Original Paper

Molecular survey of gastropods as intermediate hosts of protostrongylid nematodes in Uzbekistan

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ABSTRACT

Protostrongylid species are causative agents of pulmonary protostrongyliasis in caprine species in Uzbekistan. These nematodes typically require one intermediate host, terrestrial mollusks, to complete their life cycle. In this study, eight species of gastropods were found to be positive for protostrongylid larvae. Haplotypes of larvae corresponding to sequences of *Protostrongylus rufescens* and *Muellerius capillaris* were detected. Morphological identification of gastropods, based on shell characteristics, revealed six different morphotypes. Anatomic-morphological and molecular results confirmed the membership of these gastropods to the Buliminidae, Hygromiidae, Agriolimacidae and Parmacellidae and revealed eight different species: *Pseudonapaeus maydanika*, *P. sogdiana*, *P. albiplicatus*, *Pseudonapaeus* sp., *Angiomphallia regeliana*, *Xeropicta candacharica*, *Candaharia levanderi* and *Deroceras reticulatum*. This study displays the first report of *P. maydanika*, *C. levanderi* and *D. reticulatum* as natural intermediate hosts of *M. capillaris*. The infection rate of snails with protostrongylids was 27.9% (616/2,207) and the infection rate of slugs was 6.5% (18/279).

Key words : protostrongyliasis, caprine, gastropod, molecular identification, epidemiology.

1. INTRODUCTION

The superfamily of Metastrongyloidea (Lane, 1917) is composed of approximately 200 nematode species divided into seven families. Most of the Metastrongyloidea use gastropods (primarily terrestrial species) as intermediate hosts in their life cycle [1, 3, 14]. One such family of these Metastrongylid nematodes is Protostrongylidae (Leiper, 1926), or lungworms. Protostrongylid nematodes are widespread in Uzbekistan and Central Asia, causing significant disease in wild and commercial animals [18].

Protostrongylid life cycles are complex and gastropods of the genera *Xeropicta*, *Pseudonapaeus*, *Bradybaena*, *Macrochlamys*, and *Pupilla* are essential for their development and transmission. These gastropods serve as intermediate hosts for development of the second (L2) and infective third (L3) stage larvae [19]. Life cycles are then completed when L3 are ingested by the definitive bovid host. Development of eggs and the hatching of first stage larvae (L1) occur within the definitive hosts. The larvae, which are released to abiotic environments, actively penetrate the foot of gastropods. Within the gastropod, the larvae undergo two molts, finally becoming L3 and, under favorable conditions, leaving the gastropods during active movements and becoming disseminated on the grass. Definitive hosts become infected while swallowing infected gastropods, or the L3, together with the grass during grazing.

Identification of L1 in both feces and in the environment, and L2-L3 in the intermediate host has not generally been possible [18]. Species identification using morphological features is only possible on male adult worms [14]. Improved detection capabilities would be a significant contribution to studies in epizootiology, providing a means of identifying infected animals and the

geographic distribution for various species of parasites without direct collections of definitive hosts. In order to obtain a diagnostic tool to differentiate L1 or L3 of Protostrongylidae, therefore, a molecular approach must be utilized. Among available molecular markers, several studies on nematode species have investigated specific identifications using ribosomal DNA, essentially the internal transcribed spacers (ITS-1 and 2), known for their inter-specific variability [5, 6, 10, 11, 18], and the D2 domain (part of 28S) [22].

Similarly, species identification of snails is not trivial, when based essentially on morphological criteria. The morphological polymorphism, both inter and intra-specific, is significant in terrestrial snails depending on many parameters such as age, season, environmental conditions and biochemical factors [7, 35, 38]. Recently, the use of molecular tools, studying domains such as internal transcribed spacer and 18S rDNA or the COI and 16S mtDNA, has facilitated the recognition of snails at a specific level. The combination of the morphological and molecular approaches has helped with the identification of snails difficult to distinguish purely on morphological criteria or only characterizable by specialized malacologists [8, 27, 40].

The aim of our study is to investigate and identify the intermediate hosts of protostrongylids in natural conditions, using a morphological and molecular approach to their parasite larvae and gastropods.

