

## Short Communication: Morphological and molecular description of *Contracaecum* sp. larvae (Nematoda: Anisakidae) in common rudd fish of the Shurkul Reservoir of Uzbekistan

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**Abstract.** Soatov BB, Kuchboev AE, Karimova RR, Amirov OO. 2023. Short Communication: Morphological and molecular description of *Contracaecum* sp. larvae (Nematoda: Anisakidae) in common rudd fish of the Shurkul reservoir of Uzbekistan. *Biodiversitas* 24: 2031-2036. The common rudd (*Scardinius erythrophthalmus* Linnaeus 1758) is freshwater fish widely spread in Uzbekistan, including reservoirs of the Bukhara Province, Uzbekistan. The study aimed to perform the morphological and molecular identification of the larvae of nematode *Contracaecum* sp. found in common rudd fish in the Shurkul Reservoir of the Bukhara Province. These larvae were similar to species of the genus *Contracaecum* based on their morphological characteristics. The molecular data, according to the results of the phylogenetic tree based on nucleotide sequences of the 18S gene of rDNA, the samples of larvae showed a close relationship with haplotype *Contracaecum rudolphii* Hartwich, 1964 E. Therefore, to increase the reliability of this information, nucleotide sequences of the gene 28S of rDNA were verified. As a result of this study, these larvae were very close to the haplotype of *C. rudolphii* B (AF 226579). Also, the common rudd fish was registered as an intermediate host for the larvae of this nematode. The prevalence of this fish with larvae of *C. rudolphii* B was 10.5%, and the intensity of infection was 1-18 copies. Thus, the larvae of the species *C. rudolphii* B are registered for the first time in Uzbekistan, and the common rudd fish was noted as an intermediate host.

**Keywords:** *Contracaecum* sp., common rudd, intermediate host, morphology, nematode, prevalence, ribosomal DNA

### INTRODUCTION

Currently, great attention is being paid to conducting new research on the species composition and taxonomy of parasites in marine and freshwater fish. As a result, identifying species through classical morphology and modern molecular taxonomic methods is becoming standard (Guo et al. 2020). Furthermore, it should also be noted that quantitative and qualitative changes in recent years have occurred in aquatic ecosystems and adversely affected the epizootic situation of water bodies. In addition, intensive human economic activity has played an important role, resulting in significant economic damage caused by parasites and diseases of fish and birds, which are a dangerous factor for human health (Kuchboev et al. 2021a; Madumarov et al. 2021).

Nematodes from the family Anisakidae are parasites that have a worldwide distribution (Shamsi 2019). The genus *Contracaecum* Railliet & Henry 1912 is a parasitic nematode that includes about 50 species (Moravec 2013). Their adult forms parasitize fish-eating endothermic animals, i.e., mammals and birds. Their eggs and various larvae stages use invertebrates and fish as intermediate hosts (Zhang et al. 2018). These parasitic nematodes are the only genus in the Anisakidae family that infect terrestrial,

marine, and freshwater animals (Kjøie and Fagerholm 1995).

*Contracaecum rudolphii* Hartwich 1964 is a common bird species, parasitizing in the pre-gastric region of pelicans, mergansers, cormorants, and herons. The parasites enter the pre-gastric mucosa, causing profuse hemorrhages and severely ulcerated eosin granulomas. In addition, that parasite sometimes has large and extensive necrosis, leading to emaciation and death of birds (Strøm et al. 2015). Humans are infected by larval stages of *Contracaecum* spp. from accidental consumption of raw or improperly prepared fish and suffer from symptoms of vomiting, diarrhea, and abdominal pain (Corrêa et al. 2015). Therefore, this is currently considered a foodborne zoonotic disease. WHO launched a monitoring group to assess the global extent of foodborne diseases caused by microbial pathogens, parasites, and biotoxins to increase awareness of these diseases' public health importance worldwide (Mehrdana et al. 2014).

