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Morphological and Molecular Identification of Nematode Parasite *Eustrongylides* sp. Isolated from *Channa punctatus*: A Preliminary Study

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ABSTRACT

Eustrongylides sp. is considered as a freshwater zoonotic nematode which uses birds as final hosts and other vertebrates (fish, amphibians and reptiles) as intermediate hosts. In this study, a total of 200 specimens of *Channa punctatus* were collected from different fish markets of Mymensingh city to identify *Eustrongylides* sp. with determining prevalence, mean intensity and abundance of this species using morphological and molecular tools. Larvae of *Eustrongylides* sp. were recovered from the abdominal cavity, musculature and ovaries of 101 *C. punctatus* specimens. Light microscopy (LM) was conducted to observe the surface anatomy of the worm. The worm was slender, elongated and cylindrical, medium to large in size and sometimes broader in the middle portion. The mouth was slit-like, surrounded by two lateral rows of somatic papillae, cephalic end was conical with 12 labial papillae arranged in two circles of 6 papillae each. Oesophagus was leaded into highly coiled intestine and was fairly long, without posterior bulb and posterior extremity with spike like tail process. The parasitological investigations revealed that the prevalence, mean intensity and abundance of *Eustrongylides* sp. Infestations were 50.5%, 2.06 and 1.04, respectively and varied depend on the size of fish and difference of fish markets. The number of nematodes per fish was ranged from 1 to 13. The prevalence of *Eustrongylides* sp. also varied from 26.0% to 61.32% in different fish markets and 26.0% to 59.82% in different sizes of the hosts. *Eustrongylides* sp. was also confirmed by amplifying ITS gene. This is the first report on the identification of larval stage of *Eustrongylides* sp. from *C. punctatus* in Bangladesh which provides basic information for further detailed epidemiological and molecular study.

Introduction

Bangladesh is a riverine country and blessed with many rivers-canals, depressions and ox-bow lakes, ponds and floodplains, covering a huge area of water resources of 4.70 million hectares (DoF, 2019). Bangladesh is ranked 3rd in inland fisheries and 5th in aquaculture production (excluding aquatic plants) in the world (FAO, 2018). The country is enriched with 260 species of fishes (DoF, 2019). Among the freshwater fishes, snakeheads are important group of fish and it is getting increasingly popular showing a promising future for culture (Barua, 1989). Snakeheads, commonly known as spotted snakehead (*C. punctatus*), striped snakehead (*C. striatus*) and great snakehead (*C. marulius*), have been contributed greatly as popular protein source of country's people. These are commercially important fishes in Bangladesh and comparatively cheaper than other indigenous fish (Das *et al.,* 2018). Among these snakeheads, *C. punctatus,* locally known as 'Taki', is one of the most popular fish because of it's delicious taste and the meshed food item made from it has a great acceptability among Bangladeshi. Aquaculture of *C. punctatus* has not been started yet and hence, capture from natural water body is the main of this fish. It is mud-loving fish and due to its food habit, it can act as an intermediate host for many helminth parasites (Chandra *et al.,* 1997).

Parasites are important groups of pathogens since it evolved independently in nearly every phylum of animals, from protozoa to arthropods and chordates, as well as in many plant groups (Das *et al.,* 2018). Fish parasites of different systematic groups recorded in Bangladesh are protozoa, helminths, monogenea, digenea, cestodes, acanthocephala, nematodes and crustacea (Chandra, 2006). Like other freshwater fish, *C. punctatus* can also be infested with large number of

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parasites. Seven different species *i.e., Allocreadium handiai*, *Argulus bengalensis*, *Camallanus intestinalus*, *Euclinostomum multicaecum*, *Procamallanus mysti*, *Pallisentis ophiocephali* and *Sanga ophiocephalina* have already been identified of which six were endoparasites and one was ectoparasite (Das *et al.,* 2018).