2. MATERIALS AND METHODS

2-1. Gastropods

Terrestrial gastropods (Gastropoda, Pulmonata, Stylommatophora), representing potential intermediate hosts of protostrongylid nematodes, were collected in open scree areas, in shrubs, or in grasses between May and October of 2015 and 2016 (except during the summer months, when snails are inactive and buried), in the foothill and mountain zones of the Namangan, Tashkent, Jizzakh, and Surkhandarya regions of Uzbekistan (Fig. 1, Table 1). The sampled sites were selected for their high density of gastropods and ruminants according to results previously obtained [19]. Visits were performed in the early morning, between 6:00 a.m. and 8:00 a.m., when gastropods showed high activity and were therefore easier to observe. Studies were also performed on or after a rainy day.

Gastropods, collected under planks or on a plot, were

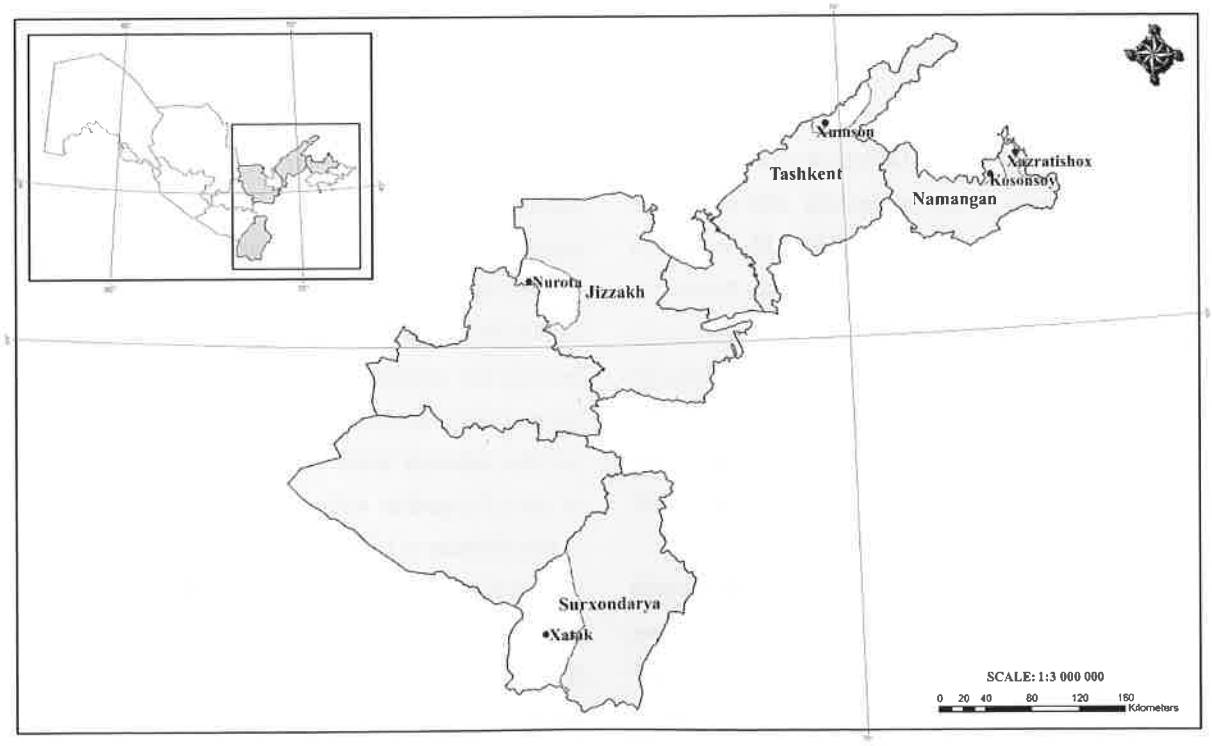

Fig. 1. Study sites divided into four regions (Surkhandarya, Jizzakh, Tashkent, and Namangan) in the foothill and mountain zones of Uzbekistan.

Table 1. Isolates of larvae and gastropods from this study used for molecular analysis and obtained Genbank accession numbers.

