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Short Communication

Occurrence of Larval *Dicrocoelium dendriticum* and *Brachylaima* sp. in Gastropod Intermediate Hosts from Fergana Valley, Uzbekistan

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AK, MA and ME analyzed and wrote the manuscript. ME, RK and OA collected materials, preserved tissues and extracted DNA for nucleotide sequencing.

Key words
***Dicrocoelium dendriticum*, *Brachylaima* sp., Gastropoda, Intermediate host, Ribosomal DNA.**

ABSTRACT

The occurrence of larval *Dicrocoelium dendriticum* and *Brachylaima* sp. is described with molecular evidences in gastropod intermediate hosts from Fergana Valley, Uzbekistan. Larvae of *D. dendriticum* were detected in 28 (10.7%) out of 262 *Xceropicta candacharica*, and 8 (9.7%) of 82 *Angiomphalia gereliana*. *Brachylaima* sp. larvae were found in 3 (1.6%) of 95 *Pseudonapaeus sogdiana*. The total number of larvae per snail varied from 8 to 110 individuals. Alignment of the first four sequences of 28S rDNA was revealed a 99-100% similarity to *D. dendriticum*. Larvae from *P. sogdiana* snails were 98% similar to *Brachylaima* sp. In this study, it was confirmed that 2 species of terrestrial snail, *X. candacharica* and *A. gereliana*, act as the first intermediate hosts of *D. dendriticum*, and *P. sogdiana* snail play a role of intermediate host of *Brachylaima* sp. in the Fergana Valley, Uzbekistan.

Terrestrial gastropods (Gastropoda, Pulmonata, Stylommatophora), potential intermediate hosts of parasitic cestode, trematode and nematode species, are represented by about 50 species in Uzbekistan (Pazilov and Kuchboev, 2017). Few studies have reported ecological relationships between these gastropods and their internal parasites (Oniyishi et al., 2018).

Dicrocoeliasis, a parasitic disease of grazing animals, is common in Europe, Asia, North Africa, and America, where conditions are suitable for intermediate host species of terrestrial gastropod and ant (Panin, 1984; Gürelli, 2017). In Uzbekistan, infection is common in sheep, goats, cattle, and horses (Shakarboev, 2009). This disease, mainly caused by infection by the lancet fluke *Dicrocoelium dendriticum*, is responsible for severe economic losses through infection reduce the value of milk and meat produced from infected animals. The larval stages of *D. dendriticum* and their first intermediate hosts gastropods have been previously reported Ernazarov, (1972), and Salimov (1974); this study aimed at determining the presence and prevalence of trematode in the species genus of the *Xceropicta* and *Bradybaena* snails.

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Species of *Brachylaima* Dujardin, 1843 (Brachylaimidae), another genus of fluke, are the main endoparasites of endothermic vertebrates, including birds, mammals, and humans (Butcher and Grove, 2001; Suleman and Khan, 2016). Studies on the intermediate hosts of *Brachylaima* sp. have not been studied in Uzbekistan. The first and second intermediate hosts in the life cycle of *Brachylaima* sp. are terrestrial gastropods; first intermediate hosts carry sporocysts and cercariae, and second intermediate hosts carry metacercariae. Infection occurs when the definitive hosts consume raw gastropods containing metacercariae (Kose *et al*., 2015).

Identification of larval trematodes using classical morphological methods is difficult and requires high levels of expertise. Identifying taxa from larval stages only is often not possible. In order to obtain a diagnostic tool to differentiate larval trematodes, therefore, a molecular approach must be utilized. Among available molecular markers, have investigated specific identifications dicrocoeliid and brachylaima species using sequences of partial 28S and the second internal transcribed spacer of ribosomal DNA (Maurelli *et al*., 2007; Nakao *et al*., 2017). The goal of this study describes with molecular evidences the occurrence of larval trematode and prevalence in gastropods in natural and synanthropic zones Fergana Valley, Uzbekistan.

Materials and methods

Several species of gastropods were collected in the Fergana Valley at AM in rainy day of September and October, 2017. To detect the larval trematodes, collected snails were individually crushed and dissected in a petri dish with 0.85% buffered saline, and the hepatopancreatic portion was mainly observed under a stereomicroscope. Some collected larvae were morphologically observed under a light microscope (ML 2000 microscope, Meiji) (Panin, 1984) and some of them were used in the molecular study (Kuchboev *et al*., 2017a). Gastropods were collected around Baharstan and vicinity of Kasansay reservoir, Kasansay district (41°14'12.6"N, 71°32'10.1"E), and Govasoy village, Chust district (41°00'00.1"N, 71°13'28.8"E), Namangan region, Fergana Valley, Uzbekistan (Fig. 1). Shells of positive and negative snails were preserved and identified on morphological and morphometric features (Pazilov and Kuchboev, 2017).

