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Molecular and scanning electron microscopy (sem) associated energy dispersive X-Ray analysis (EDXA) characterization of *Euclinostomum heterostomum* (Rudolphi, 1809) (Trematoda) from *Channa striata* (Bloch, 1793)

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Abstract

Euclinostomum heterostomum (Rudolphi, 1809) metacercaria worldwide distributed digenetic trematode parasites distinguish to infect Channidae species. Molecular analysis using partial nuclear ribosomal internal transcribed spacer (ITS rDNA) marker was carried out and resultant 1005 base pairs. Phylogeny analysis showed that the present species claded among *Euclinostomum* with 100% nodal value and came close to *E. heterostomum* (KC894799) sequence from Thailand with 99% similarity. Scanning electron microscopy (SEM) was used to document the structural details of the oral and ventral suckers. EDX peaks revealed the presence of sulphur (S), calcium (Ca), and chloride (Cl) micro-elements from the entire tegument.

Keywords: ITS rDNA, EDXA, *Euclinostomum*, channidae, phylogeny

1. Introduction

Energy dispersive X-ray (EDX-SEM) analysis is a nondestructive method for the microanalysis of element composition and is effectively implemented in many research disciplines [1, 2]. This technique is used to detect unique concentrations of chemical elements within living and non-living samples, and involves understanding the complexity of various parasitic species i.e., trematodes, cestodes and acanthocephalans [3, 4], and pathological alterations [5]. In the parasitological field, EDXA has been used for analysis of chemical elements in the body of helminths, especially the anchorage (spines, hooks) structures [6, 7].

Subfamilies Euclinostominae (Yamaguti, 1958) belongs to the family Clinostomidae (Lühe 1901), with *Euclinostomum* [8] is the type genus represents as a sole *Euclinostomum heterostomum* species. Rudolphi [9] was first described as *Distoma heterostomum* in the oesophagus of *Ardea purpurea*. Later, Travassos [8] erected *Euclinostomum* with *E. heterostomum*. Previous reports have published on the pathological effects on the liver and kidney due to metacercariae of *E. heretostomum* in the species of the Channidae family [10, 11]. Thus, the present paper is the extensive work to describe the ultrastructural details along with micro-element analysis and molecular characterization of *E. heterostomum*.

2. Materials and Methods

2.1 Sample collection

The host *Channa striatus*, were caught from nearby the markets of the Lower Lake. Fishes were euthanized, dissected following standard necropsy procedures, and their internal organs were removed and examined for the presence of encysted trematode parasites under a stereozoom microscope. Parasites collected were kept in normal saline (0.75%) for excystation. The parasites were identified according to the keys given by Jhansilakshmbai and Madhavi [12].

2.2 Scanning Electron Microscopy (SEM)

For scanning electron microscopy (SEM) - buffered glutaraldehyde (pH 7.5) fixed excysted specimens for 24 hrs, followed by three buffer washes and post-fixed in osmium tetroxide.

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The samples were dehydrated in ethanol series, dried and gold coated, and observed under a Scanning Electron Microscope coupled with energy-dispersive X-ray spectrometry (SEM- EDX, (Carl Zeiss SEM model No. EVO18) at 20 kV.

2.3 Molecular analysis

Ethanol-fixed trematode samples were used for the extraction of genomic DNA using a DNA purification kit (HIMEDIA, India), following the manufacturer's instructions. The partial ITS regions of the ribosomal RNA gene was amplified by PCR using the forward primer (BD1- 5'GTCGTAACAAGGTTTCCGTA3') and reverse primer (BD2- 5'TATGCTTAAATTCAGCGGGT3')^[13] with modifications. The thermocycler program included an initial denaturation at 96.0 °C for 3 min, followed by 35 cycles of 95.0 °C for 27 s, 50 °C for 30 s, 72 °C for 40 s and a final elongation of 72 °C for 10 min. PCR products obtained using both primers were resolved on 1.5% agarose gel stained with ethidium bromide. The positive DNA bands were sequenced (Sanger sequencing) through a commercial firm.

