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Morphomolecular Identification of *Contracaecum rudolphii* (Nematoda: Anisakidae) in Aquatic Environments of Uzbekistan

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ABSTRACT

In the present study, a total of 16 specimens of the great cormorant (*Phalacrocorax carbo*) and 19 specimens of the common carp (*Cyprinus carpio*), collected from natural freshwater bodies in the Jizzakh, Navoi, Bukhara, and Khorezm regions of Uzbekistan, were examined using both complete and partial helminthological techniques. A total of 113 first-stage (L1) and 78 third-stage (L3) larvae belonging to the genus *Contracaecum* (Railliet & Henry, 1912) were isolated. These larvae were morphologically identified as *Contracaecum rudolphii* (Hartwich, 1964) at both L1 and L3 developmental stages. The isolated larvae were then subjected to detailed morphological, morphometric, and molecular-genetic analyses.

INTRODUCTION

Contracaecum rudolphii (Hartwich, 1964), a nematode species belonging to the family Anisakidae, is an intestinal parasite commonly found in fish-eating birds such as cormorants, pelicans, and ducks **(Moravec, 2009)**. It is recognized as a highly pathogenic organism that poses a serious threat to both wildlife and human health **(Shamsi *et al.*, 2009)**. In recent years, the species has increasingly been regarded as an emerging public health concern.

Larval stages of *Contracaecum* species, including *C. rudolphii*, are responsible for human anisakidosis—a painful and severe condition that can result from the consumption of undercooked or raw fish containing third-stage larvae **(Shamsi, 2014; Takabayashi *et al.*, 2014; Bookhout & Greene, 2019)**. *C. rudolphii* has a complex life cycle involving multiple hosts. Aquatic crustaceans and insect larvae serve as the first intermediate hosts, while fish act as secondary or paratenic hosts. The parasite reaches maturity in piscivorous birds that serve as the definitive hosts **(Bartlett, 1996; Dziekońska-Rynko & Rokicki, 2007)**.

Human-induced changes to natural habitats have led to increases in wildlife population densities in certain areas, which may in turn elevate the risk of *C. rudolphii* transmission between birds and fish, and potentially to humans as well **(Daszak *et al.*, 2001; Kanarek, 2011; Barrueto *et al.*, 2014)**. *Contracaecum* is usually found in the proventriculus of piscivorous birds and pinnipeds, its definitive hosts, and has a heteroxenous life cycle. Worm eggs are expelled into aquatic environments through the feces of the definitive host. These eggs hatch into larvae, which are then consumed by copepods, acting as the intermediate hosts. When fish feed on these infected copepods, they become paratenic hosts in which the larvae are encapsulated and remain dormant within tissues such as the intestinal wall, mesentery, liver, and other internal organs. No further development occurs at this stage. However, when these infected fish are eaten by piscivorous birds or marine mammals, the larvae resume development, molt into the fourth stage, and eventually reach sexual maturity **(Dziekońska & Rokicki, 2007; Moravec, 2009)**.

This parasitic organism has been associated with various pathological conditions across a range of hosts, including fish, birds, and mammals, and is known to cause economic losses in the fishing industry. In fish-eating birds, infections can lead to hemorrhages, severe eosinophilic granulomas with ulceration in the proventricular lining, loss of body mass, and potentially fatal outcomes. In humans, consumption of raw or insufficiently cooked infected fish may result in gastrointestinal symptoms such as nausea, abdominal cramps, vomiting, and diarrhea **(Moravec, 2009; Sreedevi *et al.*, 2017)**.

Until now, the fauna of our republic has been studied at the molecular level for various groups, including fish **(Quvatov *et al.*, 2023; Ubaydullayev *et al.*, 2025)**, nematodes **(Aliyev *et al.*, 2024; Mirzaev *et al.*, 2024)**, and insects **(Kadirov *et al.*, 2024; Kimyonazarov *et al.*, 2024)**.

Despite its relevance, the genetic diversity of *Contracaecum rudolphii* remains insufficiently explored. Understanding the genetic structure of its populations is crucial for gaining insights into the species' evolutionary ecology and can provide valuable data for managing infections in both humans and wildlife **(Shamsi *et al.*, 2009; Cole & Viney, 2018)**. Prior research has indicated that the internal transcribed spacers (ITS1 and/or ITS2) within the ribosomal nuclear DNA (rDNA) serve as reliable molecular markers for differentiating among several species within the Ascaridoidea group **(Szostakowska & Fagerholm, 2012)**. Furthermore, the ITS1 and ITS2 regions have proven effective in distinguishing closely related (sibling) species **(Zhu *et al.*, 2000; Mattiucci *et al.*, 2003; Li *et al.*, 2005)**.