Genomic DNA was isolated from individual trematode larvae (cercariae) recovered from host gastropod taxa. Larvae were first rinsed in distilled water, then oven dried at 36°C, after which DNA was extracted using a Qiamp DNA mini kit (Qiagen, Hilden, Germany) following manufacturer instructions. D2 of 28S rDNA PCR (iCycler iQ Real Time PCR BIORAD, USA) amplifications were carried out in 25 µl reactions containing 1 µl of each of C2 primer (5`-GAAAAGAACTTTGRAR-3`) and D2 primer (5`-TCCGTGTTTCAAGACGGG-3`) (Gouy *et al*., 2001), 1 µl DNA template, 12.5 µl GoTaq Green Master Mix (Promega Corp., USA) and 9.5 µl nuclease-free water; amplification began with an initial denaturation at 94°C for 3 min, followed by 40 cycles at 94°C for 30 sec, 40°C for 1 min, and 68°C for 1 min, and a final extension at 68°C for 10 min. Electrophoresis of amplified DNA fragments was performed on a 1.5% agarose gel, purified using a GenEluteTM PCR Clean-Up Kit (Sigma-Aldrich). Resulting PCR products were directly sequenced in both directions using primers for DNA amplification (Genoscreen, Japan).

Our phylogenetic tree was built using a Maximum likelihood approach and MEGA version 7 Software. For ML analyses, 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. Nucleotide sequences (AF153917, JQ081965 AY222167, DQ060330 and as out groups AY222169 for *Dicrocoelium* and AF184254 for *Brachyima*) were sourced from GenBank; our sequences were deposited in GenBank (accession numbers MK796127 for *D. dendriticum*, and MK796833 for *Brachylaima* sp.).

Results and discussion

This study at determined the presence and prevalence of two trematode larvae species *D. dendriticum* and *Brachylaima* sp. in the three terrestrial snails from Fergana Valley of Uzbekistan (Table I; Fig. 2). Gastropod collections comprised 439 snails: 262 *X. candacharica* from pastures rearing sheep, goats and cattle in the vicinity of Kasansay reservoir, 82 *A. regeliana* from "Baharistan" village, and 95 *P. sogdiana* from Govasoy village. Daughter sporocysts and cercariae of *D. dendriticum* occurred in the digestive gland and hepatopancreas of 28 (10.7%) of 262 *X. candacharica*, and 8 (9.7%) of 82 *A. gereliana*; the number of *D.dentriticum* larvae per snail varied average from 14 to 110 individuals; branched sporocysts of *Brachylaima* sp. occurred in the digestive gland of 3 (1.6%) of 95 *P. sogdiana* and number of larvae per snail 8-19 individuals (Table I).

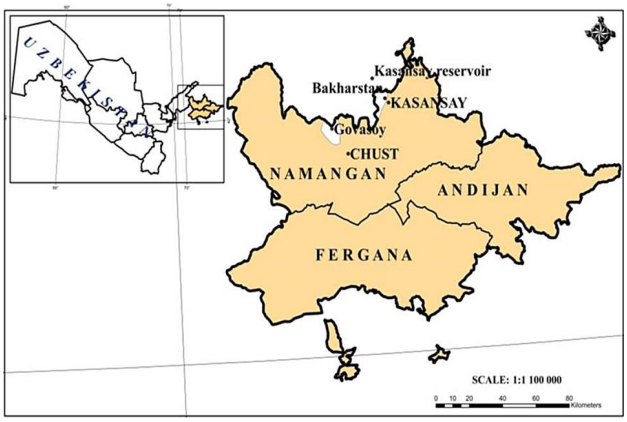

Fig. 1. Study sites divided into two districts in the foothill zone of Namangan regions Fergana Valley, Uzbekistan.

Table I.- Prevalence (%) of *D. dendriticum* and *Brachlayma* sp. larvae in gastropods in Fergana Valley, Uzbekistan.

Gastropod species	n	*D. dendriticum*			*Brachlayma* sp.		
		Infected	%	No. of larvae	Infected	%	No. of larvae
Xeropicta candacharica	262	28	10.7	54-110	-	-	-
Pseudonapaeus sogdiana	95	-	-	-	3	1.6	8-19
Angiomphalia regeliana	82	8	9.7	14-78	-	-	-
Total	439	36	10.2	14-110	3	1.6	8-19

n, number of gastropods analysed.

Fig. 2. Intermediate hosts gastropods (shells) and larval stages of trematodes: A, *Xceropicta candacharica*; B, *Angiomphalia regeliana*; C, Cercariae of *Dicrocoelium dendriticum*; D, mollusk *Pseudopanaeus sogdiana;* E, Cercariae of *Brachylaima* sp.

The most prevalent snails are *X. candacharica* (prevalence ca. 11.0 %). As one of reasons of the high prevalence shown, it might the definitive host range of *D. dentriticum* is wide, including over 20 mammalian species (Shakarboev, 2009), and the possible hosts occur throughout the survey area. In particular, the most important intermediate host is apparently *X.candacharica*, which presents a high risk factor for transmission of protostrongylid nematodes to small ruminants by being both the most abundant gastropod (>50% of samples) and the gastropod with the highest prevalence of protostrongylid larvae (Kuchboev *et al*., 2017b). The total infection of gastropods by *D. dendriticum* and *Brachlayma* sp. larvae is a ranged from 1.6 % to 10.7%, and the intensity in a snail is a ranged from 8 to 110 individuals, respectively. The species name of the larvae in Table I is given after the molecular analysis.