The establishment of *Contracaecum* spp. has been reported worldwide (Shamsi et al. 2014; Moravec and Scholz 2016; Sreedevi et al. 2017; Molnár et al. 2019; Amor et al. 2020) in countries such as cormorants and pelicans from different parts of the world.

Species of the genus *Contracaecum* of fish-eating birds can be diagnosed by morphological features, the morphology

of the distal part of the spicule, and the bifurcation of the intermediate lip. However, sometimes these features can be distinguished, so the current trend is to use molecular-genetic methods for accurate diagnosis (Mattiucci et al. 2015).

In the following years, the molecular taxonomy method was proved to be good for identifying new and cryptic species nematodes with the help of ribosomal and mitochondrial DNA markers and studying their phylogenetic relationships (Shamsi et al. 2018; Kuchboev et al. 2020; Kuchboev et al. 2021b). In particular, the use of the 18S rDNA, first (ITS-1) and second (ITS-2) internal transcribed spacers (ITS), 28S rDNA, and mtDNA COX-2 has been recognized as genetic markers for accurate species identification of ascaridoid nematodes (Garbin et al. 2013; Mattiucci et al. 2015; Younis et al. 2017; Zuo et al. 2018). Also, for *Contracaecum* species, identifying three cryptic species within *C. rudolphii* morphotypes A, B, and F used these genetic markers (Shamsi et al. 2018; Zhu et al. 2018).

Our research aims to make a morphological description and molecular identification of the larvae of nematode *Contracaecum* sp. found in common rudd fish in the Shurkul Reservoir of Bukhara Province, Uzbekistan.

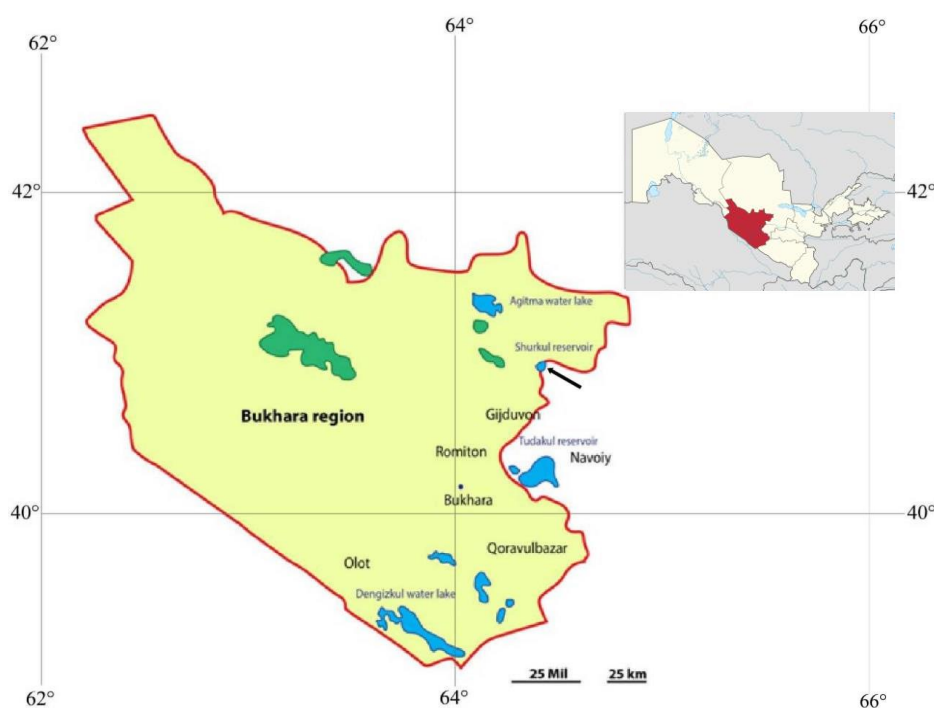
## MATERIALS AND METHODS

This research collected 76 common rudd (*Scardinius erythrophthalmus* Linnaeus 1758) parasites from the Shurkul reservoir located in the lower reaches of the Zarafshan River, Uzbekistan. These parasites were examined by helminthological examination during 2020-2021 (Figure 1). The rudd were caught by local fishermen;

their size was 18-22 cm and weight 200-300 g. In particular, parasites were examined for infection with anisakis larvae (*Contracaecum*) found in the body cavity in the common rudd. In addition, the examined body cavity was carefully examined from the outside to detect nematode larvae. When examining the body cavity, the larvae were selected with tweezers or needles, placed in clean water or saline, and then fixed in 70% ethanol.