Nematodes of the genus *Eustrongylides* (Jägerskiöld, 1909) are pathogenic parasites found as adults in the proventriculus of piscivorous wading birds (Spalding and Forrester, 1993) with larvae encysted in the body cavity and musculature of fishes (Hoffman, 1999). Three species of *Eustrongylides* are accepted as valid taxa: *E. tubifex* (Jägerskiöld, 1909), *E. ignotus* (Jägerskiöld, 1909) and *E. excisus* (Jägerskiöld, 1909) (Xiong *et al.,* 2009). Gupta (2019) identified *Eustrongylides* sp. in the abdominal cavity, musculature and ovaries of *C. punctatus* having parasite body tapering with dense transverse striation of cuticle. Goncharov *et al.* (2018) observed *Eustrongylides* sp. having dark red body, two circles of papillae with 6 papillae in each circle at the anterior end and terminal anus at the thickened and narrowed posterior portion. The partial sequences of the small ribosomal subunit (18S) and the internal transcribed spacer 2 (ITS2) region of nuclear ribosomal DNA (rDNA) were chosen as molecular markers for the taxonomic identification of the nematode species. The 18S rDNA gene represents a well-conserved gene that evolves relatively slowly and seems to be an effective marker for the barcoding of nematode worms (Floyd *et al.,* 2002). The ITS2 region is considered as an excellent tool for DNA diagnosis (Blouin, 2002), because of its high variability, and has become a popular choice for molecular analysis of closely related parasitic species and populations.

Eustrongylides sp. have complex life cycles involving a definitive host and two intermediate hosts. Definitive hosts include aquatic birds mostly from order Ciconiiformes family Ardeidae, Anseriformes, Gaviiformes and Pelecaniformes (Measures, 1988; Spalding and Forrester, 1993, 2008). First intermediate hosts for *Eustrongylides sp*. are aquatic oligochaetes (Spalding *et al.,* 1993), second intermediate hosts are planktivorous and benthivorous fish that could pass the infection on to fish (paratenic hosts) and finally on to fish eating birds (Moravec, 1998). These parasites also occasionally parasitize man and thus have zoonotic potential (Xiong *et al.,* 2009). Zoonotic infection caused by *Eustrongylides* sp. is known as human Eustrongylidosis (Cole, 2009; Deardorff and Overstreet, 1991). A plethora of scientists worked on nematode infestation in *C. punctatus* especially on *Eustrongylides* sp. (Das *et al.,* 2018; Ghani and Bhuiyan, 2011; Gupta, 2019; Miah *et al.,* 2013). They identified this parasite by microscopic as well as molecular tools. Morphological observation remains a gold standard method for the identification of nematodes. But application of both the methods such as morphological and molecular techniques would be more helpful for the accurate identification of parasite. Despite this, no published report on morphology as well as prevalence, mean intensity and abundance of *Eustrongylides* sp. is available in Bangladesh. Considering the situation, the present study was aimed to identify *Eustrongylides* sp. from *C. punctatus* using morphological and molecular tools as well as to determine the prevalence, mean intensity and abundance of collected *Eustrongylides* sp.

Methodology

Morphological identification

Collection of fish sample

Based on the previous sporadic reports, *C. punctatus* was selected as the experimental fish to investigate for nematodes. The host fishes were collected from *Pouroshova*, Kewatkhali and *Shesh More* fish markets situated at Mymensingh sadar upazila under Mymensingh district. The experimental work was performed in the Parasitology Laboratory, Department of Parasitology, Bangladesh Agricultural University, Mymensingh. The study was carried out from January to May, 2019.

Investigation of experimental fish

A total of 200 *C. punctatus* were investigated for this experiment. Live *C. punctatus* were collected from different fish markets of Mymensingh city. After collection, fish were brought to the laboratory using clean polythene bags. The sources of the fish and necessary numerical data of the collected fish were recorded. According to the length, fishes were divided into 3 groups *i.e.,* small (<12 cm), medium (12-16 cm) and large (>16 cm) to observe infestations in different length groups.

Observation of the organs and parasites

Experimental fish was first dissected and opened along the mid ventral line from the anus to the anterior portion. Body cavities, the surface of the visceral organs and mesenteries were examined carefully by naked-eyes. Further, the parasite(s) collected from each fish were kept in 0.85% physiological saline to determine the number of nematodes. Then, parasites were washed in saline for the preparation of temporary slides, kept on slides and covered with the cover slips using lactophenol to examine under light microscopic at 10× or 40× magnifications. Nematodes were then preserved in absolute alcohol and stored at −20 °C, until DNA was extracted.

