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REDESCRIPTION OF TREMATODE PARASITES FOUND IN *CHANNA PUNCTATA* BLOCH, 1793 WITH AN EMPHASIS ON SCANNING ELECTRON MICROSCOPY STUDY

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AUTHORS' CONTRIBUTIONS

This work was carried out in collaboration among all authors. Author PKB designed the study. Author IK has performed the experiments. Author DRM conducted the literature survey. Author IK has worked on data analysis, manuscript preparation and interpretation of findings. All authors read and approved the final manuscript.

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ABSTRACT

The paper presents a systematic survey of helminth parasites found in *Channa punctata* from various districts (Nadia, North 24 Paraganas and Hooghly) in West Bengal. Mainly two species of parasites *Euclinostomum heterostomum* and *Clinostomum complanatum* have been recorded during the study. Data on their morphology and site of infection are provided. Morphological studies with light and scanning electron microscopic studies as well as molecular studies have revealed some additional morphological details. New features which were observed with scanning electron microscopy in *Euclinostomum heterostomum* were aspinous and smooth tegument surface, transversely folded areas, absence of spines on both the suckers and presence of crates. Dome shaped papillae with microridges and micropores on the surface of suckers was also observed. Similarly aspinous suckers with papillae on the floor of the ventral sucker was reported in *Clinostomum complanatum.* All the helminthes have been briefly described and illustrated. The study was done on economically important fishes to establish database of various types of infection caused by helminth parasites.

Keywords: *Channa punctata*; helminth; scanning electron microscopy; taxonomy.

1. INTRODUCTION

Digenetic trematodes have a wide geographical range and are cosmopolitan [1-5]. *Clinostomum sp* and *Euclinostomum sp* (Family Clinostomidae) are parasites commonly found in piscivorous birds which acts as final host have been reported from various parts of the world [6]. Metacercarieae of these

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trematodes (Family Clinostomidae) infects different visceral organs of the fish which acts as intermediate hosts hence reduces the marketability of these fishes accounting for huge economic losses. Family Clinostomidae comprises of four subfamilies [7]: Clinostominae, Euclinostominae, Nephrocephalinae and Ithyoclinostominae. Genus *Clinostomum* Leidy, 1856 includes 14 species [8-10] which belongs to Clinostominae Lühe, 1901 and Euclinostominae Yamaguti, 1958, with *Euclinostomum* Travassos, 1928 includes eight species [11] infecting birds.

Identification and charecterisation of trematode parasites till date has been done only through morphological charecterisation but recent molecular techniques have been helpful in removing any ambiguity in regard to identification of parasites. Information in regard to trematodes infecting edible fish of the family Clinostomidae in fish from West Bengal are extremely inadequate. In the present study we have provided molecular data along with morphological data for identification and characterization of trematode parasites isolated from *Channa punctata* from West Bengal as it would enrich the databank of parasites infecting economically important fishes.

2. MATERIALS AND METHODS

2.1 Collection of Fish Samples

Fish farms from districts of West Bengal (Nadia, North 24 Paraganas and Hooghly) served as a source for providing host fishes (18- 21 cm long, weighing 55–75 g) which were brought alive in the Parasitology laboratory. They were acclimatized in aquarium and commercial feed was provided. As per CPCSEA instruction's protocol for experimentation on fishes, does not require approval from ethical committee.

2.2 Collection and Mounting of Parasites

Trematodes collected from infected organs of the host were washed in saline solution and fixed with AFA or 70% ethanol. All specimens were then preserved in coupling jars containing 70% ethanol and 5% glycerine. Identification of the parasites was done using standard methology using identification keys [12].

2.3 Sample Preparation for Scanning Electron Microscopic Study

2.5% glutaraldehyde solution (pH 7.4) prepared in 0.1 M sodium cacodylate buffer at 4°C was used for fixing trematode parasites. They were then post fixed in 1% osmium tetraoxide. Varying alcoholic grades were used for dehydration postfollowed by wash with absolute alcohol and amyl acetate mixture of varying concentrations and finally 100% amyl acetate. Using gold as coater (Quorum Q 150 TES) parasites were coated and placed on stubs. Specimens were observed in scanning electron microscope (Zeiss EVO-MA 10 Germany) operating at accelerating voltages of 15KV [13].

2.4 Ilustrations and Measurements

Stage and an ocular micrometer were used for taking measurements of the parasite while camera lucida was used for drawing figures. Camera-mounted microscope (Olympus CX41) was used for taking light microscopic photographs of parasite SPSS software was used for statistical analysis. All measurements are given in millimeters if not stated otherwise.

2.5 Deposition of Specimens

Holotype and paratypes parasite specimens were deposited in the Parasitology laboratory Department of Zoology, University of Kalyani, Kalyani in West Bengal, India.

2.6 Genotypic Characterization

The identified parasites specimens were further confirmed by modern molecular technique such as 18S rDNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA analysis. The assay involves isolation, amplification and sequencing of the gene coding for the 18S rRNA, ITS1, 5.8S rRNA, ITS2 and 28S rRNA (Eurofins Bangalore).

