Research Article

Morphological and Molecular Identification of Land Molluscs as Intermediate Hosts of Lungworms

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Abstract | Protostrongylid nematodes are important lungworms of ruminants, especially Bovids. Its life cycle is participating terrestrial molluscs as intermediate hosts. In this study, eight species of land molluscs from Uzbekistan were found to be positive larvae of protostrongylids. Based on the nucleotide sequence data, three species of nematode larvae *Protostrongylus rufescens*, *Muellerius capillaris*, and *Cystocaulus ocreatus* were identified. According to the morphological characteristics of the studied land molluscs, the shell structure was identified as six different morphotypes. After molecular genetics and morphological analyzes, these land molluscs belonged to 8 different species: *Angiomphallia regeliana, Pseudonapaeus albiplicatus, P.maydanica, P. sogdiana, Psedonopaeus sp., Xeropicta candacharica, Deroceras reticulatum* and *Candaharia levanderi.* The infection rate of Pulmonata snails by protostrongilids larvae was 28.2% and that of slugs - 6.8%. In addition, the first informs of *P. maydanica, C. levanderi* and *D. reticulatum* as natural intermediate hosts for *M. capillaris* are given.

Keywords | Lungworms, Protostrongylids, Land molluscs, Morphological, Molecular identification, Prevalence

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INTRODUCTION

Lungworms are prevalent in the world and cause significant disease in wildlife and commercial animals. The losses inflicted on agriculture are important. Lungworms are respiratory parasites of ruminants, including wild and domestic sheep, goats, and antelopes that inhabit mountain ecosystems and adjacent areas. Lungworms and other nematodes cause helminthiasis in wild and domestic ruminants, but their hosts and geographical distribution, ecology, biology, phylogenetic relationships, and integration into successful animal development we still have a limited understanding of all the critical data we have to do strategies for control (Kutz et al., 2007).

The Protostrongylidae (Nematoda: Metastrongyloidea) nematode family is widely distributed in Uzbekistan, has a complex life cycle, and land molluscs of the genera *Pseudonapaeus, Vallonia, Pupilla, Xeropicta* and *Deroceras* are essential for their development and transmission. These land molluscs serve as intermediate hosts for the development of second- and infectious third-stage larval helminthes (Kuchboev et al., 2017; 2020). The life cycle is then completed when the infectious third stage larvae are ingested by the final bovid host. Egg development and first-stage larval hatching occur within the final host. Larvae released into the abiotic environment actively penetrate the feet of land molluscs. Within gastropods, larvae undergo two molts to finally become larvae of the infectious stage, which under favorable conditions leave land mollusks during vigorous

movement and disperse on grass. The definitive host becomes infected when it swallows infected land mollusks or infected stage larvae along with grass during grazing (Kulmamatov et al., 1994).

It is generally not possible to identify first-stage larvae in both faeces and the environment, and second- and third-stage larvae in intermediate hosts (Kuchboev et al., 2015). Species identification using morphological characteristics is possible in adult males (Boev, 1975). Improved detection capabilities will greatly contribute to animal epidemiological studies, providing a means to identify the geographic distribution of infected animals and parasites of different species without directly collecting definitive hosts. Differential diagnosis using molecular markers from nuclear and mitochondrial loci is also an important contribution from new field collections of both adult parasites and larvae. Correlations of molecular sequences between adult and larval parasites provide the first means of accurately identifying first-stage larvae of Protostrongyrus and other protostrongilide species.

Therefore, molecular approaches should be used to identify larvae of the family Protostrongylidae at I, II, and III stages. Recently widely used molecular primers for the study of helminth of ruminants it is internal transcribed spacers (ITS1 and 2) and the D2 (28S) domain of ribosomal DNA (Gasser et al., 1993; Chilton et al., 2006; Lesage et al., 2015; Kuchboev et al., 2015).

Land mollusks have interspecific and intraspecific morphological polymorphism (Dieterich et al., 2013; Scheil et al., 2013; Troschinski et al., 2014). For the study of terrestrial mollusks are also used ITS, 18S rDNA and COI, 16S mtDNA, to identify mollusks at the species level.

The purpose of the study is to study the morphological and molecular identification of land molluscs as intermediate hosts and their protostrongylids larvae, parasites of bovids Uzbekistan.