2.4 Phylogenetic analysis

Forward and reverse sequences acquired were assembled to generate contigs using BioEdit^[14], and the resultant 1005 bp sequences was deposited in NCBI GenBank (Accession numbers- MT785768). NCBI BLAST-N was performed for the phylogenetic analysis carried out for the present sequence along with 8 other closest representatives *Euclinostomum* species along with 4 sequences of *Clinostomum* species retrieved. Multiple sequences alignment was done using Clustal W^[15] along with sequences of *Diplostomum* species (Accession no. AY123042; AY123044; AY123043) as an outgroup. For tree construction, multiple sequence alignment of 16 sequences including the sequences of the present study was performed in Mega X^[16]. Bayesian and Akaike Information Criteria revealed Kimura 2-parameter model with I value 0.38 as a best-fitting evolutionary model for Maximum Likelihood (ML) analysis, whereas Neighbour Joining (NJ) analysis was carried out using default parameters with 1000 replications.

3. Results and Discussion-

3.1 Scanning electron microscopy

Scanning electron micrographs showed the thick oral sucker with small round opening whereas the ventral sucker appeared with thick collar-like rim and triangular-shaped opening. The rim apparent showed the presence of irregular and uneven

protrusions (Fig.1). The ventral sucker opening also exhibited the presence of globule like structure present internally.

3.2 Energy Dispersive X-Ray Microanalysis

The micro-element peaks of sulphur (S), calcium (Ca), and chloride (Cl) were determined for the entire tegument of *E. heterostomum*. Calcium (5.56%) is the most dominant element followed by chloride (1.39%) and sulphur (1.19%). Gold (Au), Palladium (Pd) and Osmium (Os) peaks are due to specimen preparation (Fig. 2; Table 1).

3.3 Molecular and phylogenetic analysis

Molecular sequencing of the present *E. heterostomum* involving ITS1- 5.8S and ITS2 rDNA region resultant 1005 bp nucleotide was submitted to GenBank (accession numbers: MT785768). In BLAST N analysis, the present sequences showed maximum identity (99.90%) with *Euclinostomum* sp. (KC894799). This was followed by *E. hereostomum* (KP721430) with 98.99% and Clinostomidae (KF577720) with 94.01% identities. NJ and ML trees generated with concatenated ITS region sequences revealed similar clustering without any topological variations. In both the phylogenetic trees, genus *Euclinostomum* formed a monophyletic clade along with sister clade of *Clinostomum* sp. under family Clinostomidae (Fig. 3, 4). Sequences of the present parasite (MT785768) grouped with 100% nodal value and phylogeny analysis showed closed resemblance to the *E. heterostomum* (KC894799) sequence from Thailand.

Radwan *et al.*^[17] revealed three chemical elements sulphur, calcium, and phosphorus of tegumental spines of four digenean species by using energy dispersive X-ray microanalysis. Sulphur, calcium, and phosphorus are described as major element in biological molecules in animals. These elements are also known to associate with the development and function of helminths hooks and spines^[18, 19, 20]. But, in contrast with the above findings present study showed the presence of chloride (Cl) also whereas phosphorus is absent.

Molecular and phylogenetic results of the present species came close to the finding of Senapin *et al.*^[21] which showed the phylogeny of the genus *Euclinostomum* from Trichopsis and Betta fish. They also represented a monophyletic clustering of *Euclinostomum* sp. from Thailand (KC894799) and with European species (KP721439, KP721435, KP721427, KP721430, KP721437, and KP721438)^[22], whereas *Euclinostomum* and *Clinostomum* forming distinct sister monophyletic genus clade^[21].

Table 1: Showing the micro-element (wt %) composition of the *E. heterostomum*

Element	Weight %	Atomic %	Net Int.	Error %	Kratio	Z	R	A	F
CK	19.30	43.62	1191.73	8.66	0.0740	1.2587	0.8342	0.3048	1.0000
OK	25.54	43.33	1540.75	9.59	0.0570	1.2117	0.8587	0.1843	1.0000
SK	1.19	1.01	222.43	10.11	0.0085	1.0973	0.9335	0.6420	1.0099
ClK	1.82	1.39	304.94	8.02	0.0127	1.0457	0.9413	0.6577	1.0144
PdL	3.60	0.92	303.24	7.55	0.0247	0.8388	1.1237	0.7961	1.0293
CaK	5.56	3.76	761.81	4.73	0.0476	1.0651	0.9630	0.7819	1.0286
OsL	10.72	1.53	205.63	11.10	0.0951	0.6764	1.1264	1.0294	1.2754
AuL	32.28	4.45	381.05	8.84	0.2476	0.6600	1.1059	1.0263	1.1328