2.7 PCR Amplification and Sequencing

Genomic DNA isolation kit was utilized for isolation of trematode DNA. *Euclinostomum* sp. 28S rRNA gene fragment rDNA sequences was amplified by polymerase chain reaction (PCR) using 28 SF – ACCCGCTGAATTTAAGCATA and 28 SR-CCTTGGTCCGTGTTTCAAGA primers were used using BDT v3.1 Cycle sequencing kit on ABI 3730 x l Genetic Analyzer. The PCR mixture consisted of 1X assay buffer (Mg^{2+} free), 1µl of dNTP mixtures (2.5 mM each), 100 ng of each primer (forward and reverse), 200 ng of template DNA and Taq DNA polymerase (3 U/µl) in a final volume of 50 µl. PCR was performed in a thermal cycler using an initial denaturation at 94°C for five minutes, followed by thirty five cycles at 94°C for thirty seconds, 56°C for thirty seconds and 72°C for one minute and final extension at 72°C for 10 minutes. Electrophoresis was performed for separation of

amplicons which were later purified by help standard kits before sequencing.

2.8 Sequencing and Phylogenetic Analysis

In case of *Euclinostomum* sp for the PCR reaction, the DNA sequencing was carried out with 28SF and 28SR primers using BDT v 3.1 and ABI 3730xl Genetic Analyzer helped in cycle sequencing. Consensus sequence was formed from the PCR product using aligner software using data from forward and reverse sequencing.28S rDNA region sequence of *Euclinostomum* sp. was aligned using multiple alignment software program Clustal W. Distance matrix was generated and the phylogenetic trees was constructed using MEGA 7. Phylogenetic trees were inferred using the neighbour-joining method.

3. RESULTS

3.1 Description of Trematode Parasite, *Euclinostomum heterostomum* (Rudolphi, 1809) Travassos, 1928

3.1.1 Identifying characters (Figs. 1, 2, 3, 4)

Body stout and linguiform of total length 5.1-7.8(2.02 ± 2.42) and width 1.8-2.7(0.027 ± 1.02). Oral sucker round, small, subterminal of length 0.290-0.351(0.321±0.019) and width 0.488-0.586(0.066±0.004), encircled by collar like structure which may be present 1.0181-1.64(0.03632 ± 0.00613) or may be absent. The topography of the oral sucker showed that the outer rim was thick and smooth, while its floor had nodule like structure. Presence of muscular ventral sucker, larger in size than the oral sucker and placed relatively closed to it (Figs. 1a, b, 2) with length 0.992-1.23(0.366±0.06) and width 0.844-1.20(2.066± 3.45). The range of oral sucker /body width varies from 0.188-0.27 (23.121± 5.019) while ventral sucker width/body width varies between 0.40-0.59(11.67± 8.99).The distance between suckers ranges from 1.171-1.493(8.027 ± 1.12). Caeca long with numerous lateral diverticula branching from lateral walls present in between posterior sucker and caudal end. Testes irregular in shape lodged in the posterior end of trematode, anterior testes lobe U shaped while posterior testes lobe are triangular shaped. Anterior testis right arm width ranges from 0.292-0.419(0.097 ± 0.22) while anterior testis left arm width ranges from 0.283-0.322(4.77 ± 0.82). Anterior testis length varies from 0.440-0.605(1.021±5.46) while posterior testis right arm width ranges from 0.244-0.292(0.086 ± 0.99), Posterior testis left arm width varies between 0.195-0.263(0.31 ± 6.44). Posterior testis length ranges from 0.507-0.732(3.024 ± 1.02). Follicles surround caecal branches in hind body. Uterus reaches forward to ventral sucker (Fig. 1b). Vitelline folds well developed and are found to cover region extending from caudal part of the parasite to the posterior sucker (Figs. 1c, 2). Cirrus sac small oval pre-testicular (Figs. 1c, d, 2). Genital pore immediately pretesticular, median or submedian. Ovary intertesticular, submedian oval (Figs. 1c, d, 2). Ovary length ranges from 0.214-0.244 (0.59 ± 0.08), while width between 0.1464 – 0.215(0.0082 ± 0.024), ovary length/ width varies from 0.00113-0.00146 (7.97± 3.89). A comparative measurement chart of few known species of the genus is given in Table 2.

3.1.2 Scanning electron microscopic structure

A scanning electron microscopy revealed additional features. The tegument showed that surface of the fluke was covered with a smooth, aspinous layer (Fig. 3a). General body surface shows presence of transverse folds or wrinkled appearance more frequent in the anterior region than in posterior part on the ventral surface (Fig. 3a). Studies revealed that border of the oral and ventral sucker were thick, smooth, cutaneous with absence of spines on the margin of both the suckers (Fig. 3a). Dome shaped papillae with microridges, separated by micropores were present near both the rim and floor of the anterior sucker (length- 3.096 µm; width- 1.875 µm) and floor of the posterior sucker (length- 9.922 µm; width- 9.21175 µm), along with pits (Fig. 3b, c). Crates or folds on the floor of the both the suckers were absent (Fig. 3b, c). The ventral sucker was deeply seated in the tegument with a smooth, thick double walled margin and acts as a adhesive organ for attachment to the host's tissue (Fig. 4a). No spines or tubercles were observed on the tegumental surface (dorsal, ventral and lateral views) even under high resolution (Fig. 4 a, b). Papillae with microridges, with micropores were present also on the floor of the posterior sucker (length -9.922 µm; width- 9.21175 µm), along with pits (Fig. 4b, c). Post acetabular region possesses cobble stone like structure (Fig. 3d). Presence of pits on the ventral sucker (Fig. 4e, f).