MATERIALS AND METHODS

Land molluscs (Pulmonata, Stylommatophora) are intermediate hosts of protostrongylid nematodes and were found in open talus fields, shrubs and grasses from May to October 2017 and 2021, in the hilly and mountainous regions of Namangan, Tashkent, Jizzakh and Surkhandarya regions of Uzbekistan (Fig. 1-2) (Table 1). Material collection sites were selected with densities of terrestrial mollusks and ruminants according to previously obtained results (Kuchboev et al., 2017). The visit was carried out in the early morning, from 6:00 to 8:00 am, when gastropods are active and easy to observe. Surveys were also conducted on or after rainy days.

Figure 1: Survey sites divided into four regions (Surkhandarya, Jizzakh, Tashkent, and Namangan) in the foothill and mountain areas of Uzbekistan.

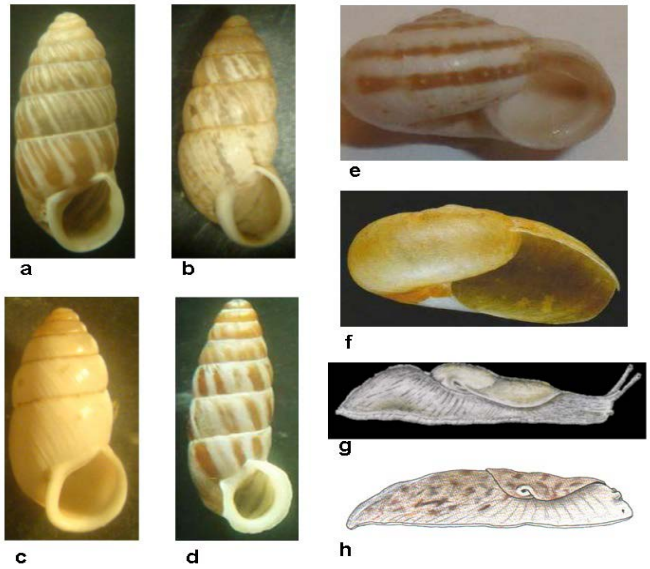

Figure 2: Shells: a - *Pseudonapaeus maydanica;* b - *P. albiplicatus*; c - *Pseudonapaeus sogdiana*; d - *Pseudonapaeus sp.*; e – *Xeropicta candacharica*; f - *Angiomphallia regeliana*; Appearance: g - *Candaharia levanderi*; h - *Deroceras reticulatum.*

Collected terrestrial mollusks were examined for the presence of lungworm larvae. Terrestrial mollusks were kept in a matchbox in the field and in a refrigerator (4 °C) under laboratory conditions. More than 2000 individuals were studied to determine the infections of land molluscs (Azimov et al., 1971), as well as using compressor methods (Boev, 1975).

Table 1: Samples of land molluscs of Uzbekistan for morphological and molecular analysis

№	Location (Region, area)	Morphotypes of molluscs	Morphological and molecular identification	GenBank accession No.	
				18S	ITS1
1	Namangan, Kosonsoy	Morphotype A White snails	*Xeropicta candacharica*	MF398539	MF398492
2	Surkhandarya, Xatak	Morphotype B Biggest oblong snails	*Pseudonapaeus maydanica*	MF398535	MF398491
3	Jizzakh, Nurota		*Pseudonapaeus sogdianus*	MF398533	MF398495
4	Tashkent, Xumson	Morphotype C Small oblong snails	*Pseudonapaeus albiplicatus*	MF351706	MF398497
5	Namangan, Xazratishox		*Pseudonapaeus sp.*	MF398532	MF398538
			Pseudonapaeus albiplicatus	KU760758	MF398497
6	Namangan, Kosonsoy	Morphotype D Brown snails	*Angiomphalia regeliana*	MF351724	MF351722
7	Surkhandarya, Xatak	Morphotype E Black slugs	*Deroceras reticulatum*	MF351707	MF398494
		Morphotype F Brown slugs	*Candaharia levanderi*	MF398531	MF398534

Table 2: Samples of infective larvae from land molluscs Uzbekistan for molecular analysis

