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The infection of *Euclinostomum sp.* in *Channa punctatus* with molecular with special and morphological study from Koderma reservoir, Jharkhand, India

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ABSTRACT

Worldwide, the parasite family Clinostomidae is widely dispersed. *Euclinostomum* sp., a common digenetic trematode, has a metacercariae stage that is ideal for infecting Channidae species because it becomes encysted in their kidneys, liver, and muscles. This paper aimed to identify *Euclinostomum sp*. in *Channa punctata* by means of a genetic and morphological combination of approaches. The morphological characteristics of the species were examined using both a light and scanning electron microscope. This parasitic organism was fascinatingly exposed by the SEM investigation, with its flattened, leaf-shaped body covered with surface characteristics. Sequencing the purified PCR results from many worms using *Euclinostomum sp.*'s 18sRNA gene produced sequences of 1700 bp nucleotides, on average. The NCBI has assigned *Euclinostomum* sp. the accession number OQ286054. The phylogenetic reconstruction demonstrated a strong genetic resemblance across the various strains of *Euclinostomum*, suggesting a common genetic ancestor. Compared to the monsoon and post-monsoon, the pre-monsoon had the highest percentage of parasite prevalence.

Figures : 05 References : 31 Table : 01
KEY WORDS : *Channa punctatus*, *Euclinostomum sp,* PCR, Prevalence, 18sRNA, SEM

Introduction

Fish is essential to human diets as a low-cholesterol source of protein and as a means of generating subsistence income[2]. Since fish are now a major source of food for humans and some parasites have an adverse effect on human health, many parasitologists are interested in studying fish parasites[30]. The spotted murrel is a species of snakehead fish in the Channidae family, scientifically known as *Channa punctata*. Native to Southeast Asia, this warm-water teleost is known by many names as the spotted murrel. It can be found in restricted water as well as freshwater environments including lakes, ponds, and rivers[10]. Nonetheless, freshwater murres support a complex biological connection in their aquatic environment by acting as a common host and habitat for a wide variety of parasites[13,24].

Digenea is a large subclass of the class Trematode under the phylum Platyhelminthes, with about 25,000 species, they primarily have an oral sucker, an acetabulum, an underdeveloped digestive system, and a syncytial tegument that can be smooth or modified by spines, channels, or microvilli[5]. Clinostomidae is a family that includes subfamilies Euclinostominae of which *Euclinostomum* is the type genus shown as a sole[15]. The species *Euclinostomum heterostomum*, was initially identified as *Distoma heterostomum* in Ardea purpurea's oesophagus[16]. Later, *Euclinostomum* and *E. heterostomum* were established. The harmful effects of *E. heterostomum* metacercariae on the liver and kidney in animals belonging to the Channidae family have been documented in the past. The metacercariae stage of *E. heterostomum* uses fish (*Channa spp*.) as their intermediate host, and adults are usually found inside

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the heron's oral cavity, mainly at the base of the tongue[7,14]. Specific morphological characteristics have been used to identify species of *Euclinostomum sp.* Their unique taxonomic placements within this fluke group can be clarified by combining molecular methods with morphological investigations[7]. Up until now, the sole method used for the identification and characterization of trematode parasites has been morphological characterization. However, the identification of parasites has become clearer thanks to the development of genetic approaches.

This work set out to provide a thorough analysis that included molecular and morphological identification of parasite *Euclinostomum sp.* in *Channa punctata* (spotted snakehead) from the DVC Dam, a Koderma reservoir in Jharkhand.

Materials and Methods

Study area

This present research work was carried out in the Tilaiya Dam reservoir (24°19'26" N 85°31'16"). Situated in the Koderma district of Jharkhand, across the Barakar River, is a power station built by DVC. Rising 30.2 meters above the riverbed, the dam spans the Barakar River 64.4 kilometers downstream. 984 square kilometres make up the DVC Dam Tilaiya, which produces power[18]. The catchment area is composed of hilly topography that includes pastures, agricultural land, wastelands, villages, and forests[27].

