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Morphology and molecular identification of the trematode *Echinostoma revolutum* metacercariae, as a new parasite of amphibians in Uzbekistan

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Abstract. *Ikromov EE, Ikromov EF, Yildirimhan HS, Sümer N, Akmuradova L, Yusupov A, Amirov OO, Rustamov I, Usmonov B. 2025. Morphology and molecular identification of the trematode* Echinostoma revolutum *metacercariae, as a new parasite of amphibians in Uzbekistan. Biodiversitas 26: 4412-4423.* This study examined the helminth fauna of two frog species, *Pelophylax terentievi*, *Pelophylax* sp., in eastern (Ferghana Valley: Andijan, Namangan and Ferghana) and central Uzbekistan (Bukhara, Navoi and Samarkand regions). We identified the metacercarium trematode *Echinostoma revolutum*, for the first time in this context, which is an intestinal parasite commonly found in various waterfowl. At the same time, we studied amphibians for the first time as new intermediate hosts of the parasite. This article discusses the morphology and molecular characteristics of *E. revolutum* found in the eye cavities, kidneys and body cavities of *P. terentievi* and *Pelophylax* sp. in the central and eastern geographical regions of Uzbekistan. Our analyses showed that the trematode, *E. revolutum*, differs morphologically from other ecomorphs in terms of body size, cyst diameter and localization. This study also provided molecular data for the genus *Echinostoma* in the ITS1+5.8S+ITS2 regions of ribosomal DNA, forming a well-supported clade in the phylogenetic tree. The sample we examined, *E. revolutum* (UZ), was in the monophyletic group together with a sample extracted from the NCBI database (*E. revolutum* MZ409811) and had a common ancestor. These results provide molecular evidence to support the identification of *E. revolutum* species in our study. In addition, our analysis showed that *Echinostoma* sp. MZ409812 is a sister group to *E. revolutum* (clade J). *E. miyagavai* (clade I), *E. nasincovae* (clade L), *E. paraensei* and *E. maldonadoi* (clade M) are the closest groups to *E. revolutum*. Our results contribute to the understanding of the species and genetic diversity of the genus *Echinostoma* and the species *E. revolutum*. The high infection rate (up to 14%) of the amphibians *P. terentievi* and *Pelophylax* sp. with these parasites indicates a significant biological threat, which requires enhanced monitoring and development of measures to protect waterfowl in the region, underscoring the importance of this study in preserving the ecological balance.

Keywords: DNA sequence, frog, helminth, parasite, second intermediate host

INTRODUCTION

When discussing transmissible diseases from amphibians to humans, studies on the mechanisms of transmission remain largely unexplored. Simultaneously, accurate parasite species identification is essential for effective diagnosis and subsequent management strategies in parasitic systems. However, the morphology and taxonomy of certain parasite groups, such as *Echinostoma*, remain poorly understood because of the limited and conflicting molecular data. Nevertheless, ongoing research and monitoring are crucial for a better understanding of the diversity and potential risks that parasites pose to wild animal populations.

According to the literature, the genus *Echinostoma* comprises approximately 120 species worldwide, but currently, 60 species are recognized as valid (Chai et al. 2020). One of these trematodes, *Echinostoma revolutum* ((Fröhlich, 1802) Looss, 1899), is widely distributed across Asia, Europe and North and South America (Chai et al. 2020). It can infect reptiles, birds and mammals, including humans (Leles et al. 2014; Sah et al. 2018).

The development of the trematode *E. revolutum* involves freshwater gastropod mollusks as the first intermediate hosts. These include species such as *Lymnaea stagnalis*, *L. auricularia*, *L. lagotis*, *L. ovata*, *L. palustris*, *L. peregra* and *Viviparus viviparus*. The same mollusks that serve as the first intermediate hosts, where the development of parthenitae occurs, often also play the role of the second intermediate hosts for *E. revolutum*. Less commonly, this role can be played by other mollusks from the genera *Anisus*, *Gyraulus*, *Physa*, *Bithynia* and *Viviparus*, as well as bivalve mollusks such as *Anodonta cygnea*, *Sphaerium corneum*, species of the genus *Henslowiana* (*Euglesa*) and various aquatic organisms such as dragonfly larvae (*Aeshna viridis*), water bugs, fish and tadpols (Yakovleva et al. 2015).

Numerous studies have asserted that adult amphibians also serve as second intermediate hosts for members of the Echinostomatidae family. Specifically, in Bulgaria,

metacercariae of *Echinostoma jurini* have been discovered in the kidneys and eye sockets of tadpoles of the common frog (*Rana temporaria*) and the marsh frog (*Rana* (*Pelophylax*) *ridibunda*) (Kanev et al. 1995). Additionally, metacercariae of *E. cinetorchis* have been discovered in the kidneys of leopard frogs (*Rana pipiens*) and green frogs (*Rana* (*Lithobates*) *clamitans*) in the United States (Martin and Conn 1990). The metacercariae of *Echinoparyphium rubrum* have been discovered in the kidneys of adult wood frogs (*Lithobates sylvaticus*) in North America (Pulis et al. 2011).

Under experimental conditions, the life cycle of *Echinostoma macrorchis* has been studied in frogs. Additionally, infections in tadpoles have been observed while studying the life cycles of other genera, such as *Echinoparyphium*, *Hypoderaeum*, *Isthmiophora* and Petasiger, in both laboratory and natural settings. Identifying *Echinostome* species is a challenging task. The classification can be based on detailed morphological studies and molecular genetic analyses. Many species resemble each other and can be considered cryptic, where distinct lineages are regarded as the same species because of their high morphological similarity. Several *Echinostome* species have undergone multiple reclassifications. For instance, the species currently known as *Echinostoma caproni* was previously identified under various names, including *Echinostoma liei*, *Echinostoma paraensei* and *Echinostoma togoensis*. *Echinostome* species within this group are currently classified based on shared morphological and biological characteristics, such as the presence of 37 collar spines. Presently, molecular methods such as mitochondrial and ribosomal DNA sequencing have become the primary techniques for parasites. In light of this, the study of *E. revolutum* in Uzbekistan, which parasitizes aquatic domestic and wild birds, holds significant practical relevance. This study provides crucial insights from an epizootiological perspective, contributing to our understanding of disease transmission and control.