№	Location (Region, area)	Molluscs species	Larvae studied	Parasite species	GenBank accession (rDNA 28S)
1	Namangan, Kosonsoy	*Xeropicta candacharica*	5	*Protostrongylus rufescens*	MF398496
2	Surkhandarya, Xatak	*Pseudonapaeus maydanica*	3	*Muellerius capillaris*	MF398493
3	Jizzakh, Nurota	*Pseudonapaeus sogdianus*	3	*Protostrongylus rufescens*	MF351860
4	Tashkent, Xumson	*Pseudonapaeus albiplicatus*	3	*Protostrongylus rufescens*	MF398536
5	Namangan, Xazratishox	*Pseudonapaeus sp.*	2	*Muellerius capillaris*	MF398498
		Pseudonapaeus albiplicatus	5	*Cystocaulus ocreatus*	MF398493
6	Namangan, Kosonsoy	*Angiomphalia regeliana*	3	*Muellerius capillaris*	MF398537
7	Surkhandarya, Xatak	*Deroceras reticulatum*	3	*Muellerius capillaris*	MF405156
		Candaharia levanderi	3	*Muellerius capillaris*	MF399038

Infection was confirmed macroscopically, as indicated by the formation of melanistic spots (from brown to black) 2-3 mm in diameter in the dense tissues of the foot of the host molluscs (Kuchboev et Hoberg, 2011). Larvae of the third invasive stage were preserved in 70% ethanol for molecular analysis (Table 2). Larvae were counted to establish the prevalence and intensity of infection in molluscs. Helminthological and molecular genetic analysis was carried out by the laboratory of the Molecular Zoology of the Institute of Zoology, Academy of Sciences of the Republic of Uzbekistan.

DNA for larvae and tissue of molluscs was extracted using a DNA Purification kit (Qiagen, New Dehli, India) and eluted twice with 100 µl AE buffer provided in the kit. PCR amplification used 0.25 µM of each primer C2 rDNA (5`-GAAAAGAACTTTGRARAGAGA-3`) и D2 (5`-TCCGTGTTTCAAGACGGG-3`) (Lesage et al., 2014). Polymerase chain reaction (iCycler iQ Real Time PCR BIORAD, USA) was performed in a volume of 20 µl with 2 µl of DNA and 1 µl of each primer with GoTaq Green Master Mix (Promega Corp., USA). PCR products were detected on a 1.5% agarose gel and purified using the GenEluteTM PCR Clean-Up Kit (Sigma-Aldrich). PCR products were purified with DNA Clean & Concentrator TM–5 columns (Zymo Research) and Sanger sequencing from both ends was performed by Syntol JSC (Moscow, Russia) and Eurofins Sequencing (Japan). The resulting chromatograms (ab1 format) were analyzed using the Chromas 2.6.6 program (Technelisium Ltd.) and translated into FASTA format. The resulting nucleotide sequences were analyzed using the BLAST algorithm, which made it possible to determine the range of species of larval nematodes and their terrestrial molluscs closest to the studied forms. The alignment was built using the Clustal X program (Thomson et al., 1994). Larval samples of Protostrongylidae were deposited in GenBank (NCBI): *Protostrongylus rufescens* (KF811493 to KF811499), *Muellerius capillaris* (AY292798), and *Cystocaulus ocreatus* (EU595593).

The collected land molluscs were identified by morpho-

logical features (Pazilov and Azimov, 2004). Molluscs infected with nematode larvae were also counted for prevalence of infection.

The primers and PCR conditions to obtain the small subunit sequence of the 18S and ITS1 region of molluscs of rDNA was used by described Steinke et al. (2004). The resulting sequences were deposited with GenBank (Table 1). The rectangular matrix was exported to aln format 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 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.

RESULTS

Overall, 2761 terrestrial molluscs were examined from the studied regions of Uzbekistan, including 2452 snails and 319 slugs (Table 3). Using on external morphological features doesn't allow identification of mollusks at the species level, but only at the level of families, and sometimes even at the level of genera. The land molluscs studied by us belong to the class Gastropoda, Pulmonata group, the families of Buliminidae and Hygromiidae.

Of the 2452 Pulmonata snails, 691 (28.2%) were positive for larval parasites. We observed that the total number of third instar larvae per snail ranged from 1 to 39.3 (Table 3). The species name of the molluscs in this table is given after the molecular analysis and pre-morphological analysis of the specialist-malacologist (Figure 2).