Sample collection and preservation

Fish samples were collected from the sapling site for the study and were brought alive to the university's laboratory for fish identification and analysis to recover helminthic endoparasites. To do this, the fish body's superfluous mucus was removed, and the samples were dissected through the abdomen by cutting with a sterile blade along the midventral line from the anus (HiMedia, India) (Fig. 1). The liver, gills, and alimentary tract were separated and elongated, and the latter was divided into the stomach, esophagus, and intestine. A portion of this alimentary canal was examined under a microscope (Magnüs MLXi PLUS, India) at a magnification of 10× while being placed in 5 different Petri dishes with saline (0.6%). Next, using a brush to remove the helminthic endoparasites from a salt solution, PBS was used for washing (HiMedia, India). It was stored at -20°C in 95% alcohol (HiMedia, India) for molecular research.

Morphological examination of parasite

For the morphological examination, the cleaned worms were maintained in an AFA solution (a mixture of alcohol, formalin, and acidic acid) for a few days, after being flattened and fixed overnight semichon's acetocarmine was used for staining. Following fixation, whole-mount preparation involved dehydrating in a series of alcohol grades (70%, 80%, 95%, and 100%), cleaning in xylene, and mounting in dammar gum.

TABLE-1 : Morphometric measurements of metacercariae of *Euclinostomum sp*. isolated from the infected *Channa punctatus*

Euclinostomum sp.	Measurements (mm)
Body length	2.26
Body width	4.31
Oral sucker width	1.18
Ventral sucker length	1.12
Ventral sucker width	1.08
Anterior testes length	0.16
Anterior testes width	0.65
Posterior testes length	0.19
Posterior testes width	0.59
Ovary width	0.35
Ovary length	0.39

Scanning electron microscopy

The worms were ready for SEM after being treated and kept in 5% formalin. They were dried using an ethanol series and acetone, and gold-palladium was coated (4:1; thickness 20 lm), and exposed to critical point drying. After that, they were investigated at a 15 kV accelerating voltage using a Hitachi 2500 SEM.

Extraction of DNA from parasite

The kit, DN easy Blood and Tissue (Qiagen, Germany) was used to extract the sample's whole genomic DNA. In order to isolate DNA, the samples were cleaned using 1.5 ml micro tubes and phosphate-buffered saline (PBS) (HiMedia, India). The TAE buffer was added in an amount of around 300 µl. After that, the sample was homogenized using a homogenizer and incubated for an additional night at 37 °C in the lysis buffer that contained 10 µl of proteinase K and 0.2% sodium dodecyl sulphate (Sigma-Aldrich, Germany).

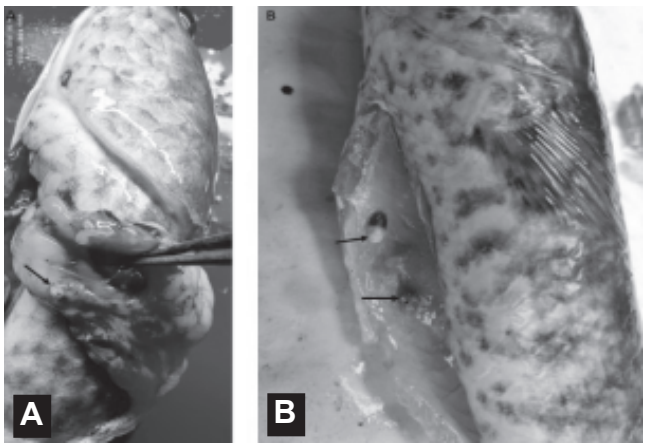

Fig. 1 : Photograph of the fish species parasitized: A) *Channa punctatus* infected by the encysted *Euclinostomum sp.* In liver (black arrow); B) Muscle infected by the encysted *Euclinostomum sp.* (black arrow).

Next, we added equal parts of isoamyl alcohol (250 µl), chloroform, and Tris saturated phenol (250 µl) (Sigma-Aldrich, Germany).