3.1.3 Molecular property

28S RNA region of the nuclear rDNA was amplified using PCR primers and the lengths of the PCR products was found to be 860 bp. The analysis further shows that the nucleotide contains contains 155 adenine bases, 174 cytosine bases, 281 guanine bases and 250 thymine bases. The sequence has been submitted to Genbank and accession number KY284847 has been provided.

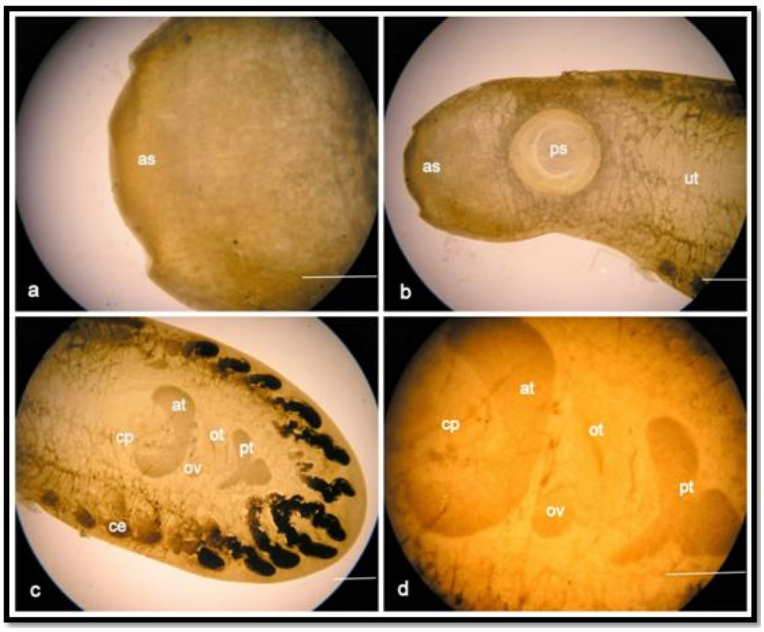

Fig. 1. Light microscopic structures of *Euclinostomum heterostomum*
a. Anterior portion of *E. heterostomum* showing the anterior sucker (as). Scale bar - 500 µm
b. Anterior portion of *E. heterostomum* showing the anterior (as), posterior sucker (ps) and uterus (ut). Scale bar- 500 µm
c. Posterior portion of *E. heterostomum* showing the anterior testis (at), posterior testis (pt), ovary (ov), cirrus pouch (cp), ootype (ot) with caeca (ce) forming numerous diverticulum. Scale bar -100 µm
d. Higher magnification of the reproductive structures at, pt, ov, cp in posterior end. Scale bar -100 µm

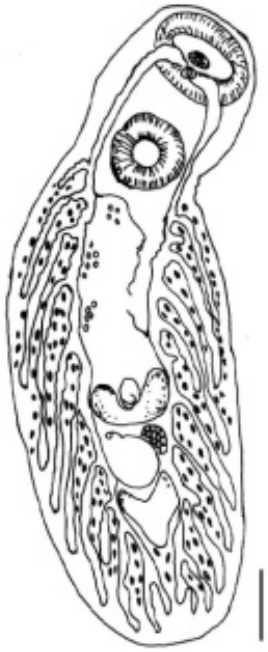

Fig. 2. Camera lucida diagram of *E. heterostomum.* Scale bar- 500 µm

The 28S rRNA gene amplified resulted in 860 bp fragment (Fig. 5). As there is no data available for 28S rRNA in the NCBI database for *E. heterostomum*, it was compared with the *Clinostomum* sp. Result of the BLAST search with consensus sequence obtained gave a 97% identity with *Clinostomum* sp L5 (MH159727), *Clinostomum tataxumui* (MF398321) and *Clinostomum marginatum* (MF398323). 91.3% *Clinostomoides brieni* (KF781299) depicted by phylogenetic tree made by neighbor joining method (Fig. 6). *E. heterostomum* formed a separate branch from the other *Clinostomum* sp. with significant boot strap values (98%). The analysis of the rRNA of the mentioned sequences from various species ranged from 0.002 % to 0.087 % (Table 1).

3.1.4 Taxonomic affinity

Site of infection: Liver.

Type locality:

Krishnanagar, Nadia, West Bengal 23.4009° N, 88.5014° E.

Barasat, North 24 Paraganas, West Bengal 22.7228° N, 88.4806° E.

Prevalence and intensity:

9/30 (30.0%), 2-3 specimens per fish.

Specimens deposited:

Slide bearing number PR/IK/TR/E-15 and PR/IK/TR/E-21 have been deposited in Parasitology laboratory, Department of Zoology, University of Kalyani, West Bengal, India.