However, comprehensive studies on the molecular identification and genetic variation of *C. rudolphii* have not been extensively conducted in Iraq. One exception is a study in Thi-Qar Province (southern Iraq), where ITS1 was utilized to detect fourth-stage larvae of *Contracaecum microcephalum* (Rudolphi, 1809) and *C. septentrionale* (Kreis, 1955), extracted from the proventriculus of *Nycticorax nycticorax* (Linnaeus, 1758) in the Al-Sanaf marshes **(Mohammad & Hbaiel, 2019a)**. Additionally, the presence of *C. rudolphii* in Iraq has been morphologically confirmed in earlier works **(Al-Moussawi & Mohammad, 2011; Al-Moussawi, 2017; Mohammad & Hbaiel, 2019b)**.

Phalacrocorax carbo sinensis has been identified as a host for two anisakid nematode species: *Contracaecum rudolphii* A and *C. rudolphii* B, both of which have been documented in brackish and freshwater environments across Central and Eastern Europe **(Szostakowska & Fagerholm,**

2007). These two taxa frequently coexist within the same host individuals across various regions of the European Boreal zone **(Mattiucci *et al.*, 2002)**. Evidence suggests a certain degree of ecological separation between them: *C. rudolphii* A appears to be more prevalent in cormorants inhabiting brackish coastal lagoons, whereas *C. rudolphii* B is primarily associated with cormorants nesting in freshwater bodies **(Mattiucci *et al.*, 2002; Szostakowska & Fagerholm, 2007)**.

Additionally, molecular analyses have confirmed the presence of *C. rudolphii* A in *Phalacrocorax aristotelis* from the western Mediterranean region, specifically off the coast of Sardinia **(Farjallah *et al.*, 2008)**. The full life cycle of *C. rudolphii* sensu lato remains incompletely understood. Several experimental studies have been conducted in an attempt to identify its early-stage hosts, involving organisms such as copepods, amphipods, and isopods **(Moravec, 2009)**. These investigations suggest that the parasite's life cycle might be facultatively direct, involving no mandatory intermediate hosts. Although aquatic invertebrates and fish serve as carriers of the infective third-stage (L3) larvae, they are likely functioning as paratenic hosts, despite being ecologically critical to the transmission cycle **(Moravec, 2009)**.

This research aimed to conduct morphological and molecular-genetic characterization of *Contracaecum rudolphii*, a species belonging to the genus *Contracaecum* (Railliet & Henry, 1912).

MATERIALS AND METHODS

Helminthological research methods

To conduct this research, 16 great cormorants (*Phalacrocorax carbo*) and 19 common carp (*Cyprinus carpio*) were examined from natural water bodies located in the Jizzakh (40.895539, 66.196784), Navoi (40.340829, 64.901274), Bukhara (39.916970, 64.851098), and Khorezm (41.272101, 60.495396) regions of our republic, using both complete and incomplete helminthological methods. A total of 113 L1-stage larvae and 78 L3-stage larvae of *Contracaecum rudolphii*, belonging to the genus *Contracaecum* (Railliet & Henry, 1912), were collected and preserved in 70% ethanol.

Morphological and morphometric analysis

The samples were placed on glass slides, stained with the necessary dyes, examined under Nexcope NE930-FL and Nexcope NSZ818 microscopes, and photographed using a ToupCam camera. Morphological and morphometric measurements of *Contracaecum* larvae collected from great cormorants and common carp were determined following the works of Nadav *et al.* **(2023)** and Atel *et al.* **(2018)**.

Molecular-genetic analysis

Total DNA was extracted from nematode tissue samples using the DNeasy Blood and Tissue Kit (Qiagen Inc., November 2023), a commercially available DNA isolation kit. For amplification of the internal transcribed spacer (ITS) regions of ribosomal DNA, the primers TW81 (forward: ata tgc tta agt tca gcg ggt) and AB28 (reverse: gtt tcc gta ggt gaa cct gc), commonly used in molecular taxonomy **(Curran, 1994)**, were employed.