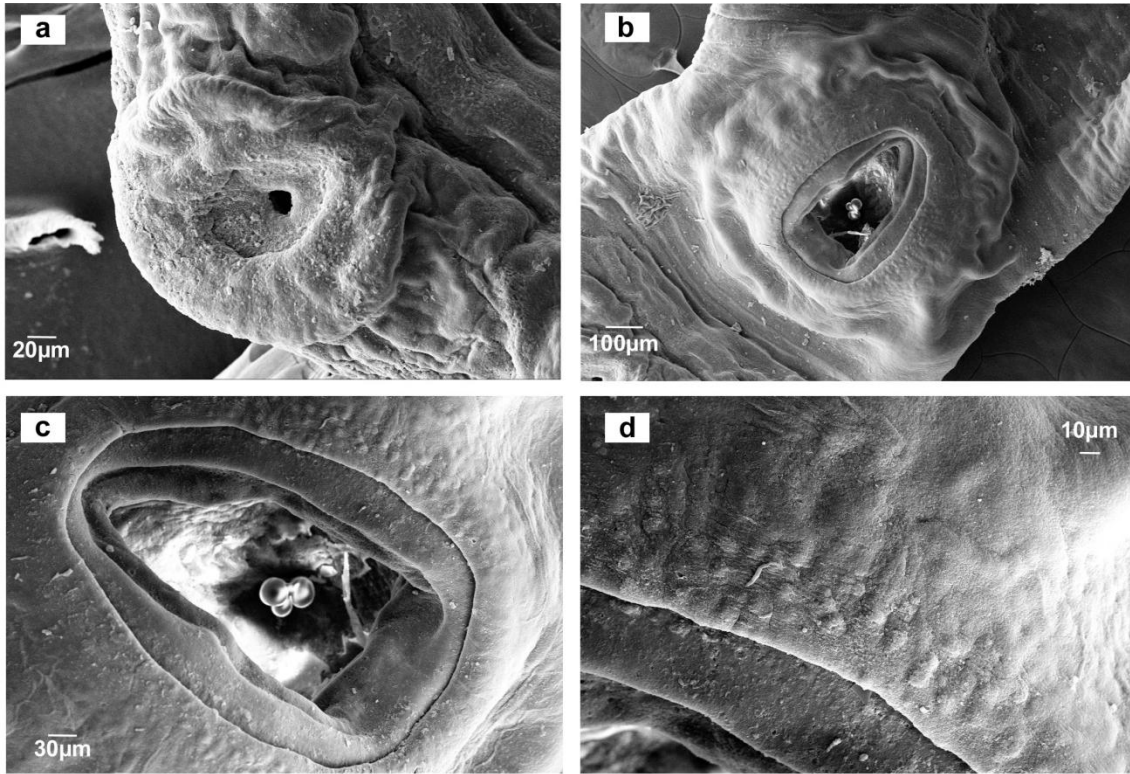


Fig 1: SEM images of *E. heterostomum* a) oral sucker; b) ventral sucker; c) enlarged view of ventral sucker; d) irregular and uneven protrusions of ventral sucker