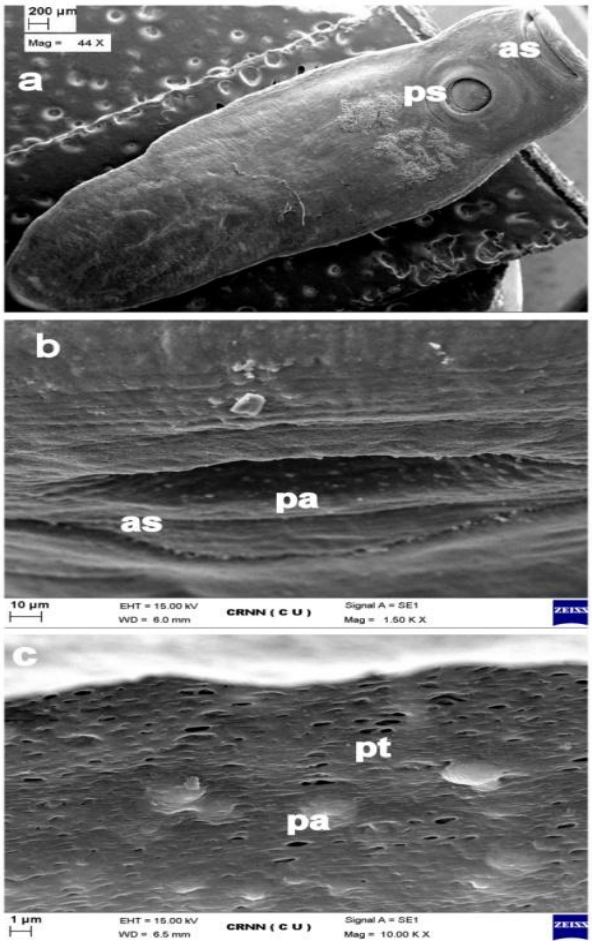

Fig. 3. Scanning electron microscopic structures of *Euclinostomum heterostomum*
a. Excysted metacercaria showing the anterior sucker (as) and acetabulum (ps)
b. Excysted metacercaria showing the arrangement of papillae (pa) around anterior sucker (AS)
c. Enlarged oval shaped papillae (pa) and pits (pt) present in anterior sucker

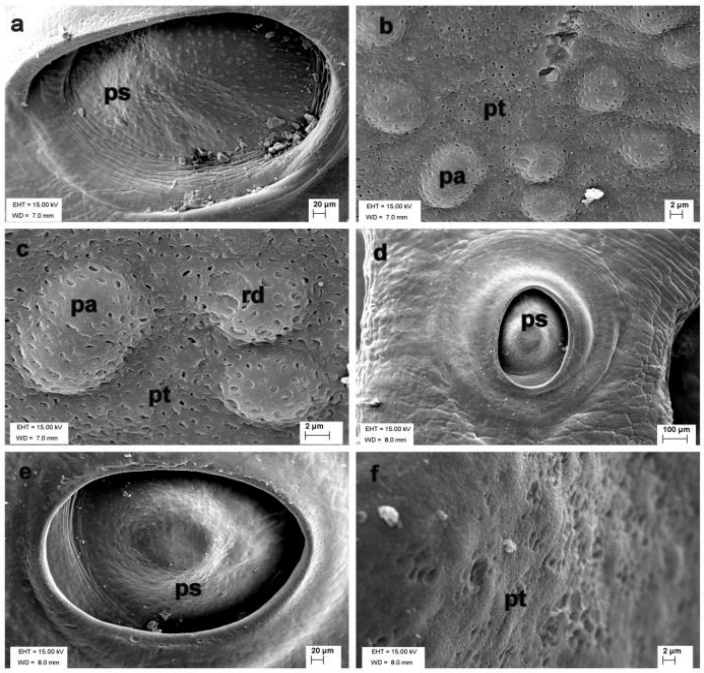

Fig. 4. Scanning electron microscopic structures of *Euclinostomum heterostomum*
a, d and e. Metaceraria showing the position of posterior sucker (ps)
b and c. Enlarged view of papillae (pa), pits (pt) and ridges (rd) present on the posterior sucker
d and e. Indicates the double walled deeply seated acetabulum (ps) with post acetabular region cobble shaped
f. Pits (pt) and ridges present in the acetabular region

Table 1. Distance matrix: Estimate of evolutionary divergence between sequences

Trematode parasite	1	2	3	4	5
KY284847.1					
MH159730.1	0.056				
MF398323.1	0.058	0.027			
MH159727.1	0.06	0.015	0.029		
MF398321.1	0.063	0.017	0.029	0.002	
KF781299.1	0.087	0.057	0.047	0.06	0.061

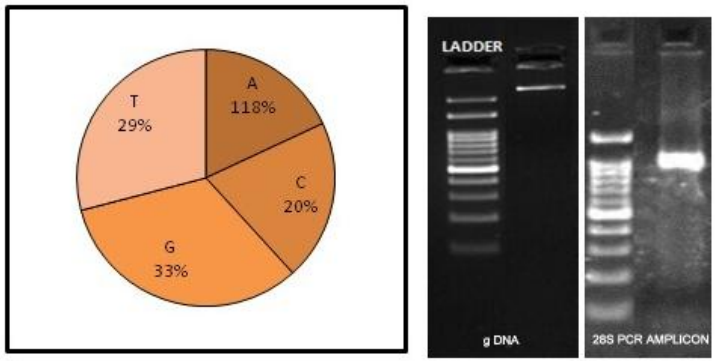

Fig. 5. Nucleotide composition of *E.heterostomum* isolated from *Channa punctata* (KY284847) and agarose gel electrophoresis showing the PCR amplified product

Table 2. Comparative chart of morphometric charecters of metacercariae stage of *Euclinostomum heterostomum* in fishes