Gastropods						Parasite		
#	Locality	Morphological identification	Molecular identification	18S	ITS-1	Larvae studied	Species	D2
1	Namangan, Kosonsoy	Morphotype A (White snails)	*Xeropicta candacharica*	MF398539	MF398492	3	*Protostrongylus rufescens*	MF398496
2	Surxondarya, Xatak	Morphotype B (Biggest oblong snails)	*Pseudonapaeus maydanika*	MF398535	MF398491	2	*Muellerius capillaris*	MF398493
3	Jizzakh, Nurota		*Pseudonapaeus sogdiana*	MF398533	MF398495	3	*Protostrongylus rufescens*	MF351860
4	Toshkent, Xumson	Morphotype C (Small oblong snails)	*Pseudonapaeus albiplicatus*	MF351706	MF398497	3	*Protostrongylus rufescens*	MF398536
5	Namangan, Xazratishox		*Pseudonapaeus* sp.	MF398532	MF398538	2	*Muellerius capillaris*	MF398498
6	Namangan, Kosonsoy	Morphotype D (Brown snails)	*Angiomphalia regcliana*	MF351724	MF351722	3	*Muellerius capillaris*	MF398537
7	Surxondarya, Xatak	Morphotype E (Black slugs)	*Deroceras reticulatum*	MF351707	MF398494	3	*Muellerius capillaris*	MF405156
8	Surxondarya, Xatak	Morphotype F (Brown slugs)	*Candaharia levanderi*	MF398531	MF398534	3	*Muellerius capillaris*	MF399038

examined for lungworm larvae. All gastropods were stored in separate labeled plastic boxes with ventilation openings and refrigerated (4℃) for a maximum of three to four weeks. To determine the prevalence of infection in terrestrial gastropods, more than 2,500 individuals were studied as described by Azimov et al. [2]. Feet were recovered and crushed between two heavy glass slides to spread the snail tissue for observation with a stereomicroscope [3, 8].

Shells of positive and negative snails were preserved and identified on morphological and morphometric features, according to the collection of continental gastropods available at the Malacological Collection of Gulistan State University of Uzbekistan. In addition, for positive gastropods infested by nematode larvae, a part of the foot was conserved in 70% ethanol for molecular analysis.

2-2. Larval nematodes

Larvae were observed in some gastropods in the musculature of the foot. After pre-identification based on morphological criteria (size, sheath, and darkened cuticle), some larvae were extracted out of muscle after dissection or pressing between a slide and a cover slip. The larvae were then preserved in 70% ethanol for molecular analysis (Table 1). By examining the feet of these gastropods with a magnifier, we established levels of L3 infection. We also counted the larvae and thus established the intensity of the infection of the intermediate hosts.

2-3. Molecular analysis

After rinsing larvae in distilled water, then drying in an oven at 36℃ , DNA extraction was performed with a Qiamp DNA mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Sequencing of the D2 domain of the 28S was used for the identification of nematodes as described previously C2 (5`-GAAAAGAACTTTGRARAGAGA-3`) and D2 (5`-TCCGTGTTTCAAGACGGG-3`) [10, 21]. The resulting PCR products were directly sequenced in both directions with the primers used for DNA amplification (Genoscreen, Japan). Sequence alignment was performed using the ClustalW routine included in the MEGA version 6.1 software and checked by eye. Haplotypes of the larvae were compared to sequences of Protostrongylinae available in GenBank: *Protostrongylus rufescens* (KF811493 to KF811499) and *Muellerius capillaris* (AY292798).

Gastropod DNA extraction was performed using the same method as for nematode larvae. Sequencing of the ITS-1 and 18S of rDNA were performed using primers and conditions described by Steinke et al. [36]. Polymerase chain reaction (iCycler iQ Real Time PCR BIORAD, USA) was performed in a 25 μl volume using 1 μl of DNA and 1 μl of each of the primers with

GoTaq Green Master Mix (Promega Corp., USA). The PCR products were detected on 1.5% agarose gel and purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich) . The PCR products were directly sequenced in both directions with the primers used for DNA amplification (Euro fins Sequencing, Japan) . Our sequences were compared with sequences of terrestrial gastropods available in GenBank for all these domains. Our sequences were deposited in GenBank under accession numbers MF351706 to MF398539 (Table 1). The gastropod sequences defined (with the exception of *Deroceras reticulatum*) was not previously deposited in the electronic database of GenBank and is new to it.

Phylogenetic trees were constructed using the Maximum Likelihood (ML) and Neighbor Joining (NJ) methods using the MEGA 6.1 software [37]. For all NJ and ML, the most appropriate nucleotide substitution model was determined, gaps were treated as missing data and internal node support was assessed by bootstrapping over 500 replicates.