Parasite specimens were placed on glass slides, examined under ML 2000 (Meiji) microscope, and photographed using a digital camera for the microscope. Next, the fish autopsy was carried out according to the method of Shamsi and Suthar (2016). All fish were transferred in ice to the laboratory and subjected to complete parasitological dissection within three days post-arrival. The number of *Contracaecum* nematode larvae was recorded and fixed for further identification (light microscopy), and determining the species of parasite larvae work of Dubinina (1987) was used. The intensity of infection was recorded by counting larval nodules in the abdominal cavity, whereafter the mean intensity and range were noted. The infection level was calculated as prevalence (percentage of investigated fish infected) and mean intensity (mean number of parasites per infected fish). For morphometric parameters of nematode *Contracaecum* sp., larvae were used in 10 specimens.

For DNA isolation, two specimens of *Contracaecum* fixed in 70% ethyl alcohol were thoroughly washed with distilled water, and the head of the parasites was cut off and placed in an Eppendorf test tube containing 200 µL of lysis buffer of each. Genomic DNA isolation was performed using the collection of the ThermoScientific GeneJet DNA purification kit (ThermoFisher com) and its protocol.



**Figure 1.** Map of the collection site of fish parasites of water reservoirs in the lower reaches of the Zarafshan River in Uzbekistan, including the Shurkul reservoir (indicated by arrow)

In the amplification of genomic DNA isolated from the nematode genotype, ribosomal DNA large subunit 28S (F 5' ACCCGCTGAATTTAAGCATAT 3' and 1500R 5' GCTATCCTGAGGGAACTTCG 3') and small subunit 18S Nem18S F (5' CGCGAATRGCTCATTACAACAGC 3'), Nem18S R (5' GGGCGGTATCTGATCGCC 3') (Bhadury et al. 2006) based on primers. Furthermore, for polymerase chain reaction (PCR), an Invitrogen (ThermoFisher com) reagent kit was used. First: 10x PCR buffer, dNTP solution, Taq - polymerase, sterilized water, and amplification were performed at the appropriate temperature using the two forward and reverse primers listed above. Next, PCR was performed using an automatic programming amplifier (Touchgene Gradient Thermal Cycler). Next, 1 µL of the product was evaluated by electrophoresis on a 1.5% gel to verify PCR results. In the next step, DNA purification was performed, and the concentration of PCR products was determined using a NanoDrop 2000/2000c Spectrophotometer (Thermo Scientific™).

Sequencing was carried out at the center of collective use, «Genome» at the Institute of Molecular Biology of the Russian Academy of Sciences (Moscow, Russia). The chromatograms (in ab1 format) obtained from the «Genome» center were analyzed using the Chromas 2.6.6 program (Technelisiu Ltd.) and translated into fast alignment (FASTA) format. The resulting nucleotide sequences were analyzed using the basic local alignment search tool (BLAST) algorithm, which made it possible to determine the range of species of parasitic organisms closest to the studied forms. The data selection for analysis assumed the inclusion of all related forms in the compared species and nucleotide sequences of representatives of another genus for use as an outgroup. The alignment was built using the Clustal X program. The resulting matrix was converted into MSF format to obtain a rectangular matrix. After that, the flanking sequences that did not match in different forms were deleted using the Genedoc program. Finally, the rectangular matrix was exported to all formats and analyzed using the MEGAX program (Stecher et al. 2020).

The resulting trees were directly copied from MEGAX and transferred to a graphics editor for the final design. The analysis was performed by three different methods: maximum parsimony (MP), nearest neighbor-joining (NJ), and maximum likelihood (ML). For the latter method, a suitable model was determined using the option available in the MEGAX package.