Based on this, the present study aimed to characterize the ecomorph morphology, conduct molecular genetic identification and perform a phylogenetic analysis of the trematode *E. revolutum* under the conditions of Uzbekistan.

MATERIALS AND METHODS

Sample collection

From 2020 to 2024, helminthological investigations were conducted in the central (Bukhara Region, Karakul 39°30'51.8"N 63°43'31.0" E) and eastern (Fergana Valley, Tyura-Kurgan 41.028310"N, 71.500459"E) parts of Uzbekistan. Specifically, *Pelophylax terentievi* specimens were collected from a small collector belonging to the Central Bukhara drainage system located in the lower delta of the Zeravshan River, near human settlements in Karakul District. In addition, *Pelophylax* sp. Mazepa (2013) individuals were collected individuals from the Kosonsoy stream in Tyura-Kurgan District of Namangan Province. A total of 68 anuran specimens were collected, comprising 33 individuals of *P. terentievi* and 35 individuals of *Pelophylax* sp. The identification of frogs was based on the work of Mazepa (2013) and Ualiyeva et al. (2022).

In the surveyed areas, numerous bird species that may serve as participants in the biological cycle of *Echinostoma* spp. were observed. These included wild ducks (*Anas platyrhynchos*), pheasants (*Phasianus colchicus*), pygmy cormorants (*Phalacrocorax pygmaeus*) and great cormorants (*Phalacrocorax carbo*). In household farms, domestic birds such as chickens, guinea fowls and turkeys are commonly raised.

The water of small collectors within the Central Bukhara drainage system and the irrigation canals of the Fergana Valley is rich in organic matter. These habitats support diverse intermediate hosts and food resources for amphibians, including mollusks (*Lymnaea*, *Planorbis*, *Anisus* spp.), insect larvae (e.g., *Odonata* nymphs, *Coleoptera* larvae) and various species of annelid worms.

Examination

The collected anurans were euthanized via injection of 20% benzocaine hydrochloride into the abdominal cavity in accordance with the AVMA Guidelines for the Euthanasia of Animals (Leary et al. 2020). The amphibians were dissected using standard methods. Metacercariae were mechanically extracted from cysts using dissection needles under a stereomicroscope and the specimens were observed alive. The collected and fixed trematodes were immersed in distilled water for approximately 15 minutes, cleared with glycerin for 5 minutes and examined under a microscope (N-300M). Illustrations of the trematodes were created using an attachment to the N-300M microscope and documented using a digital camera (FMA050). All measurements are given in millimeters (mm). *Echinostoma revolutum* metacercariae were identified based on morphological features described by Chantima et al. (2013), with emphasis on the presence of 37 collar spines, as confirmed by Butboonchoo et al. (2020). Identification followed the diagnostic criteria outlined by Faltýnková et al. (2015), including collar spine pattern, cyst structure, body shape and measurements of key internal organs. Specific diagnostic criteria for helminths were based on those reported by Faltýnková et al. (2015).

The collected metacercariae (mcc) were fixed in 70% ethanol and labeled for storage in the collection (No. 170424) at the Aquaculture Laboratory of Namangan State University, Uzbekistan. The obtained nucleotide sequence was deposited in the International GenBank (NCBI) database under accession number OR600232.1.

Molecular analysis

Data collection

For phylogenetic analysis, DNA sequences of species belonging to the genus *Echinostoma* were downloaded from the international open-access database, National Center for Biotechnology Information (NCBI, https://www.ncbi.nlm.nih.gov). The species *Echinoparyphium recurvatum* was used as an outgroup. For each species, sequences matching the respective phylogenetic marker (e.g., ITS2, COI or 28s rRNA) were selected based on high

quality, completeness and proper annotation. All sequences were saved in FASTA format (Table 1).

DNA extraction, amplification and sequencing

Genomic DNA was extracted from the three trematode samples using the DNeasy Tissue Kit (Thermofisher.com). We amplified the internal transcribed spacers ITS1+5.8S+ITS2 regions in nuclear DNA via Polymerase Chain Reaction (PCR) using primer set AB28 (5′-ATATGCTTAAGTTCAGCGGGT-3′) and TW81 (5′-GTTTCCGTAGGTGAACCTGC-3′). The PCR was conducted in a 20 µL reaction mixture containing 40 ng of each template, 1.5 µL of each primer, 0.6 µL of dNTPs, 0.6 µL of Taq polymerase, 2 µL of PCR buffer (Sileks M, Moscow, Russia) and 13.8 µL of distilled water using the ProFlex PCR system (Applied Biosystems). The thermal cycling program comprised an initial stage at 95°C for 3 minutes, followed by 35 cycles of 20 seconds at 93°C, 30 seconds at 55°C and 2 minutes at 72°C and a final extension at 72°C for 10 minutes (Kuchboev and Krücken 2022; Ikromov et al. 2023). PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide and purified using the Sileks M kit (Moscow, Russia). DNA sequencing was performed using the ABI PRISM BigDye™ Terminator v. 3.1 and reaction products were analyzed on an ABI PRISM 3100-Avant Genetic Analyzer (Moscow, Russia).

Multiple sequence alignment

The selected sequences were aligned using the Multiple Sequence Alignment by CRUSTALW online platform (https://www.genome.jp/tools-bin/clustalw). CRUSTALW settings were configured as follows: Alignment type - DNA; Gap opening penalty - default value (15); Gap extension penalty - default value (6.66); Transition weight - 0.5.

Upon completion of the alignment, the result was downloaded in .aln format. This alignment phase allowed for the identification of homologous positions among the sequences, essential for phylogenetic tree construction.

Phylogenetic tree construction

Based on the aligned sequences, a phylogenetic tree was constructed using IQ-TREE version 2.3.5 software (http://www.iqtree.org). The following command parameters were applied: iqtree2 -s aligned to .aln -m MFP -bb 1000 -alrt 1000 -nt AUTO, where: -s alignment, .aln - aligned sequence file; -m MFP - automatic selection of the best-fit evolutionary model using ModelFinder Plus; -bb 1000 - ultrafast bootstrap with 1000 replicates for node support evaluation; -alrt 1000 - SH-aLRT test with 1000 replicates for additional support values; -nt AUTO - automatic detection and use of available CPU cores.