Amplification of target gene

For PCR amplification, the extracted DNA samples were utilized. 18S rRNA regions are the genetic markers that are employed in this process. Using the PCR procedure, the forward primer and the reverse primer were used to amplify the 18S rRNA gene for *Euclinostomum sp.* detection. 14 µl Water, 1 µl Taq buffer, dNTPs (2.5 µl), $MgCl_2$ (2.5 µl), genomic DNA (2 µl), and Taq polymerase (1 µl) made up the PCR master mixture. The PCR technique consists of an initial denaturation step of 5 m at 94 °C, 35 cycles of 30 m at 94 °C, 30 m at 54 °C, and 30 m at 72 °C, with a final extension step of 5 m at 72 °C. DNA concentration was measured with an Eppendorf UV-visible spectrophotometer (India). The parasite sample's genomic sequence bore 100% sequence similarity to that of the *Euclinostomum sp.* species. The accession number has been assigned as a representation after the sequence was submitted to the NCBI GenBank. The NCBI has assigned *Euclinostomum sp.* the accession number OQ286054.

Sequence and phylogenetic analysis

A different genomic sequence that is accessible in GenBank has been compared to the one that was created. Using the BLAST Search program, a similarity search was performed for the generated *Euclinostomum sp.* sequence. MEGA11 software was used to align with the produced sequence. The 18S rRNA sequencing data for *Euclinostomum sp*. that are in the public domain were all retrieved from GenBank; the taxa that are now accessible have been determined to represent important *Euclinostomum* genera. For a dataset containing 18S rRNA sequences from parasite species of *Euclinostomum sp.*, phylogenetic analysis was performed.

Parasite Prevalence, abundance and intensity estimation

Prevalence, intensity, and abundance were calculated and used a simple percentage (%). Prevalence of parasite infestation: The prevalence of parasite infestation was calculated using the formula[19] .