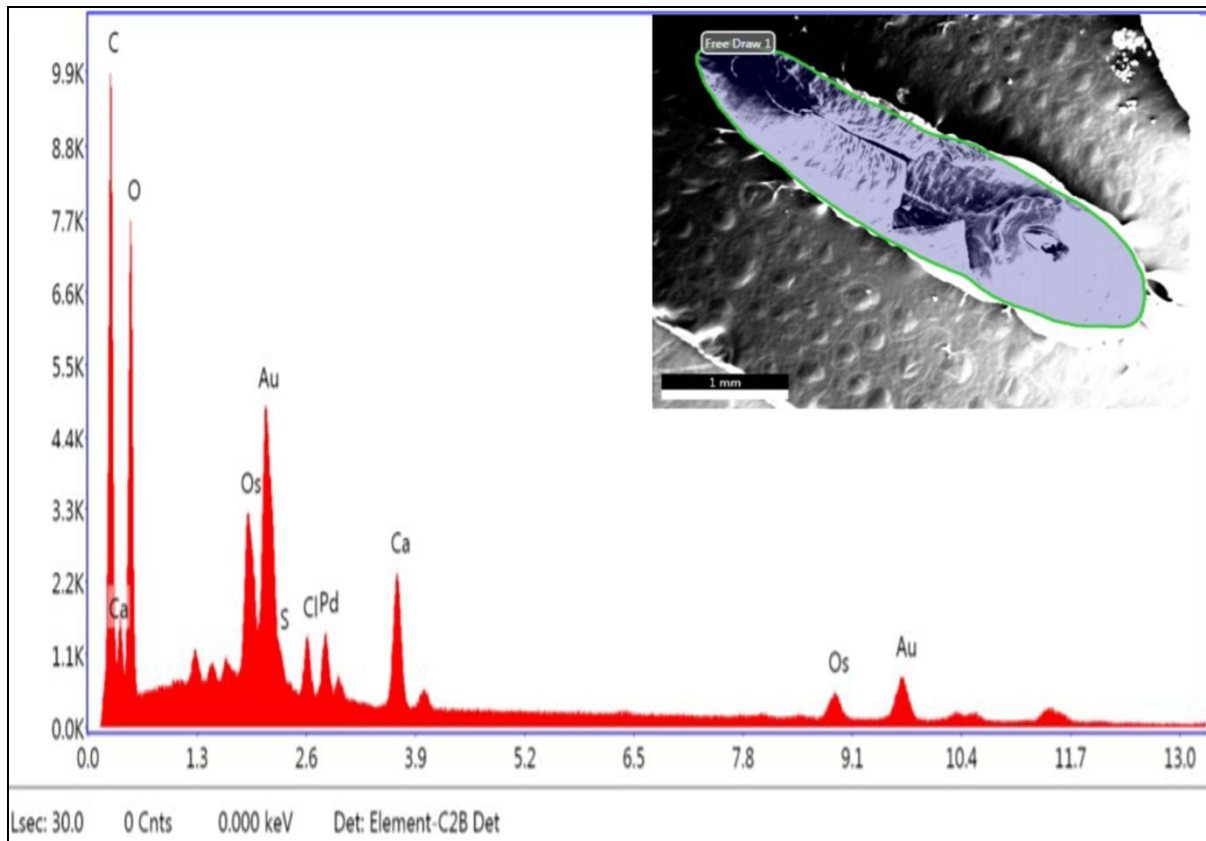


Fig 2: Energy dispersive X-ray microanalysis (EDXA) of the *E. heterostomum* showing micro elements representing their respective KeV peak and wt %

