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Morphological and Molecular-Genetic Classification of the Nematode *Rhabdias engelbrechti* Found in the Amphibian *Pelophylax terentievi* in the Aquatic Basins of South Uzbekistan

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ABSTRACT

For this molecular genetic research, helminthological samples were collected from the lungs of 17 samples of *Pelophylax terentievi* (Mezhzherin, 1992) distributed in Kashkadarya and Surkhondarya regions in the spring and summer of 2024. The difference between the nucleotides of the *R. engelbrechti_uz* (PQ219671) sample belonging to the *Rhabdias* genus collected from the southern regions of our republic and the *R. engelbrechti* (MG428406) sample obtained from the NCBI database was 0.4%. Nucleotide sequence obtained as a result of molecular-genetic study of *engelbrechti* belonging to *Rhabdias* genus was placed in the National Center for Biotechnology Information. This species was recorded for the first time to the best of our knowledge in the territory of the Republic of Uzbekistan.

INTRODUCTION

Rhabdias engelbrechti Kuzmin, Halajian, Tavakol, Luus-Powell and Tkach, 2017 belongs to the genus *Rhabdias*, whose representatives are distributed across all zoogeographic regions, except Antarctica. These parasites inhabit the lungs of amphibians and reptiles, and approximately 80 species have been identified to date **(Kuzmin & Tkach, 2017)**.

Three species of the genus *Rhabdias* are found in Madagascar (*R. madagascariensis* **Chabaud *et al.*, 1961**, *R. vencesi* **Junker *et al.*, 2010**, and *R. blommersiae* **Kuzmin *et al.*, 2013)**, and six species **(***R. sylvestris* **Baker**, **1982**, *R. collaris* **Baker, 1987**, *R. africanus* **Kuzmin, 2001**, *R. ohlerae* **Junker *et al.*, 2010**, *R. picardiae* **Junker *et al.*, 2010**, and *R. tanyai* **Junker *et al.*, 2010)** are distributed in the African

continent and the Sahara, which are parasites of the Artholeptidae, Bufonidae, and Microhylidae families **(Chabaud *et al.*, 1961; Baker, 1982)**.

Two species belonging to the genus *Rhabdias* (*R. bufonis*, *R. rubrovenosus*) are distributed in our republic, and these species were recorded in the lungs of representatives of the *Bufotes* and *Pelophylax* families **(Ikromov *et al.*, 2023)**.

According to **Kuzmin *et al.* (2017)** mitochondrial DNA sequence *COI* and ribosomal DNA sequence 12S, ITS and 28S region of the species R. *engelbrechti*, belonging to the genus *Rhabdias*, which was identified in the frog *Phrynomantis bifasciatus* (Microhylidae) distributed in Africa in 2017, were characterized based on the nucleotide sequence.

The purpose of this research was to provide a molecular-genetic description of *Rhabdias engelbrechti* found in the lungs of *Pelophylax terentievi* **(Mezhzherin, 1992)**, a species distributed in the southern region of our republic.

MATERIALS AND METHODS

Morphological research method

In order to carry out these morphological and molecular-genetic studies, helminthological samples were collected from the lungs of 17 *P. terentievi* **(Mezhzherin, 1992)** species distributed in Kashkadarya and Surkhondarya regions of the southern regions of our republic in the spring and summer seasons of 2024. The collected nematodes were fixed by placing them in 70% alcohol vials, and permanent and temporary drugs were prepared from the collected samples for morphological analysis.

The morphological and morphometric analysis of nematode samples were analyzed based on the literature of the following authors: **Chabaud *et al.* (1961)**, **Baker (1982, 1987)**, **Kuzmin *et al.* (2001, 2003, 2013, 2014, 2017, 2020)**, **Bursey *et al.* (2003, 2007, 2012)**, **Tkach *et al.* (2006, 2011, 2014)**, **Yildirimhan *et al.* (2006, 2007, 2012)**, **Junker *et al.* (2010)**, and **Morsy *et al.* (2018)**.

Molecular genetic method

ITS fragments of ribosomal DNA were isolated from the nematode species mentioned above for molecular genetic research. For this, samples of 3 individuals of *Rhabdias engelbrechti* nematodes were taken, and genomic DNA was isolated.

The GeneJet Genomic DNA Reagent Kit was used to extract genomic DNA from nematode tissue samples **(Vogelstein, 1979; Marko, 1982; Boom, 1990)**.