Table 1. Species names, accession numbers, host organisms and countries of origin of Echinostomatidae family members were obtained from the GenBank database

Species name	Accession numbers	Hosts	Countries
Echinostoma revolutum ((Fröhlich, 1802) Looss, 1899)	OR600232	*Pelophylax* sp.	Uzbekistan
Echinostoma revolutum ((Fröhlich, 1802) Looss, 1899)	MZ409811	*Radix auricularia* (Linnaeus, 1758)	USA
Echinostoma bolschewense ((Kotova, 1939) Nasincova, 1991)	KP065591	*Viviparus acerosus* (Bourguignat, 1862)	Slovakia
Echinostoma miyagawai (Ishii, 1932)	MH748722	Duck	China
Echinostoma miyagawai (Ishii, 1932)	KY436408	*Anas platyrhynchos* (Linnaeus, 1758)	New Zealand
Echinostoma miyagawai (Ishii, 1932)	KT956916	*Anas platyrhynchos* (Linnaeus, 1758)	Ukraine
Echinostoma miyagawai (Ishii, 1932)	OR509027	*Anas platyrhynchos* f. *domesticus*	Thailand
Echinostoma sp.	MZ409812	*Ampullaceana balthica* (Linnaeus, 1758)	Ireland
Echinostoma nasincovae (Faltýnková, Georgieva, Soldánová & Kostadinova, 2015)	MZ409809	*Planorbarius corneus* (Linnaeus, 1758)	Ireland
Echinostoma nasincovae (Faltýnková, Georgieva, Soldánová & Kostadinova, 2015)	OP709706	*Planorbarius corneus* (Linnaeus, 1758)	Russia
Echinostoma paraensei (Lie & Basch, 1967)	EU025867	Hamster	USA
Echinostoma caproni (Richard, 1964)	MK482490	*Biomphalaria sudanica* (E.von Martens, 1870)	Kenya
Echinostoma novaezealandense (Georgieva, Blasco-Costa & Kostadinova, 2017)	KY436407	*Cygnus atratus* (Latham, 1790)	New Zealand
Echinostoma cinetorchis (Ando & Ozaki, 1923)	KX817348	-	South Korea
Echinostoma paraulum (Dietz, 1909)	KP065604	*Lymnaea stagnalis* (Linnaeus, 1758)	Germany
Echinostoma maldonadoi (Valadão, Alves & Pinto, 2023)	OQ132569	*Meriones unguiculatus* (Exp.)	Brazil
Echinoparyphium recurvatum ((von Linstow, 1873) Lühe, 1909)	OM956186	*Indoplanorbis exustus* (Deshayes, 1833)	Vietnam
Echinostoma aconiatum	MZ409801	*Lymnaea stagnalis* (Linnaeus, 1758)	Ireland
Moliniella anceps ((Molin, 1859) Hübner, 1939)	MZ409815	*Stagnicola fuscus* (C.Pfeiffer, 1821)	Ireland
Patagifer vioscai (Lumsden, 1962)	KT956946	*Eudocimus albus* (Linnaeus, 1758)	USA
Patagifer bilobus ((Rudolphi, 1819) Dietz, 1909)	OR532446	*Little egret*	Vietnam

Tree visualization

The resulting phylogenetic tree in .treefile format generated by IQ-TREE was uploaded to the Interactive Tree of Life (iTOL) online platform (https://itol.embl.de). Using the iTOL interface, tree branches were colored, species names labeled, the outgroup specified and graphic elements added. The final phylogenetic tree was exported in high-resolution SVG and PNG formats.

Genetic distance analysis

Pairwise genetic distances based on the p-distance model were calculated in IQ-TREE (V2.3.5) for 21 analyzed sequences of the genus *Echinostoma*, including the studied sample *Echinostoma_revolutum_UZ*. These values were used to assess overall genetic variability and levels of phylogenetic relatedness.

The taxonomic positioning of *E. revolutum* was established according to the Global Biodiversity Information Facility (https://www.gbif.org/). Statistical analysis of the results was performed using Biostat 2007 software and Microsoft Office Excel 2003.

The ITS sequences of the collected samples were compared using the Multiple Alignment online tool from the Kyoto University Bioinformatics Center [https: //www.genome.jp/]. Phylogenetic tree construction was performed using the IQ-TREE v1.6.12 (Trifinopoulos et al. 2016) software based on Maximum Likelihood (ML) algorithms with 1000 bootstrap replicates. The command line used was: path_to_iqtree -s *E._rovolutum*.aln-st DNA-m TEST-bb 1000-alrt 1000-*Echinoparyphium_recurvatum* _OM956186. The phylogenetic tree was interpreted using the iTOL web tool.

Representatives of the genera *Patagifer* and *Moliniella* were considered as outgroups in the phylogram. The taxonomic positioning of *E. revolutum* was established according to the Global Biodiversity Information Facility (https://www.gbif.org/). Statistical analysis of the results was performed using Biostat 2007 software and Microsoft Office Excel 2003.

Ethical statement

The field studies conducted in the inland waters of Uzbekistan complied with the current environmental legislation of Uzbekistan, specifically the Law of the Republic of Uzbekistan "On Protection and Usage of Wildlife" (No. 545-I dated 26.12.1997; https://lex.uz/docs/-31719). The research also adhered to the ethical guidelines outlined in the "Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research" (2004) (Section 6), published by the American Society of Ichthyologists and Herpetologists (ASIH) (https://asih.org/ animal-care-guidelines/), ensuring the ethical treatment of the studied animals.

RESULTS AND DISCUSSION

Morphological characteristics

In the present study, metacercariae of the trematode *E. revolutum* were detected for the first time in *Pelophylax terentievi* and *Pelophylax* sp. in Central and Eastern Uzbekistan. These amphibian species have been identified as new secondary intermediate hosts for this parasite.

The Prevalence of Infection (PI) in *P. terentievi* was 13.0% (n = 3), the Intensity of Infection (II) ranged from 2 to 14 Mcc, with an abundance index of 1.1 Mcc. In *Pelophylax* sp., PI was 14.2% (n = 5), II ranged from 3 to 21 Mcc and the abundance index was 1.2 Mcc. A total of 72 helminths were collected.