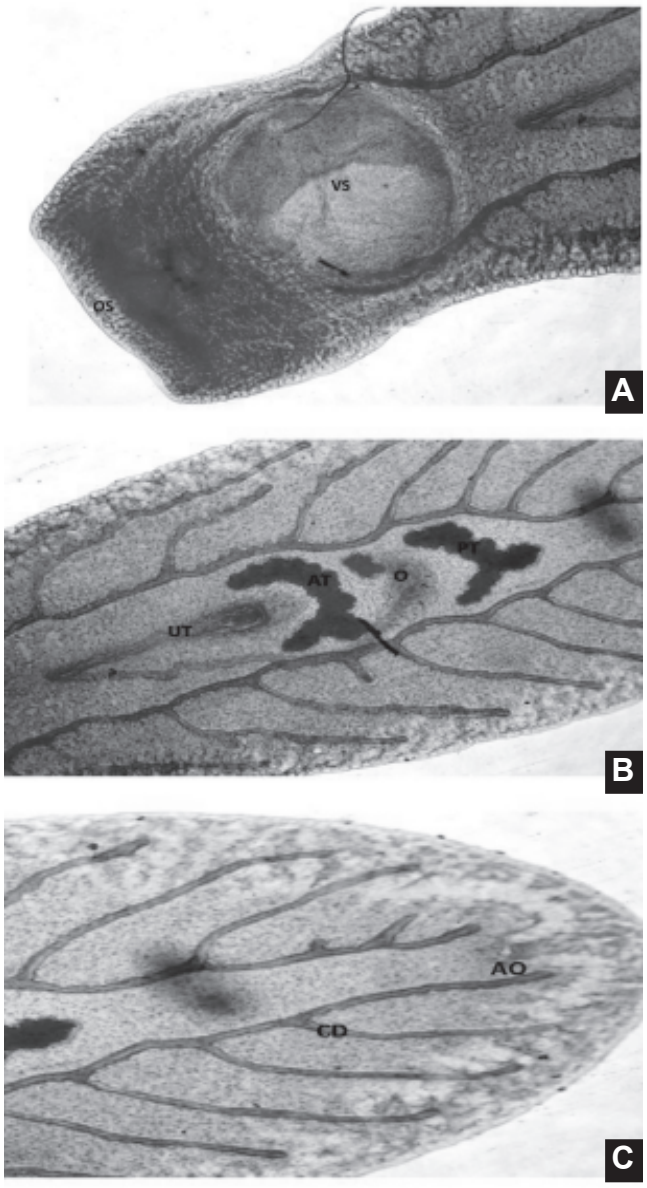

Fig. 2 : *Euclinostomum sp.* Isolated from the liver of the *Channa punctatus*, (A) Anterior part of the parasite (B) Middle part of the parasite, (C) Posterior part of the parasite, OS- Oral sucker; AT-Anterior testis; VS-Ventral sucker; UT-Uterus; PT-Posterior testis; O-Ovary; CD- Caecal diverticula; AO-Anal opening.

$$\text{Prevalence}(\%) = \frac{No.\,of\,fish\,infected}{total\,no.\,of\,\,fish\,host\,examined} \times 100$$

Abundance of parasites: The abundance of parasites was calculated using the formula[19].

$$\text{Abundance} = \frac{No.\,of\,\,parasites\,recovered}{Total\,no.\,of\,\,host\,examined}$$

Intensity of parasite: The intensity of parasites has been calculated using the formula[19].

$$\text{Intensity} = \frac{No.\,of\,\,parasites\,recovered}{No.\,of\,hosts\,infected}$$

Results

Morphological character

The thick, tight fibrous capsules surrounding the *Euclinostomum sp.* cysts have been stuck to the liver tissues. Cysts were visible as big, 2.5–5.0 mm in diameter, greyish-whitish peas lodged in tissues (Table-1). In comparison to other known metacercariae, *Euclinostomum* sp. was big and leaf-shaped. The bodies were broad rounded at the back, slightly truncated anteriorly, and robust. There was a little constriction that separated the bodies into wider posterior sections and narrower preacetabular regions. Larger intestinal ceca in the pre-acetabula region stretch laterally to the posterior ventral sucker of the body. There are pairs of interracial testes in the front and rear thirds of the body. The form of the front testis resembles a crescent or U shaped. The posterior testis has a larger, concave anterior border. The testes are amorphous and positioned at the posterior end of trematodes; the lobes of the anterior testes are U-shaped, while the lobes of the posterior testes are triangular. There is a genital hole opening on the edge of the anterior testis. The spineless tegument surface is visible (Fig. 2).

Molecular characterization of metacercariae

The 18sRNA gene of *Euclinostomum sp.* was used to directly sequence the purified PCR products from several worms, yielding sequences of 1700 bp nucleotides, respectively. OQ286054 is the accession number for the current investigation (Fig. 3). These DNA fragments were compared to other sequences in order to confirm that the parasites were *Euclinostomum sp.* (OQ286054) shared 99.74% nucleotide identity with *Clinostomum sp*. (KC894793) from Thailand, according to BLAST analysis. This specimen's nucleotide shared 97.38% of its similarities with the *Euclinostomum sp.* (KC894794) from Thailand and 97.33% with the *C. marginatum* (AY245760) from the USA.