Identification and infestation of parasites

The identification of the collected parasites was determined according to the keys and description of

Yamaguti (1962), Abe (2011) and Gupta (2019). Infestations were determined following formulae (1-3) of Margolis *et al.* (1982).

$$\text{Prevalence (\%)} = \frac{\text{Total No. of hosts infected}}{\text{Total No. of hosts examined}} \times 100$$

$$\text{Mean intensity} = \frac{\text{Total No. of parasites collected}}{\text{Total No. of infected hosts}}$$

$$\text{Abundance} = \frac{\text{Total No. of parasites collected}}{\text{Total No. of hosts examined}}$$

Molecular identification

Isolation of DNA

Total genomic DNA was extracted from individual worm using a commercial kit, QIAamp DNA Mini Kit (Qiagen, Germany). Each nematode was placed into a clean 1.5 ml microcentrifuge tube, where it was incubated at 56 °C. The tissues were disintegrated, lysed and proteins were digested by proteinase K, and washed extensively. Following brief vortexing, the solution was centrifuged at 8000 rpm to remove drops from the lid. Finally, trapped DNA was eluted by elution buffer. Extracted DNA was measured by a nanodrop spectrophotometer and stored at −20 °C until further use.

PCR amplification

The primers 18SF (5-TTGGATGATTCGGTGAGGT-3) and 28SR (5-AACCGCTTAGTAATATGCT-3) were designed to amplify the ITS rDNA region (Xiong *et al.*, 2013). The PCR was performed in a 25 µL of reaction volume containing 12.5 µL of master mix (0.05 U/µL Taq DNA polymerase, reaction buffer, 0.4 mM of each dNTP: dATP, dCTP, dGTP and dTTP, 4 mM MgCl2), 2 µL (above 30 ng/ µL) of template DNA, 1 µL of each primers (10 pmol/ µL) and 8.5 µL of double distilled water. The thermo-cycling conditions were used at 94 °C for 5 minutes for the initial denaturation followed by 29 cycles of denaturation at 94 °C for 40 seconds, annealing at 56 °C for 30 seconds and extension at 72 °C for 90 seconds and with a final extension at 72 °C for 10 minutes. The PCR products were visualized by agarose gel electrophoresis on 1.5% (w/v) agarose gels to purify that they represented single band.

Results

Gross observation and identification of nematode

The infected fish showed no pathological effects on the body surface. On autopsy, *C. punctatus* revealed the presence of highly coiled nematode parasite in the abdominal cavity, musculature and ovaries, lumen of the stomach and encapsulated in the stomach wall of 101 individuals (Figures 1 and 2). The number of nematodes per fish ranged from 1 to 13. The anterior end of the parasites was pink, remaining part of the body was red with deepest coloration in mid-anterior part of body (Figures 1 and 2). Based on its morphology, the parasite was identified as larval stage of *Eustrongylides* sp.

Taxonomic summary

Order: Enoplida
Sub-order: Dioctophymina
Super-family: Dioctophymatoidea
Family: Dioctophymatoidae
Genus: Eustrongylides
Species: Eustrongylides spp.

Morphology Eustrongylides sp. larvae isolated from C. punctatus

The isolated larva of *Eustrongylides* sp. were cylindrical and slender, surface with delicate transverse striations, medium to larger in size and sometimes broader in the middle portion (Figure 3). The anterior one-third of the larval body was pinkish to red and the rest was bright red (Figures 1 and 2). Three cuticle layers were visible at the anterior and posterior extremities of the worms. The cuticle of body was coarsely striated but least at both the extremity and without spines (Figure 4 A-B). The cephalic extremity was conical and showed 12 labial papillae, arranged in 2 circles of 6 papillae each. The inner and outer papillae were approximately the same size (Figure 4D). Head was somewhat swollen and almost globular (Figure 4A). Oesophagus was fairly long, without posterior bulb leading to highly coiled intestine (Figure 4C). The posterior portion of the body was thickened and narrowed toward the end. The anal aperture was located terminally. Genital organ was not observed (Figure 4E). The ranges of total length, maximum body width, length of the oesophagus and length of the intestine of *Eustrongylides* sp. were measured 34-49 mm, 0.38-0.42 mm, 7.94-14.93 mm and 18.50- 22.75 mm, respectively (Figure 3).

Infestation of Eustrongylides sp. in C. punctatus

The overall prevalence, mean intensity and abundance of *Eustrongylides* sp., the larval stage of nematode, was 50.5%, 2.06 and 1.04, respectively.

Area-wise infestation of nematode

The prevalence, mean intensity and abundance of larvae of *Eustrongylides* sp. was varied among different fish markets of Mymensingh city. The prevalence of *Eustrongylides* sp. varied from 26.0% to 61.32% in different fish markets whereas the mean intensity varied from 1.83 to 2.61 and abundance ranged from 0.68 to 1.25. The prevalence of *Eustrongylides* sp. was the highest in *Pouroshova* fish market than that of Kewatkhali and *Shesh More* fish markets whereas, the mean intensity and abundance were the highest in Shesh More and Kewatkhali fish markets, respectively (Table 1).

