenBank (NCBI) database, revealing 99.8% identity with *C. quadripapillatum* (ON714982) isolates and 92.8% identity with *C. multipapillatum* (ON723788), thereby confirming the molecular identification.

The evolutionary history was inferred using the Maximum Likelihood (ML) method based on the Tamura model **(Tamura, 1992)**, with analyses conducted in MEGA11 software **(Tamura *et al.*, 2021)**. Phylogenetic analysis of nucleotide sequences from the ITS region, alongside related sequences obtained from the GenBank, demonstrated that nematodes of the genus *Contracaecum* clustered into four distinct clades (Fig. 3).

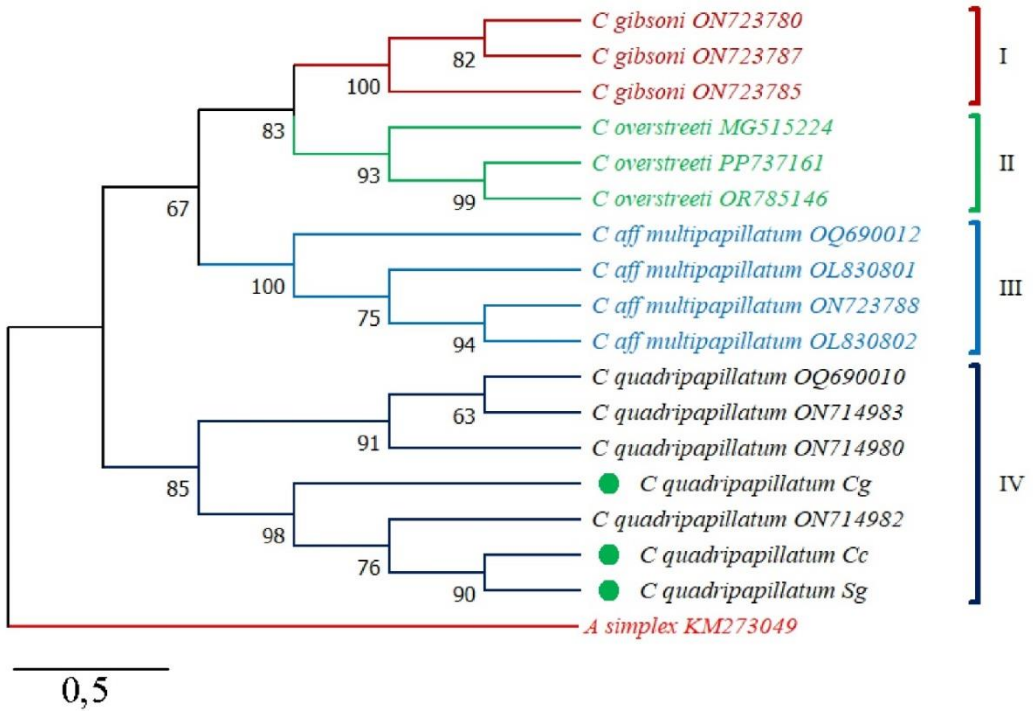

Fig. 3. Phylogenetic tree of nematode species of the genus *Contracaecum* constructed using the maximum likelihood algorithm (1000 bootstrap replicates). Bootstrap support values are shown at the corresponding nodes

First group – *C. gibsoni* species, forming a cluster with 82-100% bootstrap support.

Second group – *C. overstreeti* species, forming a cluster with 93-99% bootstrap support. Phylogenetically, *C. gibsoni* and *C. overstreeti* were closely related with an 83% similarity.

Third group – *C. aff. multipapillatum*, forming a cluster with 75-100% bootstrap support.

Fourth group – *C. quadripapillatum*, forming a distinct cluster with 85% support in relation to the main clade, and 63-98% support within the cluster.

DISCUSSION

The species *Contracaecum quadripapillatum* was first described by **Saad *et al.* (2018)**. They experimentally infected American white pelicans with third-stage larvae of *Contracaecum* fish *Clarias gariepinus* (North African catfish) collected from Lake Nasser, Egypt, and then they studied the morphology of larvae and adults in detail using light and scanning electron microscopy. In addition, molecular analysis of the internal transcribed spacers (*its1* and *its2*) of ribosomal DNA confirmed that this species is a new species and named it *C. quadripapillatum* n. sp. At the same time, molecular studies of adults showed 100% similarity to third-stage larvae collected from North African catfish.

Earlier, in the work of **Younis *et al.* (2017)**, it was also studied that they are identical in morphological features and ITS1 and ITS2 sequences, showing very high similarity (98-100%) with the studied *Contracaecum* sp. larvae that infected *Hydrocynus forskahlii* and *Lates niloticus* fish from Lake Nasser (Egypt). In addition, **Shamsi and Aghazadeh-Meshgi (2011)** collected third-stage *Contracaecum* larvae from Iranian fish, which may also belong to the same species *C. quadripapillatum*. However, the authors are needed to confirm this with further experimental host infestation and compare it with adults. **Davidovich *et al.* (2023)** conducted a study of the nematode *Contracaecum* larvae in fish from the Sea of Galilee (Palastine). They also used morphological and molecular methods to differentiate the larvae to the species level. The study showed that two wild native cyprinids, *Carasobarbus canis*, and *Luciobarbus longiceps*, were infested with *C. quadripapillatum* larvae in the abdominal cavity. One specimen of *Oreochromis aureus* was infested with two *C. multipapillatum* morphotype E larvae localized in the pericardial cavity.

Our study also found the species *C. quadripapillatum*, which belongs to the genus *Contracaecum*, which was first discovered, to the best of our knowledge, in the Republic of Uzbekistan as a larva in the abdominal cavity of *Cyprinus carpio*, *Carassius gibelio*, and *Silurus glanis*. The species *C. quadripapillatum* was characterized using morphological and molecular genetic methods.

CONCLUSION

Third-stage larvae of the nematode *Contracaecum* sp. were detected in the abdominal cavities of fish species *Cyprinus carpio*, *Carassius gibelio*, and *Silurus glanis*, collected from water bodies in the Khorezm and Jizzakh regions of Uzbekistan. These larvae were examined using morphological and molecular genetic methods, which confirmed their identification as *Contracaecum quadripapillatum*.

Molecular genetic analysis of the rDNA ITS1-5.8S-ITS2 region revealed only two nucleotide differences (0.24% total divergence) between the studied *C. quadripapillatum* samples and the reference *C. quadripapillatum* sequence (ON714982) from the GenBank (NCBI). Additionally, phylogenetic analysis based on ITS1-5.8S-ITS2 sequences showed that the examined *C. quadripapillatum* larvae grouped into a single clade, supported by bootstrap values ranging from 90 to 98%.

ACKNOWLEDGMENTS

This research work was carried out within the framework of the fundamental project "Morphological and molecular analysis of endemic, rare and taxonomically controversial species of invertebrate of the fauna of Uzbekistan: nematodes, crustaceans" of the Institute of Zoology Uzbekistan Academy of Sciences of the Republic of Uzbekistan, No. FZ-2020092633.

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