Polymerase chain reaction (PCR) was conducted under the following thermal cycling conditions: initial denaturation at 94 °C for 5 minutes; followed by 35 cycles of denaturation at

95°C for 45 seconds, primer annealing at 55°C for 45 seconds, and extension at 72°C for 1 minute and 40 seconds; with a final extension at 72°C for 5 minutes.

The presence and quality of PCR products were verified by electrophoresis on a 1.0% agarose gel under a voltage of 120 V. DNA bands were excised and purified using a gel extraction kit (Sileks M, Moscow, Russia), following the manufacturer's protocol. Sequencing of the amplified DNA fragments was performed using the ABI PRISM® BigDye™ Terminator v3.1 Cycle Sequencing Kit. Sequence data were obtained with an ABI PRISM 3100-Avant automated sequencer (Moscow, Russia).

Phylogenetic tree construction

To explore the evolutionary relationships within the *Contracaecum* genus, ITS gene sequences from 27 different *Contracaecum* species were analyzed, with one ITS sequence of *Anisakis simplex* (GenBank accession: PP189870) included as an outgroup. Multiple sequence alignment was performed automatically using the MAFFT algorithm **(Katoh *et al.*, 2002)**, with minor adjustments made manually in BioEdit version 7.0.5.2 **(Hall, 1999)** to improve alignment accuracy. A phylogenetic tree was then inferred using the maximum likelihood (ML) approach implemented in the IQ-TREE2 software package **(Minh *et al.*, 2020)**. The most appropriate nucleotide substitution model was determined using the integrated ModelFinder function, and the reliability of the tree's branching structure was assessed through 1,000 ultrafast bootstrap replicates, ensuring statistical robustness in the resulting topology.

RESULTS AND DISCUSSION

Results of the morphological examination

The nematode specimens possessed three prominent lips—one dorsal and two subventral—separated by distinct interlabia. Each lip was large and fleshy, with the anterior surface exhibiting a radially digitated pattern and a pronounced central depression. Two well-defined flanges were present on each lip. The dorsal lip bore two pear-shaped (pyriform) cephalic papillae, whereas each subventral lip carried a single papilla. The interlabia were robust and fleshy, broad at the base, and abruptly tapered into bifurcated tips. The degree and shape of the bifurcation varied among specimens. On average, the interlabia extended to approximately two-thirds of the height of the adjacent lips. Internally, the intestinal caecum was considerably elongated—approximately two to three times the length of the ventricular appendix—indicating a well-developed digestive structure (Fig. 1).