Morphometric charecters	***Euclinostomum heterostomum*** **Vijayalakshmi et al., 2011 (mm)**	***Euclinostomum heterostomum*** **Purivirojkul and Sumontha (2013) (mm)**	***Euclinostomum heterostomum*** **Present study (mm)**
Body length	3.45–8.41	5.78 -7.77 (7.10 ± 0.49)	5.10-7.8 (2.02 ± 2.42)
Body width	1.28–2.36	2.25 -2.73 (2.52 ± 0.14)	1.8- 2.70 (0.027 ± 1.02)
Oral collar width	NA	NA	1.0181-1.64(0.036±0.006)
Oral Sucker length	0.14–0.34	0.167 - 0.199(187.40 ± 9.74)	0.290-0.351(0.321± 0.019)
Oral Sucker width	0.20–0.52	0.223-0.264 (248.40 ± 11.25)	0.488-0.586(0.066±0.004)
OS width/Body width	NA	NA	0.188-0.27(23.121± 5.019)
Ventral sucker length	0.60-1.16	0.821-0.978 (917.30± 46.35)	0.992-1.23(0.366±0.06)
Ventral sucker width	0.55–1.34	0.780-0.930 (873.65± 43.87)	0.844-1.20(2.066 ± 3.45)
VS width/Body width	NA	NA	0.40-0.59(11.67± 8.99)
Distance between suckers	NA	NA	1.171-1.493 (8.027 ± 1.12)
Anterior testis right arm width	0.21–0.65	0.530-0.680 (618.20±35.900)	0.292-0.419 (0.097 ± 0.22)
Anterior testis left arm width	0.22–0.63		0.283-0.322 (4.77 ± 0.82)
Anterior testis length	0.31–0.78	0.135-0.179 (157.25± 10.54)	0.440-0.605 (1.021± 5.46)
Posterior testis right arm width	0.06–0.22	0.384–0.491 (445.65± 24.92)	0.244-0.292 (0.086 ± 0.99)
Posterior testis left arm width	0.10–0.22		0.195- 0.263(0.31 ± 6.44)
Posterior testis length	0.22-0.62	0.295-0.381 (332.40± 21.91)	0.507-0.732 (3.024 ± 1.02)
Ovary length	NA	0.136–0.179 (158.40± 11.34)	0.214-0.244 (0.59 ± 0.08)
Ovary width	NA	0.110-0.148 (126.80 ± 9.36)	0.146 -0.215 (0.0082±0.024)
Ovary length/ width	NA	NA	0.0011-0.00146 (7.97±3.89)

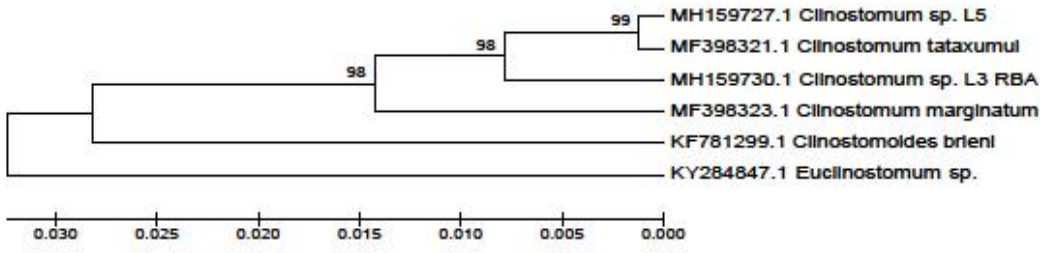

Fig. 6. Phylogenetic tree

3.2 Description of Trematode Parasite, *Clinostomum complanatum*

3.2.1 Identifying characters (Figs. 7, 8, 9)

The wormlike bodies appeared tongue shaped and were bluntly rounded at the anterior and posterior ends, Body length varies between 5.2-6.7(0.252± 0.046), while body width 1.6-2.2(2.593±0.466).Body length/width varies from 2.75-3.6(12.69 ± 6.467).The size varied from medium to large size, stout with protuberant dorsal side and saucer shaped ventral end (Fig. 7a). Oral sucker oval, subterminal, encircled by two band like wrinkles which occurs when the sucker is retracted and its surface is covered by ridges, pits, and pores of glands (Fig. 7a and b). Oral sucker length ranges between 0.094-0.170 (0.084±0.004), while oral sucker width varies between 0.200-0.380(1.77±0.92).Oral sucker width/body width ranges between 0.125-0.136(9.1121±6.034).Muscular ventral sucker, larger in size than oral sucker positioned in the anterior 1/3th of the body, exhibits sponge-like characters with ventral surface bearing grooves and folds (Fig. 7a and b). Ventral sucker length ranges between 0.702-0.953(0.321±0.019), while width varies between 0.810 -0.920(0.57 ± 0.12).Ventral sucker width/body width 0.418-0.5625(16.85 ± 8.12). Caeca long located mainly in the anterior part of the body without diverticula. Testes smooth or irregular in shape, anterior testes lobe lies in the middle and posterior lobe positioned in the rear end of the parasite. Both the testes separated by intertesticular space (Figs. 7b, 8). Anterior testis length ranges between 0.312-0.536(3.97±0.002), while anterior testis width ranges between 0.283-0.480(1.07±0.432). Posterior testis length ranges between 0.287-0.525(0.074±0.004), while posterior testis width, 0.471-0.515(2.017±0.222). The uterus intercaecal and projects from the caudal region of posterior sucker to intertesticular space (Figs. 7b, 8). The cirrus-sac and genital pore pretesticular or lateral to anterior testis. Ovary ovoid or rounded in shape, intertesticular, submedian lies on the right side of the body (Fig. 7b). Ovary length 0.156-0.168 (0.337±0.0129), ovary width, 0.112-0.152(0.972 ±0.156), ovary length / width 1.14-1.39(5.62 ±1.116). Vitelline follicles lies in between posterior extremity and level of ventral sucker. A comparative measurement chart of few known species of the genus is given in Table 3.