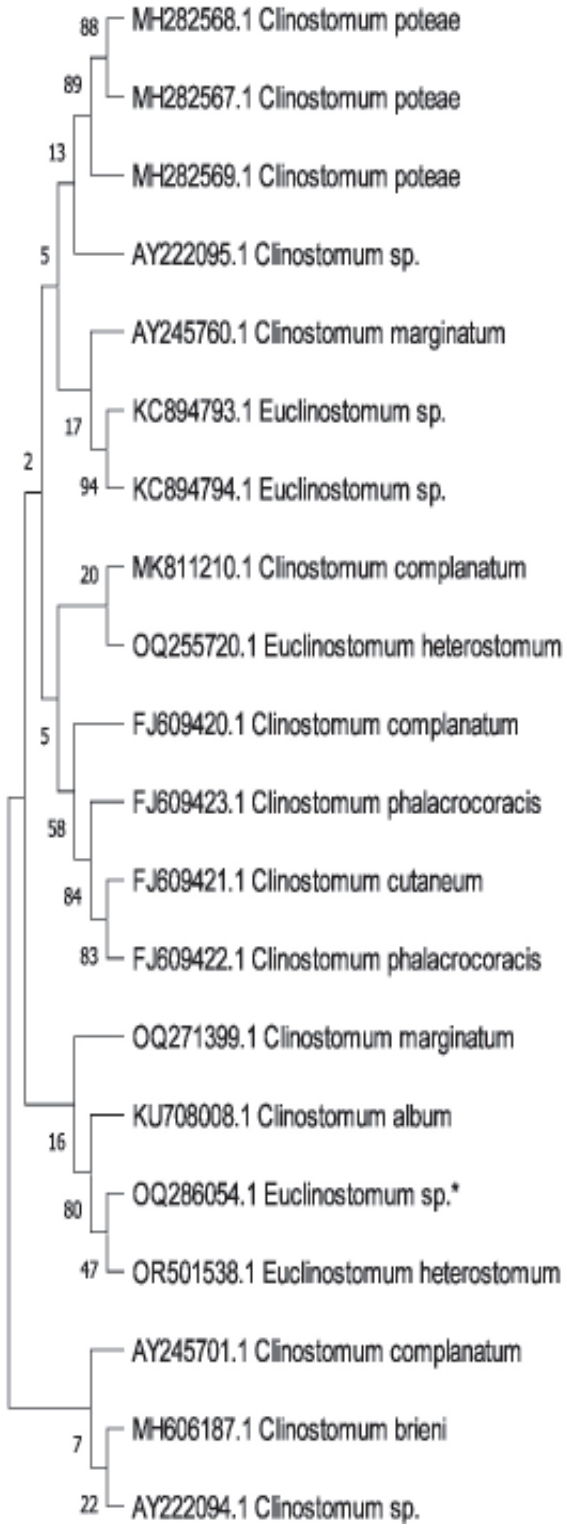

Fig. 3 : MEGA 11 is used to construct Phylogenetic by using the neighbour-joining method resultant from 1700bp 28S rRNA sequence of the distinguished *Euclinostomum sp.* The sequences were marked by names with an asterisk sign (*) and accession number.

Scanning electron microscopy of helminthic parasites

At the ultrastructure level, the ventral body surface of *Euclinostomum sp.* is smooth and devoid of scales or spines. According to (Fig. 4), the mouth opening is sub-terminal. Aggregations of irregularly shaped papillae-like structures surround the oral sucker in the tegument (Fig. 4). The ventral sucker has two domed papillae on its left bottom edge, is protuberant, rounded, and has a thick rim. It is spineless. The papillae are found anterior to the species and are smooth, spherical, and uneven in size. Situated at the extremity of the anterior part of the body, the ventral sucker has a circular form. Many papillae resembling those of the oral sucker are identified on the back of the body. It is that which might carry out a sensory task. The dorsal side's posterior end has an anal aperture.