The obtained nucleotide sequences and one rDNA 28S sequence for species *C. rudolphii* (OP912388) were deposited in GenBank.

All numerical data were processed mathematically and statistically, and some statistical analyzes were performed using the Excell 2016 program and the OriginPro 7.5 program (OriginLab Corporation, USA).

## RESULTS AND DISCUSSION

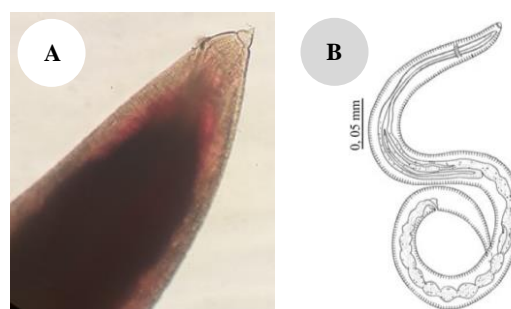
*Contracaecum* sp. larvae were recorded for the first time in the body cavity of the common rudd in the Shurkul reservoir. In this case, the prevalence was 10.5% (76 examined/8 parasitized), and the range of the intensity of infection was 1-18 copies.

The collected larvae were light brownish-yellow (Figure 2A). A larval tooth is clearly visible in the anterior body end. The mouth opened a slit-like shape, surrounded by three lips. Stoma is very small, followed by esophagus ventriculus, and its appendix is clearly differentiated. Intestinal caecum in early instar larvae may be absent or poorly developed. In larvae 1-36 mm in size, it is distinctly expressed. The length of the intestinal outgrowth varies greatly but usually exceeds that of the esophagus. Morphological examination showed that similar larvae belong to the genus *Contracaecum* (Figure 2).

Description of morphotype larvae in the third stage of development has a length of 0.78-0.91 mm, a width of 0.02-2.1 mm, and a stoma length of 0.003-0.007 mm (Table 1, Figure 2B). The length of the esophagus is 0.13-0.18 mm. The length of the ventriculus is 0.02-0.03, the width is 0.01-0.03 mm, and the length of the ventricular appendix is 0.15-0.2 mm. Intestinal caecum is 1.45-2.26 mm. The length of the nerve ring is 0.14-0.15 mm. According to morphological data, this nematode is a third-stage larva and is a species belonging to the *Contracaecum* sp. Therefore, we used molecular-genetic methods to diagnose this species accurately.

In the primary molecular identification of the studied nematode, two sequences were isolated (934 bp) based on the rDNA (haplotype UN4) of the 18S gene of larvae of *Contracaecum* sp. As a result, using the BLAST algorithm, the nucleotides of the UN4 haplotype samples were checked and indicated that they belong to the genus *Contracaecum*. A phylogenetic tree was constructed based on this sample and the 18S rDNA region sequences of *Contracaecum* nematodes in the Genbank (NCBI) database (Figure 3).