3.2.2 Scanning electron microscopic structure

Scanning electron microscopic study of the trematode revealed elongated body, aspinous, smooth and reticulated tegument with rounded extremities bearing two suckers (Figs. 8, 9a). The rim of both the oral and ventral suckers in the present metacercaria were aspinous and non-papillated while the floor of the ventral sucker exhibited papillae (Fig. 9a). Oral sucker subterminal in position with rounded opening surrounded by two collar rings and region around the sucker surrounded by ridges and pits (Fig. 9b). The postacetabular region had a spongy appearance (Fig. 9c). The ventral sucker oval in shape, larger in size and the floor exhibited wavy wrinkles with dome shaped papillae (Fig. 9c and d).

3.2.3 Taxonomic affinity

Site of infection: Swim bladder, liver, intestine.

Type locality:

Ranaghat, Nadia, West Bengal 23.1740° N, 88.5639° E.

Chandhanagar, Hooghly, West Bengal 17.4936° N, 78.3253° E.

Prevalence and intensity: 5/30 (16.66%), 1-3 specimens per fish

Specimens deposited: Slide bearing number PR/IK/TR/C-07 and PR/IK/TR/C-11 have been deposited in the Parasitology laboratory, Department of Zoology, University of Kalyani, West Bengal, India

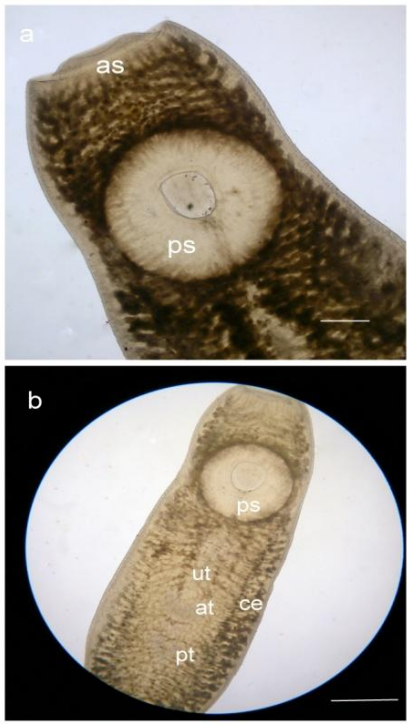

Fig. 7. Light microscopic structure of *Clinostomum complanatum*
a. Structure of *C.complanatum* showing the position of anterior sucker (AS) and posterior sucker (PS). Scale bar- 500 µm
b. Structure of *C.complanatum* showing the position of anterior sucker (as), posterior sucker (ps), anterior testis (at), posterior testis (pt), ovary (ov) and uterus (ut). Scale bar- 500 µm