Though larval dicrocoeliids have been previously reported to infest 1.5-5.2% of *X. candacharica* and *Agriolimax agrestis* snails in Samarkand region, Uzbekistan (Salimov, 1974), ours is the first to report the prevalence of larval *D. dendriticum* in *A. regeliana* snails, and, to the best of our knowledge, *Brachylaima* sp. in Fergana Valley, Uzbekistan.

So, infection of *X. candacharica* and *A. gereliana* with larvae of *D. dendriticum* in the region investigated in the present study is rather high. In the period from September to October in 2017, the density of *X. candacharica* populations on pastures fields of foothill zones reached 50 individuals per m^2. Dicrocelium cercariae are found only in gastropods collected in plants for banks of streams. They did not occur in open dry pasture areas. Therefore, focal distribution is characteristic of larval stages of *D. lanceatum*.

Metacercaria of *Brachylaima* sp. was found in the snails of *P. sogdiana* in forests along the northern slopes of the mountains, under stones. Detected metacercariae by us morphologically similar to trematode metacercariae from the Brachylaemidae family. The question of the species of *Brachylaima* sp. can be resolved only by experimental study of its further development and infection of the definitive hosts or by molecular analysis using sequences.

In this study, we showed a very simple method for preparing PCR templates. By using this method, we could determine parasite DNA sequences even in a single metacercaria or a small portion of sporocysts. From amplified partial 28S rDNA sequences we obtained fragments from five samples of larval trematode and one of nematode (534–590 bp for *A. regeliana*, 558–566 bp for *X. candacharica*, and 592 bp for *P. sogdiana*). Alignment of the first four sequences of 28S rDNA with GenBank data revealed a 99–100% similarity to *D. dendriticum*. Larvae from *P. sogdiana* were 98% similar to species of *Brachylaima*.

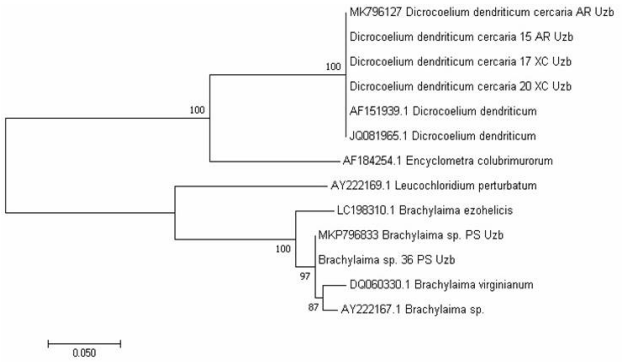

Fig. 3. Phylogenetic relationships between dicrocoeliid and brachylaima trematodes using a maximum likelihood approach.

The phylogenetic tree constructed, using our 28S rDNA sequences and those of other Dicrocoeliidae and Brachylaimidae taxa from GenBank, revealed *Dicrocoelium* sequences to be united in one clade, with three branches with nodal statistical support indicating reliable differentiation to species. Our *Brachylaima* sp., *B. virginicum* and a *Brachylaima* sp. taxon from GenBank also united in a single clade, also with clear species differentiation. The families Dicrocoeliidae and Brachylaimidae belong to different super families of trematodes. Therefore we use trematode with *Encyclometra*

colubrimurorum as outgroup (based on 28S rDNA analysis) for *D. dentriticum* and with *Leucochloridium perturbatum* for *Brachylaima* sp. Their division into two clades is very likely (Fig. 3). For these same sequences, the same clades are apparent on a phylogenetic tree constructed using a maximum economy method.

Data coded in the primary structure of different parts of trematode DNA, which significantly contributes to the solution of a number of topical issues of helminthology, contributes to an improved understanding of the systematics and evolution of parasites. Ribosomal molecular marker used by us confirms the validity of morphological features used to identify dicrocoeliid and brachylaimid species, and clearly determine the systematic position of genera in trematode phylogeny. The integration of DNA barcoding and morphological approaches (Nadler and De Leon, 2011) is essential for identifying cryptic species of the trematodes and reorganizing their systematics.

Thus, due to the difficulty to morphologically differentiate cercariae and metacercariae isolated from gastropod of snails were characterized by molecular analysis using sequences. We intend to describe with molecular evidences the occurrence of larval *D. dendriticum* and *Brachylaima* sp. in the gastropod intermediate hosts. Using a molecular approach for parasites, this study examined the natural intermediate hosts of *D. dendriticum* and *Brachylaima* sp. two parasitic nematodes responsible for dicrocoeliasis and brachylaimids animals. Understanding the life cycle of these species is essential for risk factor identification. Among the different habitats sampled, wet pasture fields foothill zones seem most favorable to the life cycle of dicrocoeliid and brachylaima species in Fergana Valley, Uzbekistan.

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Statement of conflict of interest

The authors declare no conflict of interest.

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