Taxonomic position of the parasite

Class: Trematoda

Order: Plagiorchiida La Rue, 1957

Family: Echinostomatidae Looss, 1899

Genus: *Echinostoma* Rudolphi, 1809

Species: *Echinostoma revolutum* Looss, 1899

Synonyms: *E. audyi*, *E. paraulum*, *E. ivaniosi*

First Intermediate Hosts: Mollusks - *Lymnaea stagnalis*, *Radix auricularia*, *Radix balthica*, *Radix peregra*, *Stagnicola palustris* (Faltýnková et al. 2015; Pantoja et al. 2021); *Filopaludina martensi* subsp. *martensi*, *F. doliaris*, *F. sumatrensis* subsp. *polygramma*, *Bithynia siamensis* subsp. *siamensis*, *B. pulchella*, *Anentome helena* (Butboonchoo et al. 2020).

Second Intermediate Hosts: Pulmonate and prosobranch snails, mussels, frog tadpoles, freshwater turtles and fish (Tkach et al. 2016).

Definitive hosts: Waterfowl - *Phalacrocorax carbo*, *Microcarbo pygmaeus*, *Nycticorax nycticorax*, *Tachybaptus ruficollis*, *Podiceps nigricollis*, *Ardea cinerea*, *Ciconia ciconia*, *Ciconia nigra*, *Mergus serrator*, *Mergus merganser*, *Larus argentatus*, *Larus canus*, *Larus fuscus heuglini*, *Sterna hirundo*, domestic duck and domestic goose. The fluke can also parasitize mammals (forest mouse). In Uzbekistan, mature individuals were found in the intestines of *N. nycticorax*, *T. ruficollis* and domestic chickens in various areas of Tashkent, Samarkand, Surkhandarya, Bukhara regions and the Republic of Karakalpakstan (Faltýnková et al. 2015; Toledo et al. 2019).

Localization: Sexually mature parasites were localized in the intestine, metacercariums were mainly localized in the kidneys and ocular cavities of amphibians and in rare cases, in the body cavities (2 cases).

Cysts of metacercariae of Echinostomes were found in the eye cavities and twice (2 specimens) in the body cavity of the amphibians *Pelophylax terentievi* and *Pelophylax* sp. The cyst wall has two layers: an outer and an inner layer. The inner cyst contained a larva with collar spines and excretory granules. The cysts were ellipsoidal in shape and all cysts contained highly motile larvae. The larvae were elongated and curved ventrally. Their size ranged from 0.167 to 0.194 mm (n = 72). The head collar of the metacercariae is indistinct, with 37 collar spines arranged in two alternating rows, including 5 corner spines on each side (5+6+15+6+5). The body was covered with small triangular spines and there were noticeable differences in spine sizes. The spines are comparatively longer in the anterior part of the body and become inconspicuous towards the posterior end. The oral sucker measures 0.036-0.047 mm and is located subterminally. The ventral sucker measures 0.038-0.054 mm; it is not very prominent, round,

moderately large and located in the middle of the body. The pre-pharynx is very short, the pharynx size is 0.012-0.021 mm and it is well-developed. The esophagus is somewhat elongated (Figure 1).

Comparative morphology

We compared the primary morphological characteristics of *E. revolutum* metacercariae with those of the other 2 species, *E. chankensis* and *E. cinetorchis* (Izrailskaia et al. 2021) from this genus (Table 2). As shown in Table 2, the cyst diameter of *E. revolutum* was larger than that of the other species included in the comparison. These parameters can also be used for the visual identification of the trematode.

Molecular analysis

Identifying the metacercarial stage of a parasite is challenging because it is in the larval stage and does not yet exhibit the fully developed morphoanatomical structure of the parasite. Recently, molecular genetics has been increasingly used to distinguish between morphologically similar parasites. Ribosomal DNA sequencing methods potentially allow for the identification of species and population-specific characteristics of the parasite.

After morphological identification, we conducted molecular studies on trematodes of *E. revolutum* by analyzing the nucleotide sequences of rDNA. Specifically, we obtained 1250 bp sequences of ITS1+5.8S+ITS2. Upon comparison of nucleotide sequences in the GenBank database (NCBI), metacercariae of *E. revolutum* were found to be 100% identical to *E. revolutum* (MZ409811). The differences between *E. revolutum* and other species are as follows: 10 bp with *E. miyagawai* (MH748722), 12 bp with *E. nasincovae* (MZ409809), 11 bp with *E. paraensei* (EU025867), 13 bp with *E. caproni* (MK482490) and *E. novaezealandense* (KY436407), 10 bp with *E. cinetorchis* (KX817348) and 42 bp with *Echinoparyphium recurvatum* (OM956186). Significant genetic variation was observed among species (Figure 4).

Table 2. Comparative morphometric indicators of the genus *Echinostoma* (in mm)

Organs	*Echinostoma revolutum* (metacercaria) our data	*Echinostoma chankensis* nom. nov. (metacercaria) Izrailskaia et al. (2021)	*Echinostoma cinetorchis* (metacercaria) Izrailskaia et al. (2021)
The diameter of the cyst	0.18 × 0.24	0.142 × 0.160	0.120 × 0.160
Oral sucker	0.036-0.047	0.039-0.058 × 0.054-0.062	0.039-0.042 × 0.042-0.056
Ventral sucker	0.038-0.054	0.050-0.062 × 0.058-0.062	0.050-0.057 × 0.065-0.075
Prepharynx	very short	-	-
Pharynx	0.012-0.021	0.031-0.035 × 0.021-0.025	0.022-0.039 × 0.017-0.025
Excretory bladder	V-shape	V-shape	V-shape