Nucleotide sequences of ITS fragments from nematode ribosomal DNA (rDNA) were isolated using AV28 forward (5'-ATA TGC TTA AGT TCA GCG GGT-3') and TW81 reverse (5'-GTT TCC GTA GGT GAA CCT GC-3') primers, commonly employed in molecular taxonomy.

PCR recipe

Initial DNA denaturation was performed at 94°C for 3 minutes, followed by 9 cycles of denaturation at 94°C for 1 minute, annealing at 54°C for 1 minute 30 seconds, and elongation at 72°C for 1 minute 30 seconds. This was followed by 24 cycles consisting of denaturation at 94°C for 45 seconds, annealing at 54°C for 45 seconds, and elongation at 72°C for 2 minutes. A final elongation step at 72°C was performed for 5 minutes. The results of the PCR reaction were verified by electrophoresis of 1 ml of the product in a 1% agarose gel (100 V, 80–100 mA, for approximately 30–40 minutes).

For DNA sequencing, the ABI PRISM® BigDye™ Terminator v. 3.1 reagent kit was used, and the reaction products were analyzed on an ABI PRISM 3100-Avant automatic sequencer (Moscow, Russia).

Nucleotide sequence analysis was performed using BioEdit, Clustal W, and DNAstar™ software, with phylogenetic analysis conducted via the PAUP4 program.

Constructing a phylogenetic tree

Nucleotide sequences of helminths from the genus *Rhabdias* were sequenced, and additional DNA sequences were retrieved from the International Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov). These sequences were manually aligned using Geneious Prime software, and the consensus sequences were calculated with MEGA X software. Primer data from this program, along with additional sequences from the GenBank database, were aligned using MAFFT v.7 online software with default settings and further edited with Clustal Omega 1.2.2 and Geneious Prime software.

The maximum likelihood (ML) phylogenetic tree for the ITS region of the obtained ribosomal DNA (rDNA) sequences was constructed using ultra-fast bootstrapping with 1,000 iterations in IQ-TREE version 1.6.12. The analyses were performed via the CIPRES Science Gateway v3.3. The ITS region nucleotide sequence of *R. engelbrechti* Kuzmin, Halajian, Tavakol, Luus-Powell & Tkach, 2017 (Accession number: MG428406) was included as an outgroup to assist in generating consensus trees. The resulting phylogenetic tree was analyzed and edited using iTOL v6.6 software.

RESULTS

Morphological and morphometric analyses

Based on the results of the morphometric analysis of *Rhabdias engelbrechti_uz* (n=20), the body length of this species is 5.4mm (ranging from 3.2 to 7.1mm), and the width at the middle of the body is 298µm (207–384µm), tapering gradually toward the posterior end. The body width at the junction of the esophagus and intestine is 153 µm (98–183µm), while it measures 112µm (69–151µm) at the anus. The anterior end of the body is rounded, while the posterior end is narrow. The tail tapers gradually from the anus,

with a length of 261µm (153–354µm), constituting 4.8% (2.8–6.5%) of the total body length (Fig. 1a, b, c).

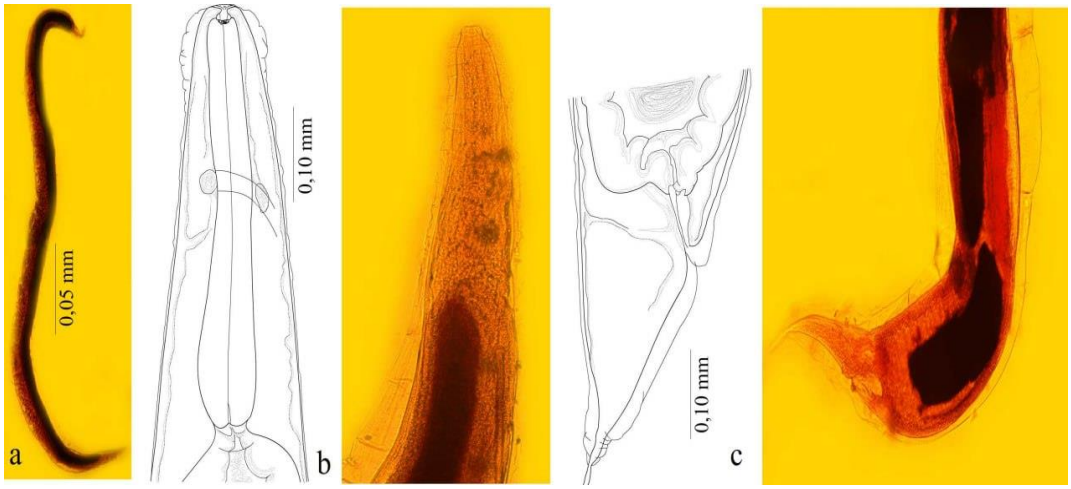

Fig. 1. *Rhabdias engelbrechti* Kuzmin, 2017 **a-** Overview; **b-** Front part of the body; lateral view; **c-** Back of the body, lateral view

During the conducted morphological and morphometric research, the morphometric dimensions of *R. engelbrechti* found in Kashkadarya and Surkhondarya regions were determined, and the obtained data were compared with the representative of this species recorded in South Africa by Kuzmin, Halajian, Tavakol, Luus-Powell and Tkach (Table 1).