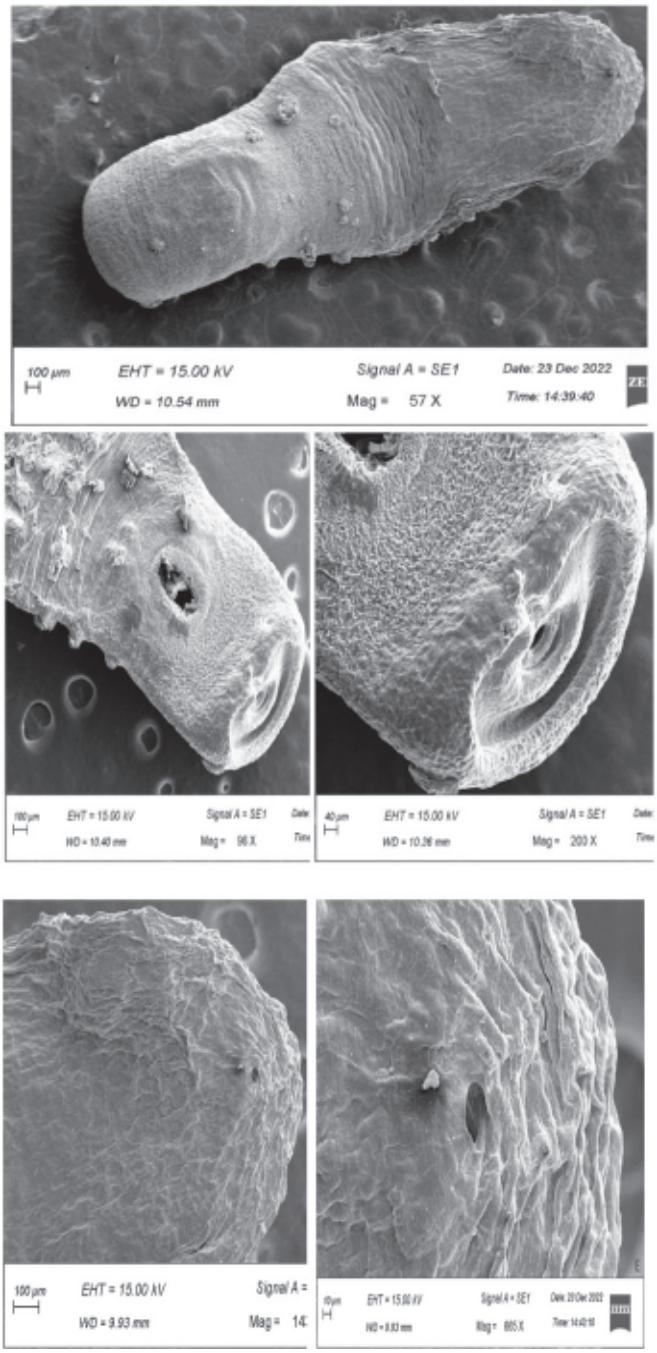

Fig. 4 : *Euclinostomum sp.* Isolated from the inner side of the liver; A, the Whole body of the parasite with a dorsal view; B&C, showing the anterior portion with the ventral and oral sucker of the parasite; D&E, show a posterior portion of the parasite with the anus.

Statistical analyses

The prevalence, intensity, and quantity of the infection were computed using a straightforward percentage (%). Examined were length range frequencies within the samples in relation to prevalence. The current investigation found differences in *Euclinostomum sp.* prevalence, quantity, and infection intensity in *Channa punctatus*. The data pertaining to the seasons is displayed in Fig. 5. Pre-monsoon had a higher proportion of prevalence of parasite than post-monsoon and monsoon periods. Compared to the monsoon and pre-monsoon, the parasite's strength was somewhat higher in the post-monsoon.

Discussion

Fish with parasite infections become malnourished and experience stunted growth because their energy is diverted into fighting the infection rather than absorbing nutrients from their food. To identify *Euclinostomum sp.* metacercariae in cichlid fish, several combined morphometric and molecular techniques were employed[7]. The observed sucker ratios are consistent with research reporting a one-third to one-half oral sucker-to-ventral sucker size ratio[9]. Numerous fish species[6,14,21], including tilapia and murrels, have been reported to be infected with *Euclinostomum heterostomum*. The present work describes the size of the ovary and the distance between the parasite's suckers, which were not elaborated in the previous study[14]. When compared to the trematode under study, the previous description[22] of the parasite's morphometric was significantly smaller, especially when it came to the size of the reproductive organs and the oral and ventral suckers.