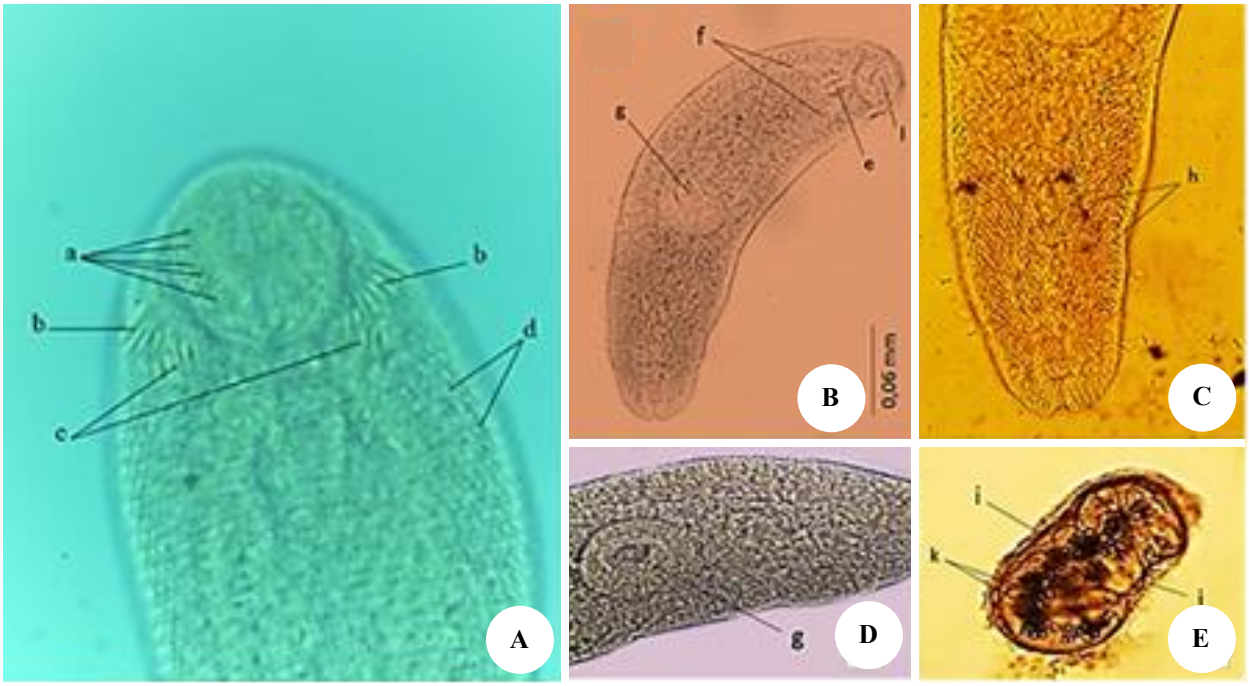

Figure 1. Morphological features of *Echinostoma revolutum* metacercariae under light microscopy. A. Anterior part of the metacercaria: a. Opening of the duct of paraesophageal glandular cells, b. Lateral spines, c. Angle spines, d. Triangular spines. B. General view of the excysted metacercaria: e. Pharynx, f. Intestine, g. Ventral sucker, C. Posterior and of the body: h. Triangular spines, D. Region containing the ventral sucker, E. Entire metacercarial form: k. Excretory granules, i. Etacercarial cyst, j. Metacercaria

Echinostoma revolutum UZ (*E. revolutum* UZ) isolate demonstrated a clear phylogenetic position within the *E. revolutum* complex based on pairwise genetic distance analysis. The closest relative was identified as *E. revolutum* (accession no. MZ409811), with a genetic distance of 0.0768 (7.68% nucleotide divergence). This moderate level of divergence suggests that both isolates may represent conspecific populations from different geographical regions. The average genetic distance between the *E. revolutum* UZ isolate and other representatives of the genus *Echinostoma* was approximately 0.135, indicating a moderate degree of differentiation from other congeners. The most divergent relationship was observed with the outgroup species *E. recurvatum* (accession no. OM956186), which exhibited a genetic distance of 0.3184 (31.84% nucleotide divergence). Despite being part of a closely related family, this significant level of divergence confirms the evolutionary gap between the genera *Echinostoma* and *Echinoparyphium*. Summary statistics across all analyzed sequences revealed a minimum genetic distance of 0.000001, suggesting nearly identical sequences within a single species and a maximum genetic distance of 0.3222, corresponding to intergeneric divergence. The overall average genetic distance among all taxa was calculated as 0.0938 (9.38% variation). These findings are further supported by the genetic distance heatmap (Figure 2), which clearly illustrates the close clustering of the *E. revolutum* UZ isolate with members of the *E. revolutum* complex and its distinct separation from other species groups. The observed genetic patterns are fully consistent with the phylogenetic tree topology and reinforce the taxonomic assignment of the *E. revolutum* UZ isolate within the *E. revolurum* lineage.

Dendrogram (UPGMA) analysis

Genetic distances calculated using the p-distance model were subjected to hierarchical clustering using the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method. The resulting dendrogram (Figure 3) clearly illustrates the phylogenetic relationships among the analyzed *Echinostoma* spp. isolates. The *E. revolutum* UZ isolate clustered primarily with the *E. revolutum* (accession no. MZ409811) isolate, indicating their close genetic relationship. This pair subsequently formed a broader clade with representatives of the *E. nasincovae* complex. The outgroup species, *E. recurvatum*, was positioned at the most distant branch of the dendrogram, reflecting a significant evolutionary divergence from all analyzed *Echinostoma* spp. isolate. This finding confirms its phylogenetic placement in a different genus within the same family.

Neighbor-Joining Tree analysis

To further assess the phylogenetic relationships, a Neighbor-Joining (NJ) tree was constructed based on the p-distance model (Figure 3). The overall branching pattern of the NJ tree supported the clustering topology observed in the UPGMA dendrogram. The *E. revolutum* UZ isolate was positioned in the closest branch with *E. revolutum* (acc. no. MZ409811), also showing a relatively short genetic distance to the *E. nasincovae* group. The outgroup species, *E. recurvatum*, was placed in a distinctly separate branch, indicating the greatest evolutionary divergence among the analyzed taxa.