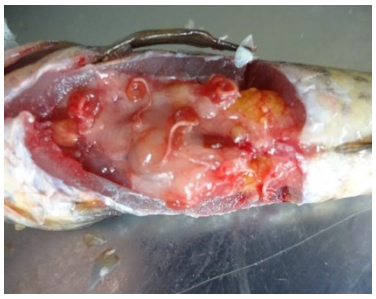

Figure 1. *C. punctatus* showing larvae of *Eustrongylides* sp. (arrow)

Figure 2. *Eustrongylides* sp. (arrow) in the lumen of the body cavity of *C. punctatus*

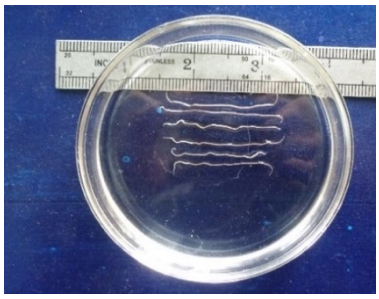

Figure 3. Length measurement of *Eustrongylides* sp.

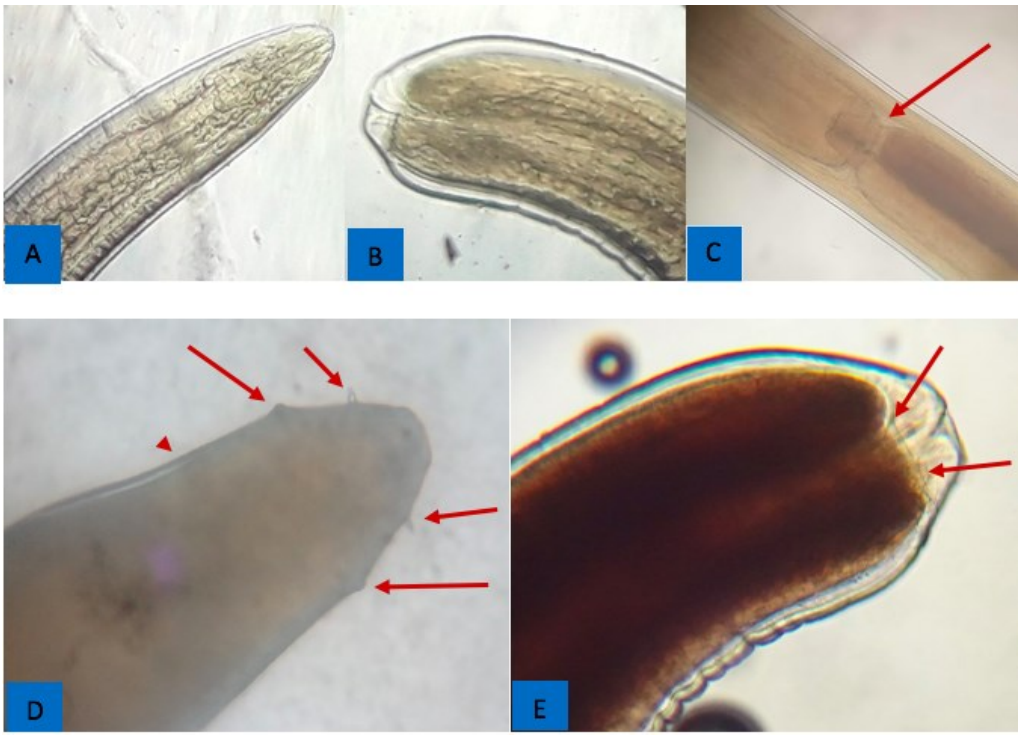

Figure 4 (A-E): Microscopic features of larvae of Eustrongylides sp. (not on scale bar). Note: A- Anterior end showing tapering end, B- Posterior blunt end, C- Oesophageo-intestinal Junction (arrow), D- Cephalic extremity, showing inner circle (short arrow) and outer circle (long arrow) papillae. The outer cuticle layer (arrowhead) from the larva is visible, E- Caudal extremity, showing a muscular sucker with cuticle (arrow).

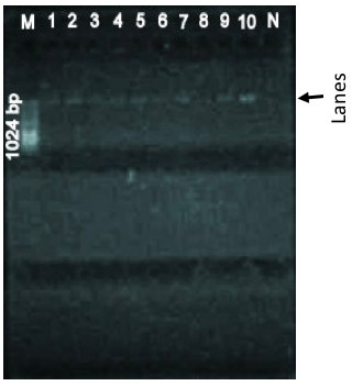

Figure 5: Molecular confirmation of *Eustrongylides* sp. Note: M= marker, Lane 1-10= positive bands, N= negative band (control).