3. RESULTS

3-1. Prevalence

In total, 2,486 gastropods were analyzed, including 2,207 terrestrial snails and 279 slugs. Gastropods were separated depending on their origin: grassland or garden. Using a morphologic approach did not allow identifying gastropods at a species level, but only at the family or sometimes at the generic level.

The gastropods examined belong to group Pulmonata and the families Buliminidae and Hygromiidae. Of the 2,207 Pulmonata snails, 616 (27.9%) were positive for larval parasites. The total number of L3 per snail varied average from 1 to 48 with average 5 larvae. The species name of the gastropods in this table is given after the molecular analysis and re-morphological analysis of the specialist-malacologist.

Identification of gastropod slugs was evoked at genus level [30]. Identification of slugs brings out two families: Agriolimacidae and Parmacellidae. A total of 6.5% (18/279) of slugs were positive for protostrongylid larvae.

3-2. Identification of larvae

Morphological studies of the L3 were based on the recovery of larvae by simple morphological criteria. The L3 of this group is generally covered by a double sheath. The upper sheath is thin, transparent, and the lower sheath is compact and rugose with rough traverse folds of brownish color. The larvae, along with its sheath, usually coils into a ring [3, 16, 33]. Macroscopically infected parts of the tissue of mollusk feet were identified as dark brown-black spots, 2 to 3 mm diameter. Our study showed that the microscopic examination detected a dark shield in Protostrongylinae larvae without dorsal

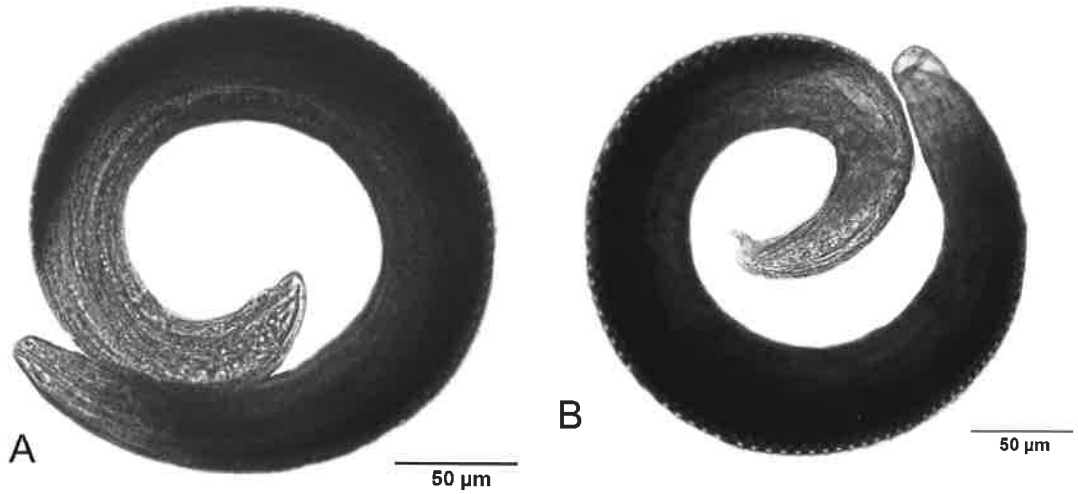

Fig. 2. Infective protostrongylid larvae from the foot of *Xeropicta candacharica* gastropods. (A) Protostrongylinae and (B) non-Protostrongylinae. Photomicrograph at 400 × magnification in differential interference contrast; scale bar in micrometers.

spine (Fig. 2A) and brown in Muelleriinae species with dorsal spine (Fig. 2B). These data require additional study.

Larvae were then analyzed using molecular markers (D2) in order to identify them at the specific level. For three positive gastropods, sequences of the larvae are 99-100% homologous with the haplotype of *P. rufescens*, whereas three snails and two slugs was positive for larvae are 96-97 % whose haplotype corresponds with *M. capillaris* deposited in GenBank.