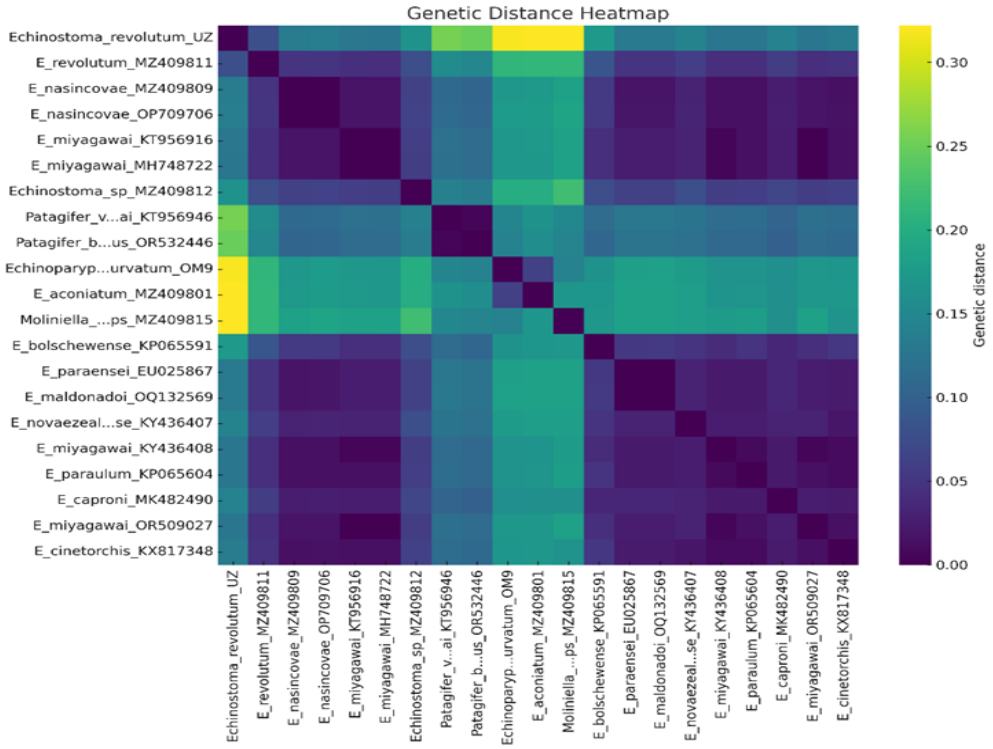

Figure 2. Heatmap of genetic distances (p-distance) among the analyzed *Echinostoma* species

Figure 3. UPGMA dendrogram and Neighbor-Joining tree based on p-distance model showing phylogenetic relationships among *Echinostoma* spp. isolates. The studied isolate (*Echinostoma revolutum* UZ) is marked in red; *Echinoparyphium recurvatum* is used as the outgroup

Various regions of DNA have been used to differentiate among 37 species with collar spines of the genus *Echinostoma* and other Echinostomatid species (Chai et al. 2020). In particular, the second Internal Transcribed Spacer region (ITS2) has been utilized for the identification of both larval and adult stages of echinostomes (Tkach et al. 2016).

Phylogenetic analysis of the examined samples showed clustering into 13 monophyletic groups (Figure 5). The sample under our investigation, *E. revolutum* (UZ), was situated in a monophyletic group along with a sample retrieved from the NCBI database (*E. revolutum* MZ409811), sharing a common ancestor (Figure 3, Clade K). This data provides molecular evidence to support the identification of *E. revolutum* species in our study. Additionally, the analysis indicates that *Echinostoma* sp. MZ409812 is a sister group to *E. revolutum* (Clade J). *E. miyagawai* (Clade I), *E. nasincovae* (Clade L), *E. paraensei* and *E. maldonadoi* (Clade M) clustered as the nearest groups to *E. revolutum*.

Discussion

Additional information on metacercariae morphology was obtained through a study of previously published data. Our analysis of morphological data revealed that the number of spines reported from various sources varied from 14 to 45. The variability in collar spines can be explained by the fact that these spines easily detach from the body and are often loosely attached, leading to inconsistent spine counts in both adult and juvenile specimens. The spines on the body of mcc are very small and relatively sparse towards the posterior part of the body (Pantoja et al. 2021). Our research confirms that the number of collar spines was 37 in all mcc examined. However, according to the literature, there are discrepancies within the "revolutum" group, which is characterized by having 37 collar spines (Faltýnková et al. 2015). The study showed significant variation in the sizes and shapes of the metacercarial cysts of this trematode species. In Bulgaria, it is 0.132-0.152 mm (Kanev 1994); in Iran, 0.150-0.170 mm (Aryaeipour et al. 2023). Cysts can be elliptical or subspherical (Kanev 1994; Butboonchoo et al. 2020) and spherical (Aryaeipour et al. 2023). Our research found that the cysts had a transparent elliptical or ellipsoid membrane, with a diameter of 0.18 × 0.24 mm. Occasionally, cysts were recorded with a diameter (0.240 mm) surpassing their longitudinal axis (0.194 mm), suggesting a dorsoventrally compressed or transversely expanded form. We observed that the shape of the cyst depends on the size of the metacercariae, as the longer larvae were found in the more ellipsoid cysts.

The size and shape of cysts in other species of the genus *Echinostoma* do not show significant differences. For instance, *E. macrorchis* measures 0.108-0.110 mm and is round (*Cipangopaludina chinensis* subsp. *malleata*) (Sohn and Na 2017); *E. maldonadoi* n. sp. measures 0.135 mm and is spherical (*Stenophysa marmorata*) (Valadão et al. 2023); *E. jurini* measures 0.140-0.160 mm and is spherical or subspherical (freshwater snails, mussels, frogs, turtles) (Kanev et al. 1995); and *E. caproni* has a diameter of 0.155 mm and is spherical (*Biomphalaria pfeifferi* (in laboratory settings) (Ataev 2023).