Phylogenetic analysis revealed a close relationship between the present parasite's (OQ286054) sequences and the Thailand *E. heterostomum* (KC894793) sequence, with the latter grouping with a 100% nodal value. Genus *Euclinostomum's* phylogeny from Trichopsis and Betta fish was demonstrated by previous workers and the molecular and phylogenetic results of the current species closely matched their findings[7]. *Euclinostomum sp.* from Thailand (KC894799)

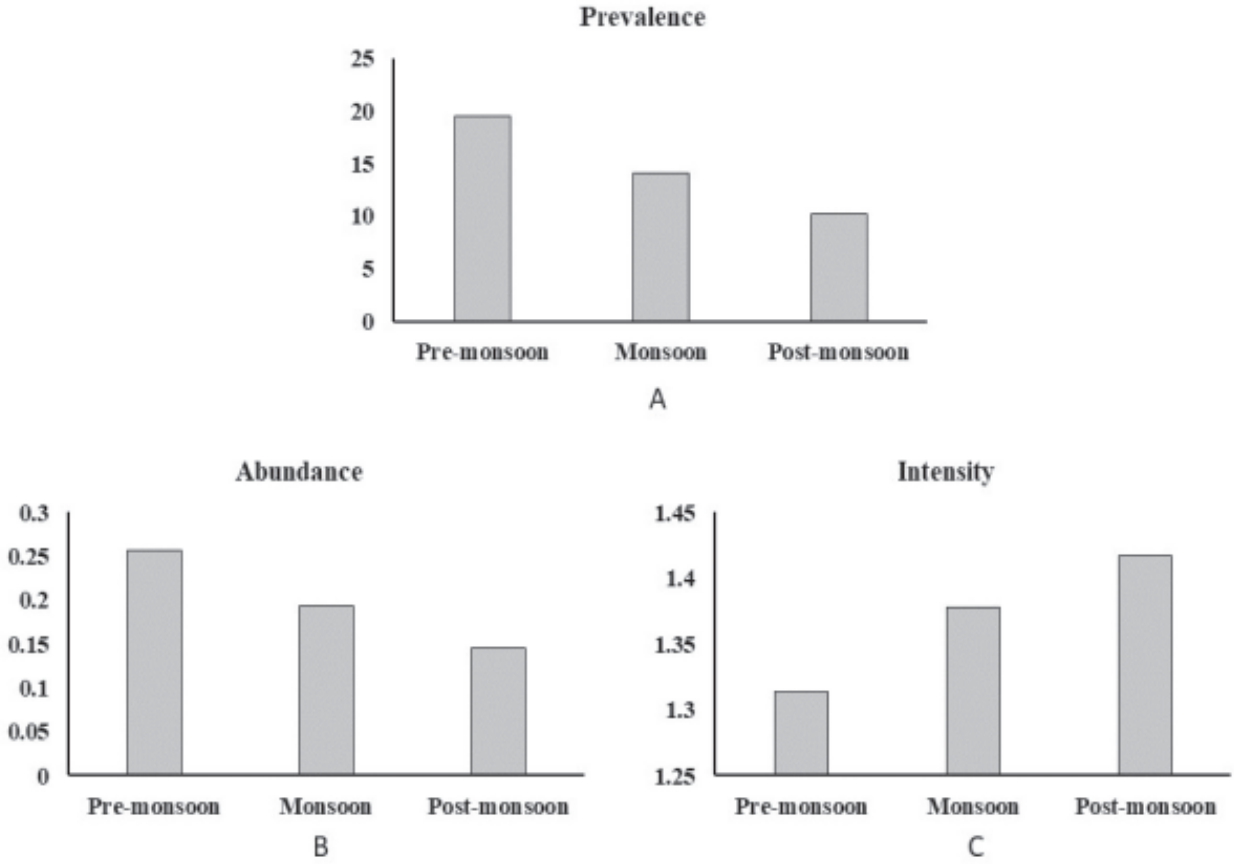

Fig. 5 : Graph showing the prevalence, abundance, and intensity of parasites found in *Channa punctatus* infected with *Euclinostomum sp.* In which prevalence and abundance are highest in pre-monsoon but the intensity is higher in post-monsoon.