3-3. Identification of positive gastropods

By pre-morphological identification based solely on shell, positive snails and slugs were all included in the Buliminidae, Hygromiidae, Agriolimacidae and Parmacellidae families. These gastropods were separated into 4 groups corresponding to their morphotype: A) white snails, including genera such as *Xeropicta* (463 snails) ; B) groups including largest oblong snails (4 and 26) and C) small oblong snails (105 and 1) with morphological features of the genus *Pseudonapaeus*; D) brown snails, with morphotype corresponding to the genus *Angiomphalia* (17 snails); E) black slugs, with morphological features of the genus *Deroceras* (9 slugs); F) positive brown snail whose morphological criteria seems to correspond to the *Candaharia* genus (9 slugs). Specimen of group A was found infected species of *Protostrongylus rufescens*, individual specimens of snails of groups B and C were found infected with *M. capillaris* and *P. rufescens*. Snail in group D and slugs E and F contained a species of *M. capillaris* (Table 1).

For each group, two gastropods were used for molecular analyses. An initial study of the combined dataset of 18S and ITS-1 sequences and additional anatomical-morphological identification by malacologists shows that all positive snails belong to the Buliminidae, Hygromiidae, Agriolimacidae and Parmacellidae families (Fig. 3). These snails can be separated into three different clades corresponding to the different morphotypes. Group A is composed of the four species of the genera *Pseudonapaeus*: *P. maydanika* (Pazilov et Gaipnazarova, 2015), *P. sogdiana* (Martens, 1874), *Pseudonapaeus* sp., *P. albiplicatus* (Martens, 1874), as well as the species of *Angiomphallia regeliana* (Martens, 1882) and *X. candacharica* (Pfeiffer, 1846). Group B is composed of *Candaharia levanderi* (Simroth, 1901) and *Deroceras reticulatum* (Muller, 1774).

It should be noted that except for species of the genera *Xeropicta* and *Deroceras* nucleotide sequences closely related genera to these gastropods, we did not find the international Genbank. In the phylogenetic tree, three well-isolated clades of gastropods of the genus *Pseudonapaeus*, *Angiomphalia* and *Candaharia* are noted. These species are noticeably different, and their groups are geographically isolated.

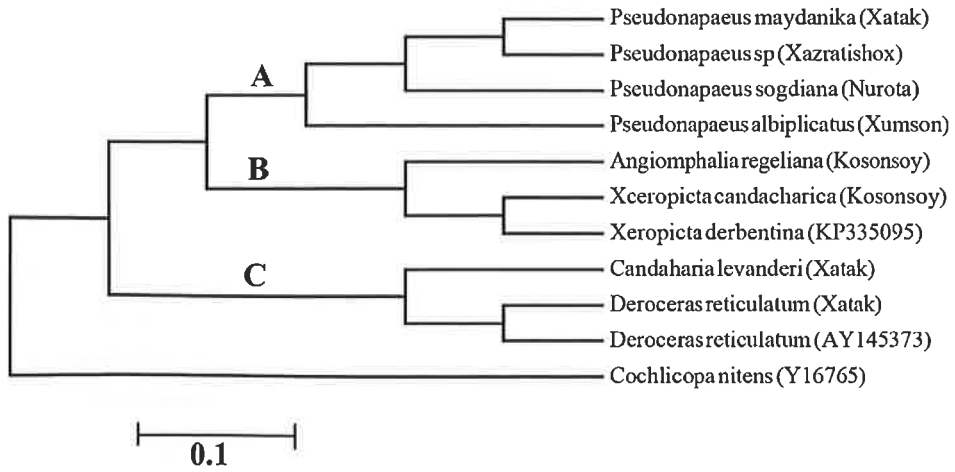

Fig. 3. Phylogenetic tree based on a combined dataset of 18S and ITS-1 sequences with a total of 1395 nucleotide sites constructed using the Maximum Likelihood method and the general time reversible model (GTR +1). The tree has been rooted using *Cochlicopa nitens* (Y16765.1). A = Buliminidae, B = Hygromiidae; C = Agriolimacidae and Parmacellidae.

The sequence we defined was not previously deposited into the electronic database of GenBank and is new to it. Morphological and molecular-genetic analysis of identified gastropods made it possible to clarify their species and spatial distribution. The data obtained show that these species differ at different morphological and genetic levels.

4. DISCUSSION

In recent years, systematic studies of protostrongylid nematodes, parasites of the ruminants in Uzbekistan, have been conducted. These studies have examined the fauna, ecology, life cycles and other aspects of the parasite-host relations of protostrongylid nematodes in mountain ecosystems [17, 19, 20].