Table 1: Comparison of the *Eustrongylides* sp. infestations in *C. punctatus* collected from various fish markets located in Mymensingh sadar

Sampling location	Number of hosts examined	Number of infected hosts	Number of nematodes collected	Prevalence (%)	Mean intensity	Abundance
Pouroshova	106	65	119	61.32	1.83	1.12
Kewatkhali	44	23	55	52.27	2.39	1.25
Shesh More	50	13	34	26	2.61	0.68

Table 2: Comparison of the *Eustrongylides* sp. infestations in different sizes of *C. punctatus* collected from Mymensingh sadar

Length classes (cm)	Number of hosts examined	Number of hosts infected	Number of nematodes collected	Prevalence (%)	Mean intensity	Abundance
<12	50	13	34	26	2.61	0.68
12-16	38	21	52	55.26	2.47	1.36
>16	112	67	122	59.82	1.82	1.08

Size-wise infestation of nematode

The prevalence of *Eustrongylides* sp. was varied from 26.0% to 59.82% in different sizes of the host whereas the mean intensity varied from 1.82 to 2.61 and abundance ranged from 0.68 to 1.36. Based on the size of the host, the prevalence of infection was maximum in larger fish and minimum in smaller fish whereas the mean intensity of infection was increased with the decrease of size of the host. The abundance of infection was maximum in the medium sized fish (Table 2).

Molecular confirmation

The PCR amplification of the ITS region yielded an approximately 1027 bp–sized. Randomly ten (10) *Eustrongylides* sp. were selected for DNA extraction and subsequently subjected for PCR amplification. All the samples regarding the PCR amplicons showed positive band on agarose gel (Figure 5).

Discussion

Eustrongylides sp., commonly known as big red worm, is one of the four genera of adenophorean nematodes belonging to the super-family Dioctophymatoidea, family Dioctophymatidae Railliet, 1915, family Eustrongylinae Chitwood and Chitwood, 1937 (Anderson *et al.,* 2009). The present study is the first report regarding the parasitism of second stage larvae of the genus *Eustrongylides* in *C. punctatus* in Mymensingh, Bangladesh. Larvae of this genus have also been reported in *C. punctatus* and amphibian hosts in different regions of the world including India (Gupta, 2019), China (Xiong *et al.,* 2013), Japan (Abe, 2011), Ukraine (Goncharov *et al.,* 2018), U.S.A (Yildirimhan *et al.,* 2012) and Brazil (Vicente *et al.,* 1991).

Eustrongylides sp. has been recognized as zoonotic parasite (Centres for Disease Control, 1982; Eberhard *et al.,* 1989; Narr *et al.,* 1996; Wittner *et al.,* 1989) that may pose a public health risk to consumers. In addition, *Eustrongylides* sp. causes a serious problem in fisheries. The larval stage of *Stroggylis's* sp. have been reported in different parts of the world including North and South America, Europe, eastern Africa, China, and Turkey (Goncharov *et al.,* 2018). *Eustrongylides* sp. usually parasitize oligochaetes, fish, and piscivorous birds. These parasites have complex life cycles, with oligochaetes as the first intermediate host and fish as the second. The predatory fish that consume infected fish serve as the paratenic or transport host. Fish-eating birds serve as the definitive host (Measures, 1988a, 1988b, 1988c). In the present study, the parasites were collected from the different parts of the body of *C. punctatus* with typical morphological characteristics including large, coiled, cylindrical body, surface with delicate transverse striations, pinkish to red coloration and the cephalic extremity having 12 labial papillae arranged in 2 circles. According to Anderson (2000) and Lezama and Sarabia (2002), these characteristics are important for diagnosis of the genus *Eustrongylides*. In this study, genital primordium was not found that indicated the second larval stage which was also confirmed by Lichtenfels and Pilitt (1986), Moravec (1998) and Xiong *et al.* (2009). It was noted that the body length, width, buccal cavity and length of esophagus of the identified larvae, *Eustrongylides* sp. collected from *C. punctatus* were 34- 49 mm, 0.38- 0.42 mm, 0.09- 0.11 mm and 7.94- 14.93 mm, respectively. These findings are in line with the report of Goncharov *et al.* (2018) and Novakov *et al.* (2013).