So, the initial definition of slugs shows two families Agriolimacidae and Parmacellidae. We registered a total of 22 (6.8%) slugs were infected with protostrongylid larvae. There are from 4.5 to 5.6 larvae of the third stage per slug (Table 3).

Our morphological studies of the larvae of protostrongylids of the third stage were based on the structure of the larvae according to morphological characters. We found that macroscopically infected areas of the leg tissue of the mollusc are presented as dark brown-black dots 1-2 mm in diameter. In our study, microscopy showed that dark shields were detected in Protostrongylinae larvae without dorsal spines and brown in Müllerlinae species with dorsal spines. These data require additional research.

Larvae of nematode were amplified using the D2 gene (28S rDNA) for identification at the species level. The nucleotide sequence of the larvae of the obtained three species of molluscs was 99-100% homologous to the species *P. rufescens*. Sample sequences of three snails and two slug's species were obtained that were positive for *M. capillaris* larvae (97-98%). In one species of snail, a larva of *C. ocreatus* was found, which was deposited in GenBank (NCBI). Identification of infected land molluscs was carried out by morphological features, based the structure and colors of shells. All infected snails and slugs belonged to the families Hygromiidae, Buliminidae, Agriolimacidae and Parmacellidae. These land molluscs are divided into six groups based on their morphotypes. A) White snails, including genera such as *Xeropicta* (509). B) Group including the largest oblong snails (5 and 28) and C) small oblong snails (113 and 1) with morphological characteristics of the genus *Pseudonapaeus*. D) Brown snails (14) with morphotypes corresponding to the genus *Angiomphalia*; E) Black slugs (10) with morphological characteristics of the genus *Deroceras*; F) Positive brown slugs (Table 1, Fig. 2) whose morphological criteria appear to correspond to the genus *Candaharia* (11).

Specimens from three groups (A, B, and C) were found to be infected with three species of protostrongylid larvae, *P. rufescens, M. capillaris*, and *C. ocreatus*. However, group D snails group E, and F slugs contained only one parasite of the *M. capillaris* species (Table 1).

For molecular analysis, for two snails and slugs were used from each group. Comparison of the obtained 18S and ITS-1 sequence data with Blast (GeneBank) and additional morphological identification by malacologists show that all infected snails and slugs belong to the families Hygromiidae, Buliminidae, Parmacellidae, and Agriolimacidae (Fig. 2). These molluscs can be divided into four different clades corresponding to different morphotypes. Group I is composed of the four species of the genera *Pseudonapaeus*: *P. maydanica* (Pazilov et Gaipnazarova, 2015), *P. sogdiana* (Martens, 1874), *Pseudonapaeus* sp., *P. albiplicatus* (Martens, 1874), group II is the species of *Angiomphallia regeliana* (Martens, 1882) and III group is species *X. candacharica* (Pfeiffer, 1846). Group IV is belonging to *Candaharia levanderi* (Simroth, 1901) and *Deroceras reticulatum* (Muller, 1774) (Fig. 3).

We did not find nucleotide sequences in the International Gene Bank (NCBI) for molluscs species studied by us, except for species of the genus *Xeropicta* and *Deroceras*. In the phylogenetic tree, three well-isolated clades of mollusks of the genus *Pseudonapaeus*, *Angiomphalia* and *Candaharia* are noted. These species are considerably varied, and their groups are geographically isolated. The sequence we defined was not previously deposited into the electronic database of GenBank and is new to it. The identification

Table 3: Prevalence of Protostrongylid infective larvae in land molluscs natural condition of Uzbekistan

Family and species of molluscs	Molluscs studied	Infected by Protostrongylidae larvae		
		Infection	%, prevalence	The numbers of 3th stage larvae
Hygromiidae: *Xeropicta candacharica*	1416	509	36.0	39,3 ± 14,1
Angiomphalia regeliana	112	14	12.8	10,2 ± 1,6
Buliminidae: *Pseudonapaeus albiplicatus*	543	113	20.8	24,2 ± 3,1
Pseudonapaeus sogdiana	161	24	15.0	8,2 ± 0,8
Pseudonapaeus maydanica	132	5	3.3	1,4 ± 0,5
Pseudonapaeus sp.	78	2	2,5	1,0 ± 0,1
Agriolimacidae: *Deroceras reticulatum*	223	10	4.5	4,5 ± 1,2
Parmacellidae: *Candaharia levanderi*	96	11	11.5	5,6 ± 1,1
Total:	2761	688	12.3	1-39,3

of morphological and molecular-taxonomic analysis of molluscs made it possible to clarify their species and spatial distribution. The data obtained in the experiment show that these species differ at different morphological and genetic levels.