Protostrongylid nematode infections are frequently found in caprine species in Uzbekistan [19], but few studies have been conducted to look for intermediate hosts of these parasites in natural conditions. This approach, however, is essential for understanding the development of the parasite life cycle, and thus to evaluate the risk of dispersion and contamination of the parasite in populations of definitive hosts. Our study aims to find these hosts in nature for different species of protostrongylids previously isolated from lungs of caprine species in Uzbekistan, in order to identify the host-parasite relationship and more precisely the gastropod-nematode relationship, by using techniques previously tested for trematodes [13, 15, 31].

Since the discovery of the role of snails in the development of *M. capillaris* by Hobmaier and Hobmaier in 1929, several studies have been implemented to identify intermediate hosts and describe infestation pathways. Life cycles of the intermediate hosts of protostrongylids of domestic ruminants, parasites involved in diseases affecting pulmonary tract of livestock and responsible for economic losses [12], have also been well studied [23, 33]. Yet, pulmonary protostrongyliasis, disease caused by these nematodes, is still frequently encountered and occasionally implicated in the cyclic decline of ruminant populations [26].

In Uzbekistan, the most important intermediate host is apparently *Xeropicta candacharica*, which presents a high risk factor for transmission of protostrongyles to small ruminants by being both the most abundant gastropod (>50% of samples) and the gastropod with the highest prevalence of protostrongylid larvae. The second most important intermediate host is *Pseudonapaeus albiplicatus*, which was the second most abundant species (approximately 20% of all samples) and had the second highest prevalence (nearly 20%) of protostrongylid infection, significantly higher than that of all other gastropods except for *Candaharia levanderi* (approximately 10%). The three above gastropod species with the highest prevalence as well as their ranking were identical to what was reported by Kuchboev et al. [17].

Our research on potential gastropod intermediate hosts was carried out at sites where positive caprine species had been located. For terrestrial snails, eight different families were isolated; from these, only gastropods belonging to the families Buliminidae, Hygromiidae, Agriolimacidae and Parmacellidae were found positive in natural conditions for protostrongylid larvae. Other families of gastropods among the Pulmonata have been referenced in the literature naturally [19], others experimentally [3, 20]. The method used to search for larvae in mollusks in this study was different from the usually-performed methodology. Although pepsin-digestion is commonly utilized [9, 32], especially for large mollusks, we utilized the method of pressing snails between two glass plates for small specimens. The proportion of infested snails in this study is high (27.9%) but similar to other previous studies [19, 25]. The number of larvae per snail varied from 1 to 48. Molecular analyzes of the larvae confirmed the presence of two species of Protostrongylinae, *P. rufescens* and *M. capillaris*. These species have been previously found in caprine hosts in Uzbekistan. The analysis of positive snails based on morphological criteria of the shell highlighted 4 groups, though identification was limited to the family or the genus level. Identification at the species level is extremely difficult, because of intra and inter-specific polymorphism. To be free from the constraints related to morphological identification, we used molecular tools, as previously described, to identify species incriminated as intermediate hosts [8, 36].

A single larva of *P. rufescens* was isolated from one individual belonging to each of the *X. candacharica*, *P. sogdiana*, and *P. albiplicatus* species. Larvae of *M. capillaris* were observed in five different species of snails and slugs: *P. maydanika*, *Pseudonapaeus* sp., *Angiomphalia regeliana*, *Candaharia levanderi*, and *Deroceras reticulatum*.

It should be noted that the gastropods *Pseudonapaeus maydanika*, *Pseudonapaeus* sp. and *Deroceras reticulatum* are natural intermediate hosts of the Protostrongylinae of caprines. Protostrongylidae are not highly specific in their use of intermediate hosts; many species of snails have been shown to be involved in their development [25]. The specificity between nematode parasites and their intermediate hosts seems less strict compared to what can be observed, especially for trematodes. However, it is likely that only some intermediate host species are important in natural transmission to the definitive host. All of these snails primarily colonize open fields with a dry environment and calcareous soil, typical of the landscape in Central Asia [30], along with a large part of Uzbekistan territory and some Central Asian countries. The snail *X. candacharica* inhabits plants in dry weather, forming accumulations on the stems of grasses. It is widely distributed throughout Central Asia. These snails are primarily found in pasture fields and gardens, where populations of snails are often very dense [19].