Table 1. Some comparative morphometric measurements of *Rhabdias engelbrechti* Kuzmin, Halajian, Tavakol, Luus-Powell & Tkach, 2017 (n=20)

Indicator	***Rhabdias engelbrechti* (µm)**		
	Research data		**Research data (Kuzmin *et al.*, 2017)**
	lim	***M±m***	***Lim***
Body length (mm)	3.2-7.1	5.4±0.3	3.8-6.1
Body width at the junction of the esophagus and intestine	98-183	153±1.7	111-158
Body width at the vulva	207-384	298±3.6	218-362
Body width at the anus	69-151	112±2.1	73-145
Buccal capsule width	16-21	18±0.8	16-18
Buccal capsule length	5-12	8.2±0.7	6-9
The length of the vestibule	3-8	4.8±0.9	3-5
Esophagus length	278-421	370±4.2	293-393
The width of the esophagus	50-68	63±1.3	53-64
The distance from the anterior end to the nerve ring	127-192	162±2.5	138-171

Distance from anterior tip to vulva (mm)	1.87-4.7	2.9±0.3	2.1-3.1
Tail length	153-354	261±3.1	166-317

Note: n-number of samples, lim-sign limit, M-arithmetic average value, m-arithmetic average error.

According to the analysis presented in Table (1), the main morphometric dimensions of the studied nematode are slightly smaller (3.8–6.1mm) compared to those of the representative found in South Africa (3.2–7.1mm). Additionally, there are differences in the genitalia, notably the vulva, which has slightly prominent lips measuring 2.9mm (1.87–4.7mm) anteriorly, and a vestibule that is slightly longer (3–5µm) compared to the South African specimen (3–8µm) (Table 1).

Molecular genetic analysis

The molecular-genetic research revealed that the rDNA of *Rhabdias engelbrechti_uz*, found in the lungs of *Pelophylax terentievi* frogs from the southern regions of our republic, belongs to the ITS-1+5.8S+ITS-2 region with a length of 665 nucleotides. To compare these sequences, data from *Rhabdias engelbrechti* (Accession number: MG428406) and *Rhabdias bufonis* (Accession number: KF999609) from the International Center for Biotechnology Information (NCBI) were used (Fig. 2).

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Fig. 2. Nucleotide sequence comparison of the rDNA ITS region of species belonging to the genus *Rhabdias* based on sequence material

Fig. (2) shows a difference of 3 nucleotides between the *Rhabdias engelbrechti_uz* sample from the southern regions of our republic and the *R. engelbrechti* sample obtained from the NCBI database. Specifically, at the 268th nucleotide position, C-cytosine in the NCBI sample is replaced by T-thymine in our sample. At the 269th nucleotide, G-guanine in the NCBI sample is replaced by C-cytosine in our sample. Additionally, A-adenine nucleotides were found to be exchanged in the *R. engelbrechti_uz* sample, resulting in a 0.4% difference in total nucleotide composition.

There is also a difference of 10 nucleotides between the *Rhabdias engelbrechti* sequences and those of *R. bufonis* from the NCBI database. Specifically, at the 30th nucleotide, A-adenine in *R. engelbrechti* is replaced by T-thymine in *R. bufonis*. At the 40th nucleotide, T-thymine in *R. engelbrechti* is replaced by G-guanine in *R. bufonis*. At the 149th nucleotide, T-thymine in *R. engelbrechti* is replaced by S-cytosine in *R. bufonis*. At the 268th nucleotide, T-thymine in *R. engelbrechti* is replaced by G-guanine in *R. bufonis*, and at the 269th nucleotide, C-cytosine in *R. engelbrechti* is replaced by A-adenine in *R. bufonis*. The total nucleotide difference between *R. engelbrechti* and *R. bufonis* is 1.5% (Fig. 2).