and European species (KP721439, KP721435, KP721427, KP721430, KP721437, and KP721438) were likewise represented in a monophyletic clustering, while *Euclinostomum* and *Clinostomum* formed a separate sister monophyletic genus clade[7]. It was feasible to distinguish between the *Clinostomum sp.* clade and the *Euclinostomum sp.* clade using the phylogenetic tree that was generated in the current study utilizing 28S data. Using rDNA ITS2, 28S, and 18S sequences from *Euclinostomum* metacercariae, conducted molecular sequencing[3]. In the phylogenetic analysis, the ITS 2 sequence formed a distinct branch from other Clinostomidae groups. There was no available phylogenetic tree for the 28S and 18S sequences.

By the SEM analysis, with its flattened, leaf-shaped body decorated with surface features, this parasitic organism is revealed fascinatingly. An elongated, broad-bodied structure with rounded ends and a sub terminal oral sucker positioned strategically in the front portion of the organism was readily seen by SEM imaging. The presence of a collar-like ventral sucker, which aids in the parasite's capacity to cling securely to host tissues, was also revealed by the SEM microphotographs. Other trematode species and even another *Clinostomid,* the common species *Clinostomum complanatum* (Rudolphi, 1814), have also been reported to possess sensory papillae[1]. These papillae most likely serve as chemoreceptors[25], identifying micronutrients and the mucous membrane of each unique microhabitat in sensitive hosts. The papillae of the ventral sucker were found to have spines[20], but this was not seen in the current investigation. While dome-shaped papillae was noted on the oral sucker[20], the current investigation found ridges and pits on the oral sucker. The oral sucker of certain digeneans, such as *Orientocreadium batrachoides*, have both sensory papillae and spines[12].

The monsoon and pre-monsoon seasons are typical when the largest numbers of parasites are observed. In our experiment, the contagion rate varied depending on the season. The pre monsoon period in the host fishes was when the cestodes were most prevalent, having ended the primary reproductive season. The parasite was most prevalent before monsoon, while its percentage was lowest after monsoon

(Fig. 5). It was discovered that the post-monsoon had the lowest rates of infection intensity and abundance, whereas the pre-monsoon had the highest rates. The three seasons: monsoon, pre-monsoon and post-monsoon had significantly differing infection rates of prevalence, according to a statistical analysis of the data. Despite no discernible difference in incidence across the three seasons, helminthic infections were more common in the pre-monsoon in the current study than in the monsoon or post-monsoon. On the other hand, helminth infection peaked in the summer and decreased in the winter[23]. The parasite infection exhibited seasonal changes, with the summer months having the highest prevalence[12]. Fish are more susceptible to disease during dry seasons because of unbalanced nutritional conditions brought on by a decrease in water volume[4]. The results are contradictory in this regard. Many authors from around the world have examined the seasonal variation of helminth parasites, including parasites of public health relevance in fish, and their existence is regulated by a variety of circumstances.[8,28,29,31] The temperature is the primary component that determines seasonality since it directly affects the parasite's ability to develop into free-living stages or inside of hosts.

This research presents the molecular and morphological characterization and identification of *Euclinostomum sp.* additionally, this study highlights the utility and worth of molecular technologies in the classification and identification of parasites of importance to medicine and veterinary practice.

Conclusion

In order to establish parasite organization strategies that target both the infection of the final host and the transfer of larval forms, a continuous survey that lasts for around a year appears useful. Therefore, it may contribute to a decrease in the quantity of intermediate hosts and, in turn, the spread of infection of parasites. It can be inferred that due to the build-up of waste and lack of water, the pre-monsoon climate seems to be conducive to the prevalence of parasites. Before the pre-monsoon, precautions should be taken to disrupt the life cycle of parasites and prevent infection of species of fish.

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