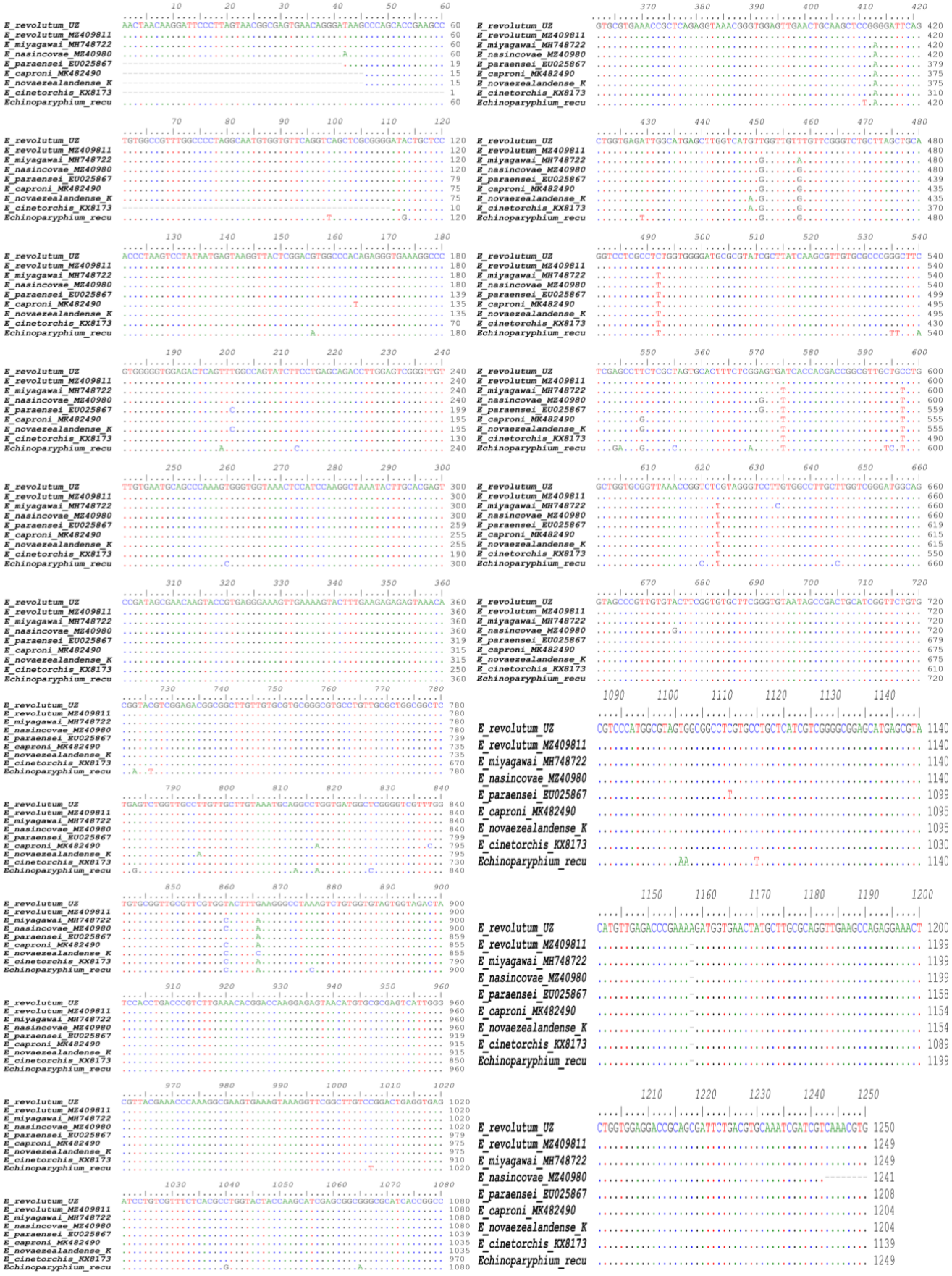

Figure 4. Multiple sequence alignment of the ITS1+5.8S+ITS2 region of *E. revolutum* and related species using ClustalW in MEGA X (Kumar et al. 2018). Accession numbers and taxa are indicated

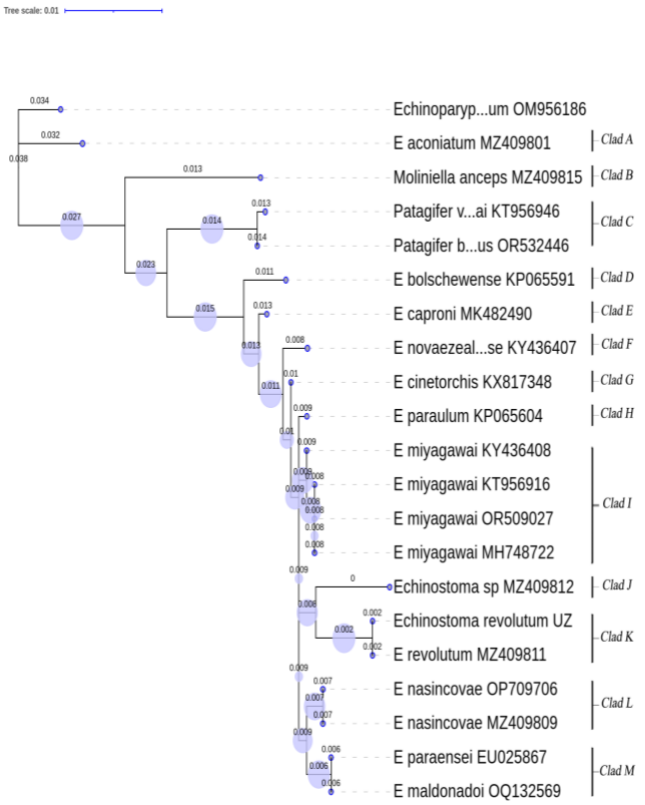

Figure 5. Maximum Likelihood (ML) trees constructed based on partial ITS1+5.8S+ITS2 sequences, illustrating the phylogenetic relationships among members of the genera *Echinostoma*, *Patagifer*, *Moliniella* and *Echinoparyphium*

The mcc of *E. revolutum* shows several distinguishing morphometric characteristics when compared with those of other *Echinostoma* species studied by Izrailskaia et al. (2021). For example, the spines located on the anterior part of the body of *E. revolutum* are longer than those of *E. chankensis*, in which the spines are uniform in length. The cyst wall of *E. revolutum* is two-layered, whereas in *E. chankensis* and *E. cinetorchis*, it is multilayered and relatively thicker. The oral sucker of *E. revolutum* is smaller in size, measuring 0.036-0.047 mm, compared to that of *E. chankensis* (0.039-0.058 × 0.054-0.062 mm) and *E. cinetorchis* (0.039-0.042 × 0.042-0.056 mm). Similarly, the ventral sucker is also smaller - 0.038-0.054 mm, compared to that of *E. chankensis* (0.050-0.062 × 0.058-0.062 mm) and *E. cinetorchis* 0.050-0.057 × 0.065-0.075 mm). Although the dimensions of the oral and ventral suckers of *E. revolutum* are close to those of *E. miyagawai* (oral sucker: 0.030-0.038 × 0.038-0.042 mm; ventral sucker: 0.039-0.042 × 0.042-0.054 mm), *E. miyagawai* differs by lacking tegumental spines on its body (Georgieva et al. 2017).