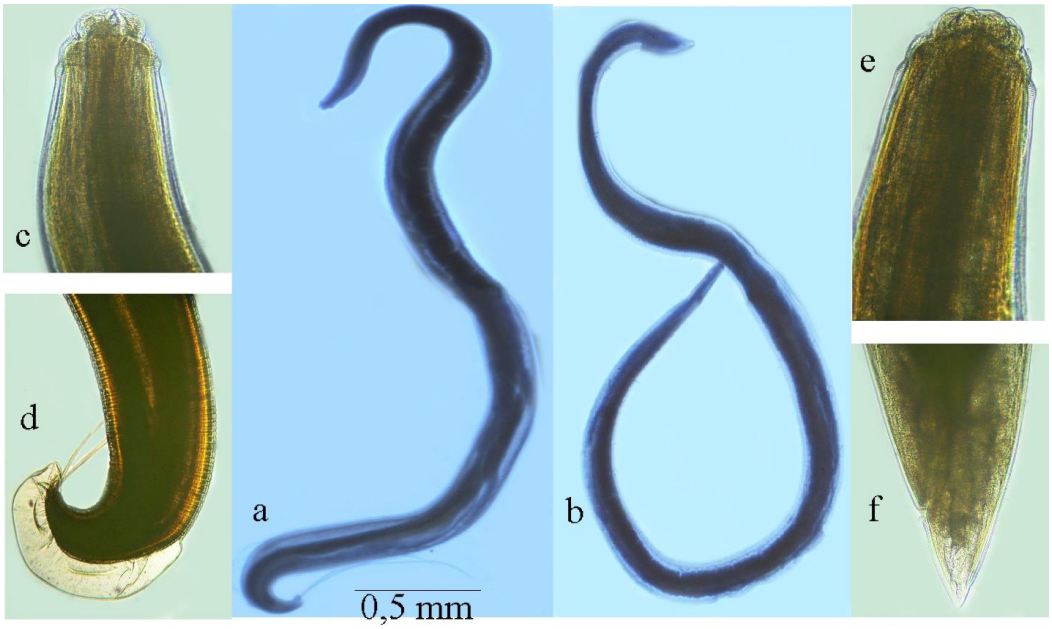

Fig. 1. Morphological appearance of the larva belonging to the genus *Contracaecum* (Railliet & Henry, 1912)
Caption:
a – general appearance of the male;
b – general appearance of the female;
c – head region of the male;
d – tail region of the male;
e – head region of the female;
f – tail region of the female.

Male – Form A: Body length 21.4mm (range: 14.9–26.1), body width 0.52mm (0.52 ± 0.04), oesophagus length 2.9mm (13.5% of total body length), ventricle length 2.1mm (9.8%), nerve ring located at 0.56mm (2.65%) from the anterior end, spicule length 5.25mm (24.5%).

Female – Form A: Body length 31.3mm (range: 25.6–37.8), body width 0.61mm (0.37–0.81), oesophagus length 4.8mm (15.3%), ventricle length 3.2mm (10.2%), nerve ring located at 0.57mm (1.8%) from the anterior end, vulva located 10.9mm (34.8%) from the anterior end.

Male – Form B: Body length 21.2mm (range: 11.3–30.7), body width 0.57mm (0.37–0.81), oesophagus length 3.2mm (15.09%), caecum length 2.2mm (10.4%), nerve ring located at 0.41mm (1.9%) from the anterior end, spicule length 5.75mm (27%).

Female – Form B: Body length 23.9mm (range: 14.8–35.7), body width 0.55mm (0.39–0.81), oesophagus length 4.1mm (17.1%), caecum length 2.8mm (11.7%), nerve ring located at 0.52mm (2.2%) from the anterior end, vulva located 9.1mm (38.07%) from the anterior end (Table 1).

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Table 1. Morphometric measurements of the larva belonging to the genus *Contracaecum* (Raillet & Henry, 1912)

Morphological characteristics	**Male**		**Female**	
	C. rudolphii A n=7	*C. rudolphii* B n=6	*C. rudolphii* A n=8	*C. rudolphii* B n=10
Body length	12.9-27.8 (21.4±2.9)	11.3-30.7 (21.2±4.3)	25.6-37.8 (31.3±3.7)	14.8-35.7 (23.9±3.9)
Body width	0.3-0.7 (0.5±0.4)	0.4-0.8 (0.6±0.5)	0.4-0.9 (0.6±0.8)	0.4-0.8 (0.6±0.7)
Esophagus length	1.7-4.2 (2.9±1.3)	2.1-4.2 (3.2±1.9)	2.4-5.8 (4.8±2.5)	2.1-5.6 (4.1±2.1)
The length of the cecum	1.4-3.7 (2.1±1.1)	1.7-4.3 (2.2±0.9)	2.3-4.8 (3.2±1.4)	1.6-4.8 (2.8±1.6)
Neck length	0.3-0.7 (0,56±0.4)	0.3-0.5 (0.4±0.9)	0.5-0.6 (0.6±0.8)	0.4-0.7 (0.5 ±0.8)
Length of right spicule	3.2-6.9 (5.2±1.9)	2.9-7.1 (5.7±1.8)	-	-
Left spicule length	3.1-7.1 (5.3±1.7)	2.4-6.8 (5.8±1.7)	-	-
Vulva length	-	-	8.3-13.2 (10.9±1.8)	6.9-11.8 (9.1±1.8)

In this study, the anisakid larvae collected from different great cormorants were found to be morphologically consistent with *Contracaecum rudolphii*. However, detailed morphological examination revealed that the specimens could be separated into two distinct forms—*C. rudolphii* D and *C. rudolphii* E. Larvae belonging to the E group appeared more robust, with greater overall body length and width. Their lips were also larger but had smaller lateral flanges. The labial region exhibited a smaller diameter compared to the adjacent body section, and the deirids were positioned farther from the anterior end than in *C. rudolphii* D. In addition, the E group displayed longer oesophagi, intestinal caeca, and ventricular appendices.