The cephalic papillae of both the inner and outer circles of the *Eustrongylides* sp. larvae were presented as large pointed apices of inner circle papillae, and outer circle papillae with nipple-like apices which is supported by previous findings of Gupta (2019), Lichtenfels and Pilitt (1986) and Xiong *et al.* (2009). However, Melo *et al.* (2016) reported that the cephalic papillae of both the inner and outer circles of the *Eustrongylides* sp. larvae isolated from *Rhinella marina* (an amphibian commonly found in the Amazon forest region of Brazil) presented sub-spherical shapes with clear cuticle delineations. This variation was found probably due to the differences of *Eustrongylides* sp. in species level and geographical location because

Eustrongylides sp. (probably, *E. ignotus*) larvae found in *R. marina* from the eastern Amazonia have larger outer circle cephalic papillae compared to papillae in the inner circle (Melo *et al.*, 2016).

Eustrongylides sp. are pathogenic parasites of piscivorous birds transmitted through two intermediate hosts: aquatic oligochaetes and fish (Karmanova, 1968; Measures, 1988). In fish, these parasites are conspicuous as long, red, coiled individuals located in the body cavity or embedded in the muscle (Mitchum, 1995; Overstreet, 2003). Unencysted larvae of these parasites migrate under the skin and in the muscles, causing extensive inflammation and necrosis. Encystation occurring in the viscera, namely liver, spleen, or gonads causes severe pathologic changes in the adjacent tissue (Kundu *et al.,* 2016; Paperna, 1974). But in the study, there was no noticeable pathological changes found on the host, *C. punctatus.* More than half of the examined fishes were infected with *Eustrongylides* sp. and the overall prevalence was 50.5%. Kundu *et al.* (2015) also reported *Eustrongylides* sp. from *C. punctatus* with a prevalence rate of 44.28%. Previous findings (Soylu, 2013; Branciari *et al.,* 2016) revealed that *Eustrongylides* sp. isolated from perch, largemouth black bass and sand smelt showed the prevalence rate of 94.5%, 6.84%, 1.89% and 0.13%, respectively. The reason behind the variation of infestation can be mentioned that the prevalence depends on many factors like host's feeding habits, the water body the fish inhabits, availability of intermediate hosts such as oligochaetes, snails and fish-eating birds etc. In addition, elevated temperatures along with organic enrichment of the water bodies, water pollution, agricultural runoff, deposition of biological wastes and over density of intermediate hosts may also plays vital role to increase *Eustrongylides* sp. infestation in *C. punctatus.*

During the period of study, the experimental fish were categorized by different length groups. The prevalence was observed higher in the length group of >16 cm fish with the prevalence of 59.82%. Mean intensity was observed higher as 2.47 in the fish of medium length group. *C. punctatus* is carnivorous and fed mainly on animal foods (Chandra and Haq, 1986). The reason behind the findings regarding prevalence and mean intensity could be due to higher or middle length group of *C. punctatus* took more food containing earlier stage of this parasite (Das, 2003). However, findings of Das *et al.* (2018) reported the highest prevalence rate and mean intensity of different groups of parasitic infestations in small and medium length groups of *C. punctatus* collected from Lalabazar and Ratargul swamp forest of Sylhet, Bangladesh

Findings of the current research also represent the first report on ITS rDNA sequences of *Eustrongylides* sp. larvae isolated from *C. punctatus* in Bangladesh that has been successfully used for the molecular identification of this nematode. Xiong *et al.* (2013) identified the nematodes *Eustrongylides* sp. collected from different fish species in China and Abe (2011) identified *Eustrongylides* sp. in Japanese smelt using the molecular markers mitochondrial cytochrome oxidase c subunit 1 (COI) gene and internal transcribed spacer (ITS) rDNA regions. In summary, *C. punctatus* of Mymensingh region, Bangladesh are infected by the larval stage of *Eustrongylides* sp.

Conclusion

The present study reports the first identification of *Eustrongylides* sp. by morphological and molecular tools as well as determines the infestation of this parasite on the popular fish *C. punctatus* in Bangladesh. A total of 200 specimens of *C. punctatus* were collected from different fish markets of Mymensingh city. *Eustrongylides* sp. was found in different organs of the body of *C. punctatus* and was prevalent with the infection rate of 50.5%. The molecular technique was performed by amplifying ITS gene to confirm *Eustrongylides* sp. and all the specimens showed positive results. Further works are recommended to amplify more genes to find out phylogeny and genetic structure of this parasite.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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