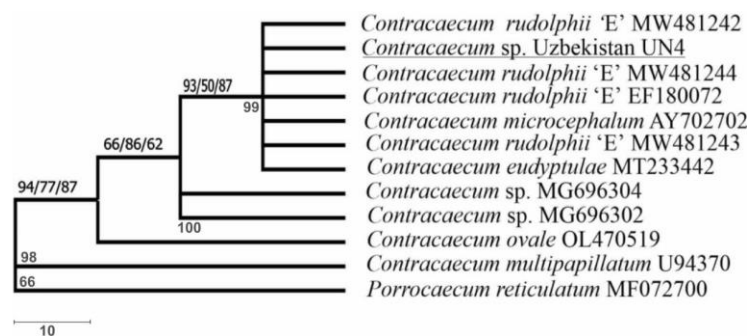
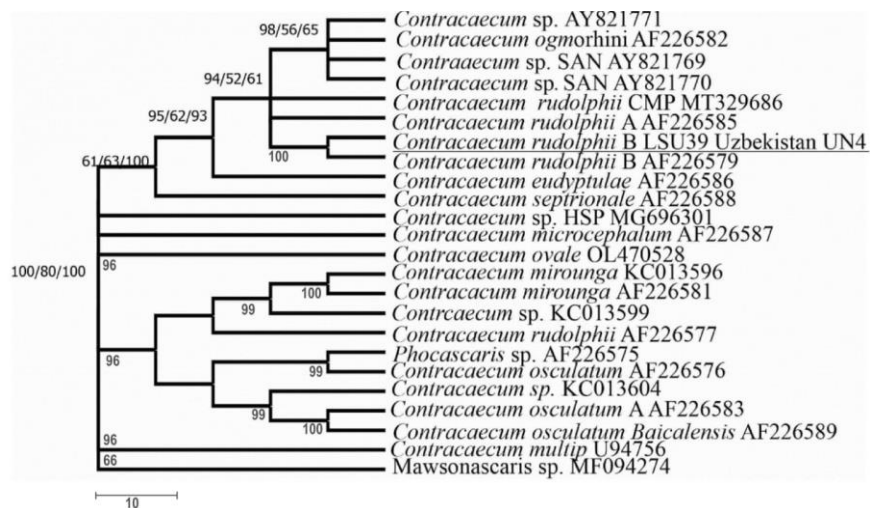
The UN4 sample showed a close relationship with other representatives of the species *Contracaecum microcephalum* (Rudolphi 1809) Baylis 1920, including haplotypes of *C. rudolphii* E. It should be noted that these forms are common in the Southern regions of Europe (Figure 3).



**Figure 2.** *Contracaecum* sp. larvae. A. The anterior end larvae, magnified 400x. B. General view (drawing own picture)

**Table 1.** Morphometric parameters of nematode *Contracaecum* sp larvae

Characters	The number of specimens	Male		
		The limit of a variable	The arithmetic mean	The coefficient of variation
Body length	10	0.78-0.91	0.843±0.014	5.4
Maximum width	10	0.02-2.1	0.919±0.252	86.7
Stoma length	10	0.003-0.007	0.0049±0.0004	29.6
Nerve ring	10	0.14-0.15	0.144±0.0016	3.6
Esophagus length	10	0.13-0.18	0.156±0.0043	8.7
Ventriculus length	10	0.02-0.03	0.028±0.0024	27.9
Ventriculus width	10	0.01-0.03	0.021±0.0026	39.6
The length of the ventricular appendix	10	0.15-0.2	0.172±0.0052	9.6

Notes: Reliability level  $P > 0.05$ **Figure 3.** Phylogenetic tree of *Contracaecum* spp. based on partial sequences 18S rDNA. The tree was rooted using the sequence of *Porrocaecum reticulatum* (MF072700) as an outgroup. Statistical (bootstrap) support values are given in MP, NJ, and ML format**Figure 4.** Phylogenetic tree of *Contracaecum* spp. based on partial sequences of 28S rDNA. The tree was rooted using the sequence of *Mawsonascaris* sp. (MF094274) as an outgroup. Statistical (bootstrap) support values are given in MP, NJ, and ML format

Therefore, to improve the reliability of the study of the phylogenetic relationships of this nematode species, the nucleotide sequences of the rDNA large subunit 28S partial region were amplified. As a result, UN4 clustered with the highest level of support into the *C. rudolphii* B (AF226579) (Figure 4). Furthermore, to our knowledge, this sequence was obtained from an adult *Contracaecum* nematode that

parasitizes cormorants living in various basins of the world's oceans, including European seas (Amor et al. 2020; Bouzid et al. 2022).