Male specimens of *C. rudolphii* E exhibited a greater distance from the cloaca to the posterior tip. The posterior end of these males was more abruptly tapered—first after the initial pair of post-cloacal papillae and again after the final row—unlike males of *C. rudolphii* D, which possessed a more uniformly conical tail. These distinguishing features were consistently observed across specimens. However, the potential influence of fixation techniques on certain morphological traits remains unclear and warrants further investigation.

Molecular-genetic identification

Phylogenetic tree analysis was performed to determine the evolutionary relationships of *Contracaecum* species, using *Anisakis simplex* (GenBank accession: PP189870) as the outgroup.

The resulting topology revealed two main clusters, reflecting genetic diversity within the genus and phylogenetic proximity among species (Fig. 2).

The first cluster included samples of *C. rudolphii* (FJ589792, FJ589791, FJ589790), *C. rudolphii* A *Bullini et al.* (OR263204, OR263201), *C. aff. rudolphii* A LG-2020 (MT385214, MT385220, MT385217). Within this clade, the species exhibited close genetic relationships with similarities of 89%, 99%, and 95%, indicating minor differentiation among *C. rudolphii* taxa. Notably, the *C. aff. rudolphii* A LG-2020 sequences formed a separate subclade with 57 and 74% similarity, suggesting partial divergence from the main *C. rudolphii* group.

The second cluster contained *C. rudolphii* sequences from Jizzakh_UZB (MW596001), Bukhara_UZB, Bukhara_1_UZB, Navoiy_UZB, as well as sequences (FJ467618, FJ467620, KX550950, MH778108, MH778114, MH778116) and *C. rudolphii* B *Bullini et al.* from Khorezm_UZB (OR263210, OR263216, OR263208, OR263212). This clade showed 100% sequence similarity, indicating that these samples are genetically identical or extremely closely related. Smaller subclades within this cluster exhibited similarity levels ranging from 48 to 81%, which appear to reflect both geographic distribution (e.g., Jizzakh, Bukhara, Navoiy, Khorezm) and genetic variation.

The two major clusters formed a single large monophyletic group with 95% bootstrap support. This group was connected via a separate branch to *C. rudolphii* F (JF424597), indicating that *C. rudolphii* F is genetically distinct from the main cluster. These results provide an important basis for understanding the evolutionary history and genetic diversity within the genus *Contracaecum*.

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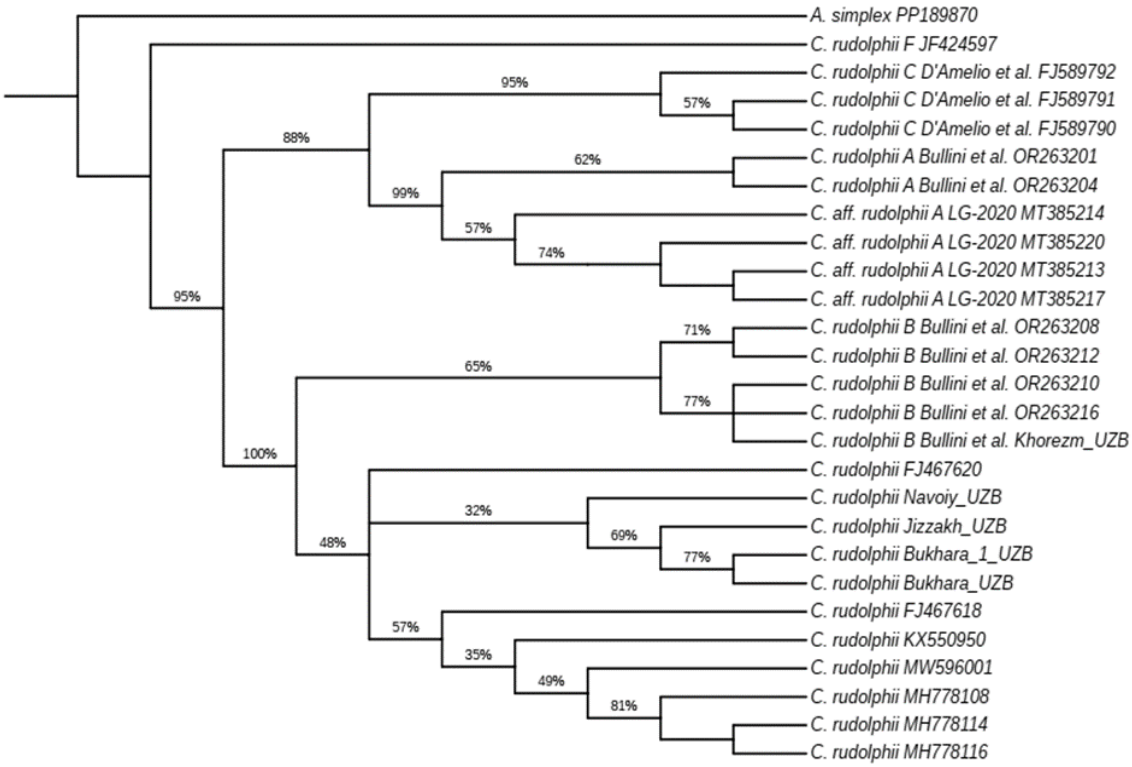