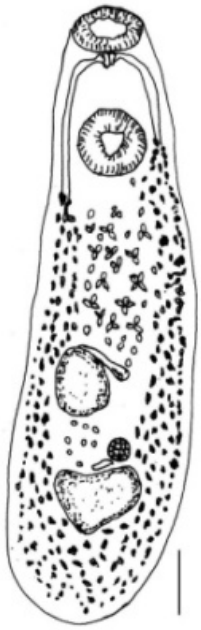

Fig. 8. Camera lucida diagram of *C. complanatum*. Scale bar-500 µm

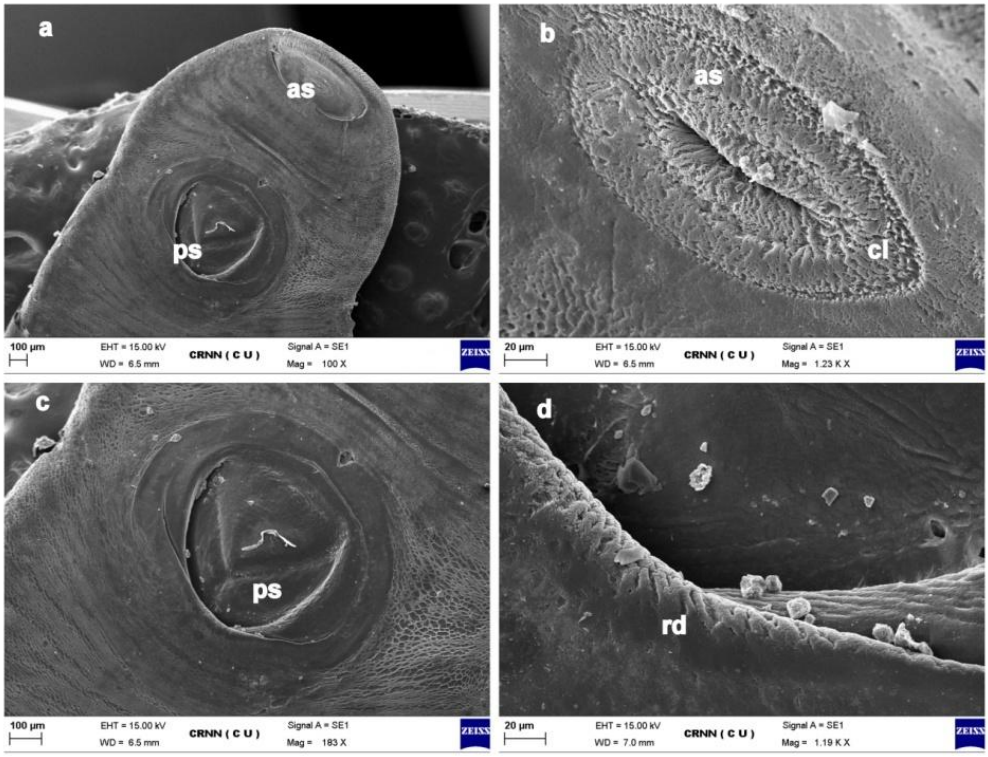

Fig. 9. Scanning electron microscopic structures of *C. complanatum*
a. Metacercaria showing presence of anterior sucker (as), posterior sucker (ps), oral sucker surrounded by double wall, smooth and aspinous
b. Subterminal oral sucker surrounded with collar ring
c. Posterior sucker surrounded by aspinous rim, smooth and deeply seated
d. Rim of posterior sucker with pits and ridges (rd)

4. DISCUSSION

E. heterostomum was erected as type specimen collected from purple heron (*Ardea purpurea*) by Travassos [14] hence resulting in the establishment of the genus *Euclinostomum*. Seventeen species of metacercariae stage of *Euclinostomum* sp. have been reported from worldwide surveys and the trematode was not found to exhibit host specificity. Although several *Euclinostomum* species have been described, Ukoli, Dennis and Sharp [15,16] regarded only *E. heterostomum* and *E. multicaecum* Tubangui and Masilungan (1935) as valid species.

The present work involves detailed study of the trematode using light microscopy, scanning electron microscopic studies as well as molecular analysis. As far our knowledge this is the first report of the scanning images of the metacercariae of *E. heterostomum* from *C. punctata* from India along with molecular charecterisation. The morphometric analysis of the parasite was found to be similar with that of Jhansilakshmibai and Madhavi [2] which was performed by light microscopic studies only. Jhansilakshmibai and Madhavi [2] studies, dimensions of ovary and distance between suckers of the parasite were not elaborated in their paper, which has been described in the present work. Purivirojkul and Sumontha [5] description on the morphometrics of the parasite were comparatively smaller in comparison to the trematode studied particularly in relationship to dimensions of the oral and ventral suckers as well as reproductive organs.

New characters which were observed by scanning electron microscopy studies of the tegument includes aspinous surface, spongy tissue and transversely folded areas. Presence of dome shaped papillae with microridges and micropores on the border as well as floor of the both the suckers, smooth margins of the suckers, absence of spines, along with presence of crates or folds were observed. Cobble stone like structure in the post acetabular region was also observed. All the new characters reported in this work by scanning electron microscopy was not observed by Jhansilakshmibai and Madhavi [2],

Purivirojkul and Sumontha [5] and Suanyuk et al. [17]. Aspinous surface of the tegument were also found in other flukes such as *Gorgoderina attenuata* [18]; *Leucochloridium* sp. [19]; *Phyllodistomum umblae* [20]; *Concinnum epomopis* [21] and *Austrobilhariza variglandis* [22]. Similarly presence of lobulated papillae in oral sucker were found in *Schistosoma mansoni* [23], *Diplostomum phoxini* [24] and *Gorgoderina attenuate* [18]. Papillae which surrounds the mouth could provides proximity for reception which would help in feeding [25]. Scanning electron microcopic studies of *E. heterostomum* obtained from guppies *Poecilia reticulata* by Suanyuk et al. [17] were morphologically similar to those observed by Jhansilakshmibai and Madhavi [2] but the characters reported in this study was not observed by them. In the present study, the parasites corresponds in morphology and measurement to that of the larval stage of *E. heterostomum* (Rud 1809) Travassos, 1928 as observed by Vijaylakhsmi et al. [26] which was performed with only with light microscopic studies. A comparative measurement account of the genus is given in Table 2.

In recent times molecular characterization has added to the identification of parasites in addition to morphological charecterisation. Although not much data of 28S RNA molecular sequences of *Euclinostomum heterostomum* are available so phylogenetic tree was constructed in comparison with 28S RNA sequence of *Clinostomum* sp which was available in Genbank. In the present study phylogenetic tree was constructed using 28S sequences and it was possible to differentiate *Clinostomum* sp clade from *Euclinostomum* sp. Athokpam et al. [27] performed molecular sequencing with rDNA ITS2, 28S and 18S sequences from the *Euclinostomum* metacercaria and the ITS 2 sequence formed a separate branch from other Clinostomidae taxa in the phylogenetic analysis. Phylogenetic tree for 28S and 18S sequences was not available. Caffara et al. [28] reported that *Euclinostomum* parasites formed a monophyletic group with sequences internal transcribed spacer 1 and 2, 5.8S rDNA and cytochrome c oxidase I collected from the specimens from Israel. They have claimed that the samples collected by them are more likely to be *E. heterostomum* than that of Purivirojkul and Sumontha [5], Athokpam et al. [27] or Senapin et al. [29]. Thus for proper identification at the species level more parasites are needed to be collected world wide from variant hosts which would shade light in the proper phylogenetic analysis and comparison.