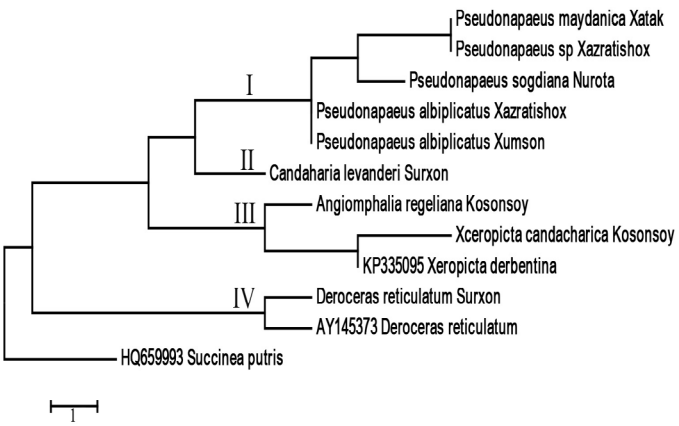

Figure 3: Phylogenetic tree based on a combined dataset of 18S and ITS-1 rDNA sequences of molluscs with a total of 1338 nucleotide sites constructed using the Maximum Likelihood method and the general time reversible model (GTR +1). The tree has been rooted using *Succinea putris* (HQ659993.1). I - Buliminidae, II - Hygromiidae; III - Parmacellidae; IV - Agriolimacidae.

DISCUSSION

Systematic studies of protostrongylid nematodes, which are parasites of ruminants, have been carried out effectively in recent years in Uzbekistan. And, in the above studies, the fauna, ecology, life cycles and other aspects of parasite-host relationships of protostrongylid nematodes in montane ecosystems have been studied and studies are continuing (Kuchboev et al., 2012, 2017).

Despite the fact that, infection with protostrongylid nematodes are frequent in domestic and wild bovids of Uzbekistan (Kuchboev et al., 2017). At present, few researches has been carried out to find the species composition, distribution and infection of mollusc by helminths as intermediate hosts in the natural and agro landscapes of Uzbekistan. In the study, we used methods previously tested on other groups of helminths to determine the host-parasite relationship, and more specifically the nematode-mollusc relationship, parasites of the lung of bovids of Uzbekistan (Korsunenko et al., 2010; Pinto et al., 2014; Kuchboev et al., 2020).

After the study of the role of snails in the development of the nematode *Muellerius capillaris* by Hobmaier and Hobmaier (1929), several studies were carried out on the identification of intermediate hosts and their mode of infection by helminths. In ruminants, transmission of protostrongylids to livestock by parasitic larvae in intermediate hosts causes damage to the pulmonary tract, causing pulmonary protostrongylosis, which causes economic losses (Jenkins et al., 2006; Rogerson et al., 2008). This disease may be related to the declines of the ruminant population (Mozzer et al., 2011).

In Uzbekistan, the most important intermediate host is clearly *X. candacharica*, as it is both the most abundant gastropod (more than 50% of the samples) and the land mollusc with the highest prevalence of protostrongylid larvae. It represents a high risk factor for protostrongyles transmission to small ruminants. The second most important intermediate host is *P. albiplicatus*, which is the second most abundant species (approximately 20% of all samples) and has the second highest prevalence of protostrongylid infection (nearly 20%), was significantly higher than all other molluscs. *Candaharia levanderi* (about 10%).

The three gastropod species with the highest prevalence above and their ranking were the same as those reported by Kuchboev et al. (2012).

Our study was carried out in places where widespread potential intermediate hosts - land molluscs and definitive hosts of bovids. This, we identified eight families of land molluscs, of which 4 families belonging to the families Hygromiidae, Buliminidae, Agriolimacidae, and Parmacellidae were found to be infected with protostrongylid larvae. Other families of land molluscs from among the Gastropoda have given in the literature (Kulmamatov et al., 1994; Kuchboev et al., 2017).