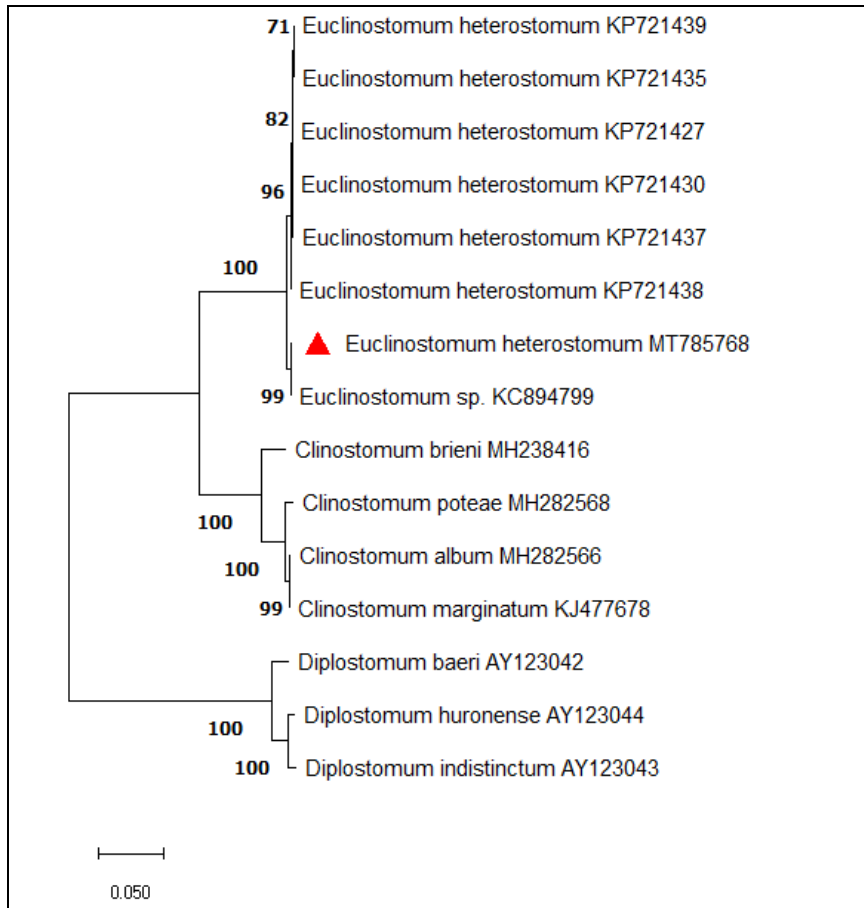


Fig 3: Phylogenetic analysis for *E. heterostomum* - Neighbour joining (NJ) tree for ITS region, present sequence is marked as

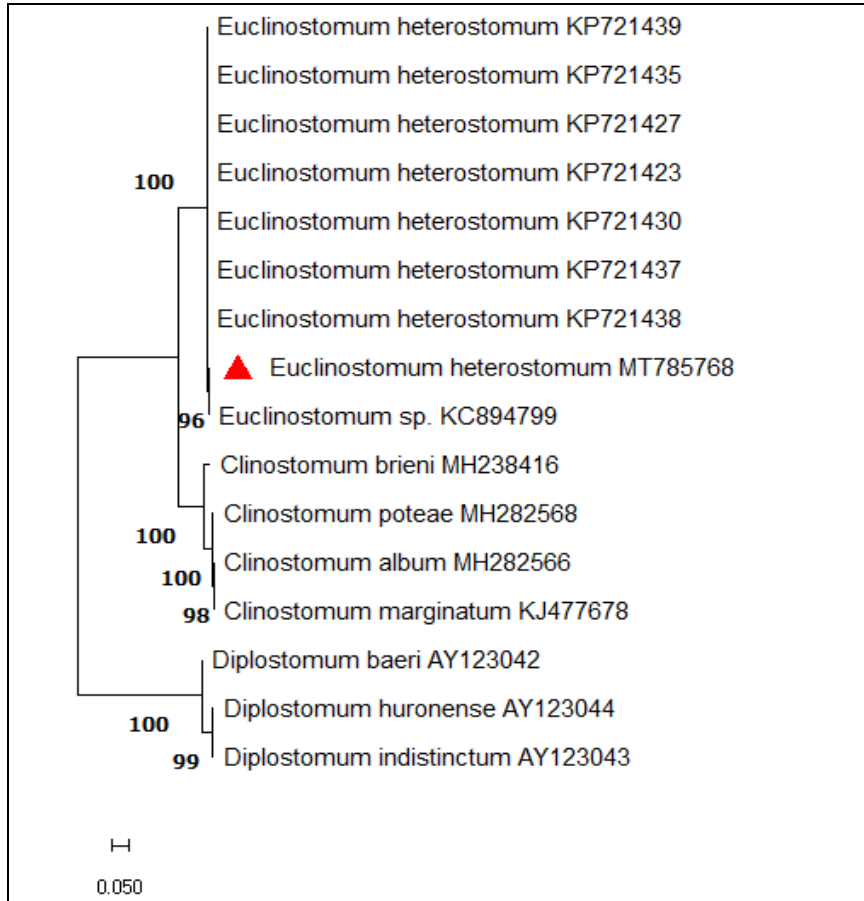


Fig 4: Phylogenetic analysis for *E. heterostomum* - maximum likelihood (ML) tree for ITS region, present sequence is marked as

4. Conclusion

The current study elucidates molecularly confirms and assigns the present genus *Euclinostomum* belongs to the single species *E. heterostomum* along with ultra-structural details and micro-element composition using advanced microscopy techniques.

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