Literature analyses have revealed that the trematode *E. revolutum* in its larval stage localizes in various organs, including the pericardium, kidneys and muscles of the host. It has been found in the ocular cavities of frogs-*Rana temporaria* and *Rana* (*Pelophylax*) *ridibunda*. Additionally, they have been identified in freshwater turtles *Emys orbicularis*, as well as in the pericardial cavity of the snails *Physa acuta* and *Planorbarius corneus* in Bulgaria (Kanev 1994) and in the pericardial cavity of the mollusk *F. sumatrensis polygramma* found in Thailand (Butboonchoo et al. 2020).

In experimental infections of tadpoles delay in the encystment process of echinostomes was observed after 13th days of life. This phenomenon is attributed to the influence of the immune system on the more mature tadpoles. However, in this study, we noted encystment in adult frogs, indicating that their immune system is insufficiently effective against echinostomes. Tadpoles infected with Echinostomes may develop generalized body edema, indicating potential renal disease or insufficiency. However, in adult specimens, significant pathological changes were not prominent in internal organs, eyes or periorbital regions.

To date, the molecular-genetic characteristics of representatives of the genus *Echinostoma* have been well studied (Saijuntha et al. 2014). Among these studies, the molecular-genetic analysis of *E. revolutum* has been conducted. In this study, the nucleotide composition of the ITS1-5.8S-ITS2 of ribosomal DNA from a specimen collected in Germany was analyzed. Significant differences in nucleotide sequences were identified when compared to the species *E. trivolvis*, *E. paraensei* and *E. caproni*.

The overall topological structures of the UPGMA dendrogram and the Neighbor-Joining (NJ) tree were highly congruent. In both phylogenetic reconstructions, the *E. revolutum* UZ isolate clustered as the closest relative to *E. revolutum* (acc.no. MZ409811) and showed a relatively short genetic distance from the *E. nasincovae* group. In both phylogenetic models, the designated outgroup *E. recurvatum* was placed on a distinct branch, clearly separated from all analyzed *Echinostoma* spp. isolates. This result confirms the appropriateness of the chosen outgroup and supports the consistent applicability of genetic distances calculated using the p-distance model in phylogenetic inference. The congruence between the UPGMA and NJ methods enhances the reliability of the phylogenetic outcomes. It provides additional confirmation that the *E. revolutum* UZ isolate is nested within the *E. revolutum* complex.

There are no genetic barriers to interbreeding for the trematode *E. revolutum* on the Eurasian continent. This has led to the identification of phylogenetic relationships between individuals distributed in Europe and Asia. In this process, aquatic birds serve as vectors (Enabulele et al. 2023).

Studies conducted in Uzbekistan have shown that a number of vertebrate and invertebrate animals are involved in the biological life cycle of *E. revolutum*. In particular, this species has been recorded in vertebrate hosts from the northeastern regions of Uzbekistan (Shakarboev et al. 2024) and in domestic birds such as chickens (*Gallus gallus domesticus*), pheasants (*Phasianus colchicus*) and guinea fowls (*Numida meleagris*) in the central part of the country (Akramova et al. 2021, 2024). In addition, several freshwater mollusks have been identified as first intermediate hosts in the northwestern and northeastern parts of Uzbekistan, including the Republic of Karakalpakstan

and the Fergana Valley. These mollusks include *Lymnaea auricularia*, *L. corvus*, *L. stagnalis*, *Planorbis planorbis*, *Anisus septemgyratus*, *A. converiusculus* and *A. spirorbis* (Shakarbaev et al. 2020, 2021). Thus, the wetlands in the central and eastern regions of Uzbekistan may contribute to the wide distribution of the parasite among wild and domestic waterfowl. The high infection rates (with an infestation rate reaching up to 14%) of amphibians *Pelophylax terentievi* and *Pelophylax* sp. by the parasites indicate a serious biological threat, necessitating intensified monitoring and the development of protective measures for waterfowl in the region.

According to an analysis of scientific literature, the prevalence of the trematode *E. revolutum* exceeds 10% among amphibians (*Pelophylax* spp.), aquatic birds (both resident and migratory) and mollusks (*Lymnaea*, *Planorbis*, *Anisus* and others) inhabiting water bodies in the eastern (Fergana Valley) and central (Navoiy, Bukhara, Samarkand and Jizzakh region) parts of Uzbekistan, where this study was conducted. In some areas, this species of trematode is reported as dominant. Such epizootological conditions can significantly increase the risk of transmission to humans. Given the zoonotic potential of *E. revolutum*, it is crucial to conduct further research on water systems located near ecotourism zones and to implement appropriate parasitological monitoring and sanitation measures aimed at reducing public health risks.

Thus, the wetlands in the central and eastern (Fergana Valley) regions of Uzbekistan may influence the widespread distribution of the parasite among wild and domestic waterfowl. The high infection rates (with an infestation extent of up to 14%) of amphibians *Pelophylax terentievi* and *Pelophylax* sp. by the parasites indicate a serious biological threat, necessitating intensified monitoring and the development of protective measures for waterfowl in the region.

In conclusion, this study revealed that Amphibians *Pelophylax terentievi* and *Pelophylax* sp. were identified for the first time as new second intermediate hosts of the trematode *E. revolutum* in the Central and Eastern parts of Uzbekistan. The discovered trematode *E. revolutum* is morphologically distinct from other ecomorphs in terms of body size, cyst diameter and localization in the host. Molecular data obtained on the genus *Echinostoma* and the species *E. revolutum* in the ITS1+5.8S+ITS2 regions of ribosomal DNA demonstrated their clear clustering in the phylogenetic tree. This finding confirms both the species identity and the genetic diversity within the genus *Echinostoma* as well as the specific species *E. revolutum*. The study demonstrated that wetlands in the Central and Eastern regions of Uzbekistan contribute to the widespread distribution of this parasite among wild and domestic waterfowl. The high infection rate of amphibians (up to 14%) indicates a serious biological threat, necessitating enhanced monitoring and the development of protective measures for waterfowl in the region.

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