Phylogenetic tree

According to the results of molecular genetic research, it was found that the representatives of this genus are grouped into 7 clades (groups) according to the analysis of nucleotide sequences belonging to the ITS region of rDNA of the studied species belonging to the genus *Rhabdias* and nucleotide sequences obtained from the GenBank database (Fig. 3).

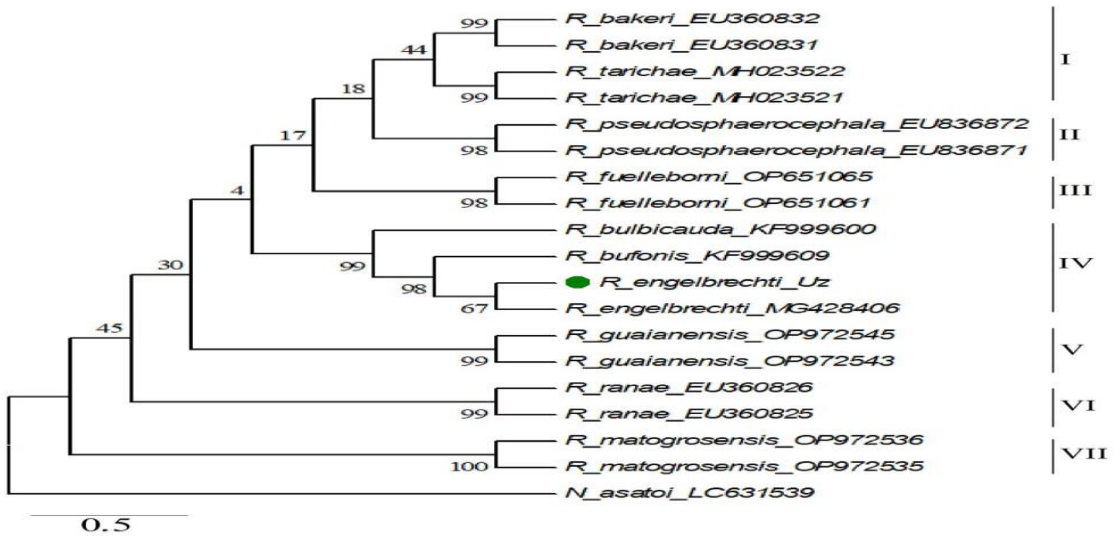

Fig. 3. A phylogenetic family tree of *Rhabdias* genus helminths developed based on the maximum likelihood-ML method

The phylogenetic analysis revealed several distinct groups with varying bootstrap support. The first group includes *Rhabdias bakeri* and *R. tarichae*, which have 44% bootstrap support relative to the main group and 99% within the species. The second group comprises *R. pseudosphaerocephala*, with 18% relative to the main group and 98% within the species. The third group includes *R. fuelleborni*, with 17% relative to the main group and 98% within the species. The fourth group features *R. bulbicauda*, showing 4% relative to the main group and 99% within the species. This group further subdivides into three subgroups: *R. bufonis* with 98% support, *R. engelbrechti_Uz* with 67% support, and *R. engelbrechti* with 67% support. In the fifth group, *R. guaianensis* has 30% support relative to the main group and 99% within the species. The sixth group includes *R. ranae*, with 45% support relative to the main group and 99% within the species. The seventh group consists of *R. matogrosensis*, which exhibits 100% bootstrap support for the main group (Fig. 3).

CONCLUSION

The difference between the *Rhabdias engelbrechti_uz* sample and the *R. engelbrechti* sample obtained from the NCBI database is 0.4%. This difference in nucleotide sequences is observed between the species found in the frog *Pelophylax terentievi* and its environmental factors. Some morphometric dimensions of *R. engelbrechti* parasitizing the lungs of *P. terentievi* in southern Uzbekistan differ from those of populations found in South Africa.

The nucleotide sequence of the rDNA 5.8S-ITS2 region of *R. engelbrechti* from southern Uzbekistan (accession number PQ219671) shows 98.62% similarity to the sequence of *R. engelbrechti* with an accession number of MG428406 in the International GenBank Database. This high level of similarity indicates that *R. engelbrechti* is a globally widespread helminth species with diverse morphoanatomical and genetic characteristics.

The nucleotide sequence obtained from the molecular-genetic study of *R. engelbrechti* of the *Rhabdias* genus has been deposited in the National Center for Biotechnology Information (NCBI). To our knowledge, this is the first record of this species in the Republic of Uzbekistan.

GRATITUDE

The work was carried out within the framework of the program “Molecular identification of hoofed animals and their parasitic nematodes” implemented by the Academy of Sciences of the Republic of Uzbekistan.

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