Leidy [30] erected the genus *Clinostomum* with *C. gracile* as its type-species from fishes and frogs. In the present study the morphometric dimensions of the parasite corroborates with the study of Caffara et al., [4] and Simsek et al. [31] however Echi et al. [3], Gholami et al. [32] and Ngamniyom et al. [33] reported structural measurements in terms of body length, anterior and ventral suckers which was smaller than the studied parasite. Scholz et al. [34] reported dimensions which were even higher than those reported from the present study. A comparative measurement account of the genus is given in Table 3. The position of the cirrus sac was found to be similar as reported by Caffara et al. [11] which helps in differentiating amongst various species of *Clinostomum*. The topographical surface of the trematode were till date mostly were reported mainly by light microscopic study in India but scanning electron microscopic study was performed on the parasite for the first time which showed additional features which were reported.

Scanning electron microscopic studies showed that the rim of both the oral and ventral suckers in the present metacercaria were aspinous and non-papillated while the floor of the ventral sucker exhibited papillae which were similar to the reporting by Marwan and Mohammed [35]. Ngamniyom et al. [33] reported the presence of spines on the papillae of ventral sucker which was not observed in the present study. On the oral sucker ridges and pits were observed in the current study but Ngamniyom et al. [33] observed dome shaped papillae on the oral sucker. In some digeneans like *Orientocreadium batrachoides* the oral sucker had both spines and sensory papillae [36].

Spines with cytoplasmic ridges in posterior part of ventral region and the dorsal region with marked cobblestone like units were observed by Ngamniyom et al. [33] and the description corresponded with the morphology described by Abidi et al. [37] but such features were not observed in the specimen studied. Marwan and Mohammed [35] reported presence of papillae on the ventral region while the dorsal surface was marked by pores and apertures but such observations was not recorded in the trematode in the present study.

The tegument of the trematode *C. complanatum* studied was charecterised by reticulated appearance which has been previously been reported on metacercariae of *Leucochloridiomorpha constantiae* [38] and maturing *S. mansoni* [39,40,41]. The reticulate nature of the tegument results in increased surface area and displays numerous loci like structure which helps in anchorage helping the trematode to slide over the visceral organs like swim bladder.

Table 3. Comparative chart of morphometric charecters of metacercariae stage of *Clinostomum complanatum* in fishes

Morphometric charecters	***Clinostomum complanatum*** **Gholami et al., 2011(mm)**	***Clinostomum complanatum*** **Caffara et al., 2011(mm)**	***Clinostomum complanatum*** **Simsek et al., 2018**	***Clinostomum complanatum*** **Present study (mm)**
Body length	2.92-5.81	4.495–7.874	3.998–6.718 (5.108 ± 0.34)	5.2-6.7 (0.252±0.046)
Body width	1.34-1.99	1.635–2.434	1.197–2.131 (1.697 ± 0.16)	1.6-2.2 (2.593±0.466)
Body length/Width	-	2.200–4.369	2.580–3.526 (3.045 ± 0.21)	2.75-3.6 (12.69 ± 6.467)
Oral Sucker length	0.10-0.18	0.259–0.337	0.243–0.319 (272 ± 11)	0.094-0.170 (0.084±0.004)
Oral Sucker width	0.28-0.49	0.284–0.507	0.261–0.483 (336 ± 36)	0.20-0.38 (1.77±0.92)
OS width/Body width	-	1.065–1.666	-	0.125-0.136 (9.1121±6.034)
Ventral sucker length	0.25-0.92	0.637–0.910	0.581–0.821 (682 ± 36)	0.702-0.953 (0.321±0.019)
Ventral sucker width	0.36-0.77	0.766–0.952	0.679–0.893 (763 ± 32)	0.810-0.920 (0.57± 0.12)
VS width/Body width	-	1.781–2.694	-	0.418-0.5625 (16.85 ± 08.12)
Anterior testis length	-	0.316–0.957	0.290–0.711 (472 ± 65)	0.312-0.536 (3.97 ± 0.002)
Anterior testis width	-	0.273–0.559	0.203–0.498 (409 ± 45)	0.283-0.480 (1.07 ± 0.432)
Posterior testis length	-	0.245–0.441	0.219–0.398 (321 ± 24)	0.287-0.525 (0.074±0.004)
Posterior testis width	-	0.408–0.602	0.384–0.564 (456 ± 28)	0.471-0.515 (2.017 ± 0.222)
Ovary length	-	0.135–0.164	0.120–0.158 (139 ± 5.7)	0.156-0.168 (0.337 ± 0.0129)
Ovary width	-	0.097–0.178	0.81–01.49 (110 ± 9.9)	0.112-0.152 (0.972± 0.156)
Ovary length/ width	-	0.589–1.086	0.67–0.94 (0.78 ± 0.01)	1.14-1.39 (5.62 ±1.116)

5. CONCLUSION

Thus along with morphological data, molecular data helps in better charecterisation of the parasites detected from fishes and would help in phylogenetic analysis and creation of data bank of parasites infecting fishes.

ETHICAL APPROVAL

Animal ethical care guidelines were followed as edible fishes were used in the study. It has been informed that as per CPCSEA instruction's protocol for experimentation on edible fishes, does not require approval.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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