*Contracaecum rudolphii* (recorded as *Contracaecum spiculigerum*) is considered a cosmopolitan nematode. This nematode is one of the main parasites of cormorants and is collected from the esophagus and stomach of *Phalacrocorax*

*carbo* Linnaeus 1758. The definitive host of *Contracaecum* is fish-eating birds, mainly the genus *Phalacrocorax* (Pelecaniformes: Phalacrocoracidae). The great cormorant *P. carbo* is a migratory, nesting cormorant. Its distribution area is very wide, including all continents worldwide except South America and Antarctica (Moravec and Scholz 2016). The subspecies *Phalacrocorax carbo carbo* is mainly found in the waters of the Atlantic Ocean and near inland areas, including the coasts of Western Europe, while the subspecies *Phalacrocorax carbo sinensis* (Blumenbach) is distributed from northern and central Europe to southern China (Moravec and Scholz 2016; Shamsi 2019).

The morphological characteristics of the adults determine *Contracaecum* species. Therefore, separating the larvae into any species is difficult, and it is impossible to design a key to identify them (Moravec 2013) reliably. Sibling species of the *C. rudolphii* (s.l.) complex are habitual endoparasites of cormorants of the Phalacrocoracidae family worldwide. Garbin et al. (2011) show that *C. rudolphii* has five congeneric complexes, *C. rudolphii* A, B, C, D, and E. Later D'Amelio et al. (2012) studied, according to molecular data, led to the detection of two new species: *Contracaecum fagerholmi* n. sp. was also supported by clear morphological evidence, and *C. rudolphii* F is a new cryptic species within the *C. rudolphii* complex. Molecular studies also presented by Szostakowska and Fagerholm (2012) identified *C. rudolphii* B in freshwater fish in Poland. This *C. rudolphii* B species was recorded in cormorants from freshwater areas, while *C. rudolphii* A species also found in brackish water areas in Finland and Poland. Pekmezci and Yardimci (2019) studied the presence and molecular identification of larval *Contracaecum* species in marine fish from Turkey. All *Contracaecum* third instar larvae were molecularly characterized and were identified as *Contracaecum overstreeti* Mattiucci, Paoletti, Solorzano & Nascetti, 2010 based on mtDNA COX2 sequence analysis. Isolated freshwater fish from Iraq morphologically and molecularly studied the *Contracaecum* larvae. The sequences of ITS1, ITS2, and COX2 reveal that all *Contracaecum* larvae from all infected fishes represented exactly one species of *Contracaecum rudolphii* B (Abdullah et al. 2021). Recently, Roca-Geronès et al. (2023) parasitologically analyzed the presence of nematodes in samples of European shag from the western Mediterranean coast of Spain. All hosts were found infected with *Contracaecum* specimens. Sequencing analysis of the mtDNA COX2 gene and the ITS1 and ITS2 regions of the rDNA revealed that all samples were genetically *C. rudolphii* sp.

Thus, morphological and molecular studies have shown that anisakid larvae found in common rudd fish of Uzbekistan defined turned out to be the species *C. rudolphii* B. They continue their development cycle, become infected by fish-eating birds, and reach their sexually mature form. For example, suppose the prevalence of the larvae of *Contracaecum* is 10.5% in the common rudd of the Shurkul Reservoir, Uzbekistan. In that case, this parasite is low prevalence in this reservoir and surrounding reservoirs.

In conclusion, according to morphological studies, nematode larvae belonging to the genus *Contracaecum* found in common rudd fish in Shurkul reservoir of Uzbekistan correspond to *Contracaecum* sp.. According to the results of the phylogenetic tree based on nucleotide sequences of the 18S gene of rDNA, the samples of larvae showed a close relationship with haplotypes *C. rudolphii* E. Therefore, to increase reliability of this information, the variable region of the 28S gene of rDNA was verified by nucleotide sequence. As a result, these larvae were very close to the haplotype of *C. rudolphii* B (AF 226579). Also, the common rudd fish (*S. erythrophthalmus*) was registered as an intermediate host for the larvae of this nematode. The prevalence of these fish with larvae of *C. rudolphii* B was 10.5%, and the intensity of infection was 1-18 copies. The larvae of the species *C. rudolphii* B are registered for the first time in Uzbekistan, and the common rudd fish is noted as an intermediate host.

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