The method (Azimov et al., 1971) we used in this study to identify larvae in land molluscs differs from the commonly used methodology. Most of the literature has used pepsin or gastric juice (Gajadhar et al., 2000; Qvarnstrom et al., 2007) and this has been done for large molluscs. For small samples, two glass plates are recommended. We used the method of pressing the snail between the glass plates (compressor).

In our work, the total prevalence of molluscs by protostrognylids larvae is high (28.2%), but similar to previous studies of investigators (Movsesyan et al., 2010; Kuchboev et al., 2017). The number of larvae in each of molluscs ranged from 1 to 39 copy. The presence of *P. rufescens*, *M. capillaris* and *C. ocreatus* species on molluscs was confirmed by molecular analysis of III stage larvae. These species were previously found in ruminant animals by researchers in Uzbekistan (Kuchboev et al., 2015).

Based on the analysis of the morphological structure of the shell, the infected molluscs were divided into 4 groups (Hygromiidae, Buliminidae, Agriolimacidae and Parmacellidae). Identification of molluscs to the species level is difficult due to internal and intraspecific polymorphism. Therefore, we used molecular markers to identify species identified as intermediate hosts (Steinke et al., 2004). As a result, the larvae of *P. rufescens* and *C.ocreatus* were detected in *P. sogdiana, P. albiplicatus* and *X. candacharica* species. *M. capillaris* larvae were identified in 5 species of molluscs: *P. maydanica, Pseudonapaeus sp, A. regeliana, C. levanderi* and *D. reticulatum*.

The specificity between the protostrogylids and its intermediate hosts is less likely to be observed, than in other group helminthes. But, only certain intermediate host species are important to the transition of infection to the definitive hosts in natural conditions. All of these snails are primarily found in dry environments and open fields with calcareous soils of the Central Asian (Pazilov and Azimov, 2003), landscape, along with most of the territory of Uzbekistan and some countries of Central Asia. The snail *X. candacharica* lives on plants in dry weather, forming accumulations on the stems of grasses. They are widespread in Central Asia. These snails are mainly found in pastures and gardens (Kuchboev et al., 2017).

All of protostrongylid larvae were isolated from molluscs collected from foothill pastures of Uzbekistan. It is known that protostrongylid larvae are well adapted to the pasture environment, and terrestrial molluscs provide optimal conditions for the development and survival of nematode larvae. In particular, III instar larvae of *M. capillaris* remain infective for at least six months at -12°C (Sauerlander, 1979), but are very sensitive to dry conditions and drier pastures are most suitable for larval survival (Morev, 1967). The third instar larvae of protostrongylids in the tissues of terrestrial molluscs usually do not leave the organism of the intermediate host and remain in their body until their natural death. Larvae that are mechanically separated from living molluscs are not important in the life cycle of protostronglid lungworms (Kulmamatov et al., 1994; Panayotova-Pencheva et al., 2015; Kuchboev et al., 2017).

CONCLUSIONS

Based on a parasite-mollusc morphological and molecular approach, this study identified three parasitic nematodes, *P. rufescens, M. capillaris*, and *C. ocreatus*, is responsible for caprinae protostrongylids. We have identified the intermediate host - land molluscs in natural conditions, and this allows us to identify factors of invasion. Grassland habitats are most suitable for the life cycle of protostrongylids of ruminants.

Morphological identification based on terrestrial mollusc shell features has revealed six distinct morphotypes. After molecular results and re-anatomical morphology, we confirm that these snails belong to the families Buliminidae, Hygromiidae, Agriolimacidae and Parmacellidae. Among them, eight species were identified: *P. maydanica, P. sogdiana, P. albiplicatus, Pseudonapaeus sp., A. regeliana, X. candacharica, C. levanderi* and *D. reticulatum*. In this study, P. *maydanica*, *C. levanderi* and *D. reticulatum* were first reported as natural intermediate hosts for *M. capillaris*. This study provides a new resource for better understanding parasites and impacts effective control of parasite-induced diseases.

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CONFLICT OF INTEREST

There are no conflicts of interest in our present study.

AUTHORS CONTRIBUTION

Materials was collected, morphological study and performed statistical analysis of data by Mehmonjon Egamberdiev. Molecular analysis and manuscript was written by Abdurakhim Kuchboev. Both author read and approved the manuscript.

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