In our study, all larvae of Protostrongylinae were isolated from gastropods collected in foothill areas. It is possible that the protostrongylid larvae are better adapted to the pasture field environment, allowing gastropods to release larvae more effectively or offering optimal conditions for larvae survival. For example, the L3 of *M. capillaris* remain infective for at least six months at -12 ℃ [34], but are considered to be very sensitive to dry conditions [24] and the pasture fields, drier, do seem best suited for larval survival. However, L3, which infect the definitive host when ingested, typically leave the snail when it dies, or larvae may be partially rejected as a random, mechanical phenomenon [20]. Additionally, Cabaret [4] reports that L3 do not usually leave the foot when the snail is alive but their output is activated when it dies. According to Trushin [39], individual L3, accidentally found outside the organism of an intermediate host, fall into new ecological conditions. In these new conditions, they exhibit little resistance, and remain viable for only 20-30 days. Meanwhile, the L3 of the *Muellerius*, remaining within the body of the intermediate host, remain viable for 1.5-2 years. Therefore, the emergence of larvae from the intermediate host to the external environment, where they die in a comparatively short time, can hardly be considered expedient from the point of view of the benefits for individuals and the species as a whole.

Thus, the L3 of protostrongylids, located in the tissues of gastropods, do not typically leave the organism of the intermediate host and instead remain in them until their natural death. Larvae mechanically detaching from live gastropods is considered to play a minor role in the lifecycle of protostrongylid parasites [20, 28, 29, 39].

Using a combined morphological and molecular approach for parasites and gastropods, this study examined the natural intermediate hosts of *P. rufescens* and *M. capillaris*, two parasitic nematodes responsible for caprine pulmonary protostrongyliasis in Uzbekistan. Understanding the life cycle of these species is essential for risk factor identification. Among the different habitats sampled, pasture fields seem most favorable to the life cycle of caprine Protostrongylinae.

Morphological identification of gastropods based on shell characters revealed 6 different morphotypes, and anatomic-morphological and molecular results confirm the membership of these snails to the Buliminidae, Hygromiidae, Agriolimacidae and Parmacellidae. Eight unique species were identified: *Pseudonapaeus maydanika*, *P. sogdiana*, *P. albiplicatus*, *Pseudonapaeus* sp., *Angiomphallia regeliana*, *Xeropicta candacharica*, *Candaharia levanderi* and *Deroceras reticulatum*. This study displays the first report of *P. maydanika*, *C. levanderi* and *D. reticulatum* as natural intermediate host of *M. capillaris*.

The present study provides novel resources for a better understanding of the parasite, which, in turn, has implications for the effective control of the disease it causes.

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ウズベク共和国における Protostrongylidae 科線虫の中間宿主－腹足類

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要　約

ウズベク共和国における羊の肺虫症の原因寄生虫プロトストロンギルス科線虫は陸棲腹足類を中間宿主としているので、この線虫症の疫学では腹足類の調査が必須であった。そこで、同国において貝殻の形態で6種に分類された軟体類を対象にプロトストロンギルス科の感染幼虫の保有状況を調査した。その結果、分子生物学的に *Protostrongylus rufescens* および *Muellerius capillaris* のハプロタイプに一致した感染幼虫を得た。また、今回、幼虫が得られた腹足類について解剖学および分子生物学的な検討によりこれらは4つの科 Buliminidae, Hygromiidae, Agriolimacidae あるいは Parmacellidae の次のような8種に同定された：*Pseudonapaeus maydanika, P. sogdiana, P. albiplicatus, Pseudonapaeus* sp. *Angiomphallia regeliana, Xeropicta candacharica, Candaharia levanderi, Deroceras reticulatum*. 本研究により *P. maydanika, Candaharia levanderi* および *D. reticulatum* が *M. capillaris* の自然中間宿主であることが証明された。全体的なプロトストロンギルス科線虫の感染幼虫の保有率はカタツムリ類で27.9% (616/2,207)、一方、ナメクジ類では6.5% (18/279) と低かった。

Key words：プロトストロンギルス科、羊、腹足類、分子同定、疫学