Fig. 2. Phylogenetic tree for *Contracaecum rudolphi* species

Pairwise genetic distances between *Contracaecum rudolphii* and 24 closely related sequences were calculated based on mitochondrial COI sequences using the Kimura two-parameter model. The analysis revealed very low genetic divergence among *C. rudolphii* isolates collected from various regions of Uzbekistan (Bukhara, Navoiy, Jizzakh, and Khorezm), with distances ranging from 0.00000 to 0.00413, indicating a high degree of genetic similarity across these populations. Notably, the isolates *C. rudolphii* Bukhara_UZB and Bukhara_1_UZB were nearly identical, with genetic distances of 0.00000–0.00001, suggesting they may belong to the same haplotype. Similarly, the sequences MH778116.1, MH778108.1, and OR263216.1 exhibited minimal genetic variation (0.00000–0.00524), further supporting their close phylogenetic relationship. In contrast, the sequence PP189870.1 displayed substantially higher genetic distances (0.29690–0.35497) from all other sequences, suggesting it may represent a distinct species or a divergent lineage within the genus *Contracaecum*. Other GenBank sequences, such as FJ589790.1, FJ589791.1, and FJ589792.1, showed moderate divergence (0.00928–0.01950) when compared to the Uzbekistan isolates, which may reflect regional or population-level differentiation.

CONCLUSION

Previous genetic analyses have indicated that *Contracaecum rudolphii* infecting *Phalacrocorax carbo sinensis* in Italy actually comprises at least two genetically distinct but closely related sibling species, referred to as *C. rudolphii* A and *C. rudolphii* B. In the present study, molecular examination of the ITS-1 region from two different populations of *C. rudolphii* further supports this classification. One population exhibited the highest sequence similarity with *C. rudolphii* B, while the other aligned more closely with *C. rudolphii* A. These results are consistent with previous findings demonstrating the coexistence of both sibling species within the same host across different geographic regions. This investigation is particularly noteworthy because it focuses on *P. carbo* specimens collected in central Iraq—a region that has not been extensively studied in this context. Given that the prevalence of *C. rudolphii* varies substantially between locations, it is likely that further genetic differentiation exists among populations from other geographic areas. While morphological studies remain important, they are often limited by challenges in specimen collection and the small number of larvae suitable for microscopic examination. Consequently, molecular markers such as ITS-1 are invaluable for assessing intraspecific genetic variation. Overall, the findings from this study add to the growing evidence that *C. rudolphii* is not a single uniform species but rather a species complex composed of at least two genetically divergent sibling taxa.

GRATITUDE

We would like to express our gratitude to the scientific team of the Laboratory of Molecular Zoology at the Institute of Zoology of the Academy of Sciences of the Republic of Uzbekistan for their practical assistance in determining the composition of nematode species, as well as to the leadership of the scientific project "Molecular-genetic characterization of wild vertebrate species of Bukhara and Navoi regions".

NOVELTY STATEMENT

Molecular identification of L1 larvae of the nematode species *Contracaecum rudolphii* Hartwich was first carried out in Uzbekistan in 1964. According to the results of molecular-genetic studies, a 650-base ribosomal DNA sequence was isolated from the *C. rudolphii* Hartwich National Deposit, which belongs to the National Center for Biotechnology and Information Systems (NCBI) and is assigned accession numbers (PV746215, PV746210).

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