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Characterization (Morphological and Molecular) and Seasonal Occurrence of the Zoonotic Anisakid Larvae from the Freshwater Catfish, *H. Fossilis*

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Abstract

The anisakid nematodes causing Anisakiasis in fishes as well as in humans are the commonly known parasites of marine fishes. In the present study we present the first report on the occurrence of anisakid larvae in the freshwater catfish, *H. fossilis* (Siluriformes: Heteropneustidae) from Tripura, North-East India. The identification and morphological characterization of the larvae was carried out using light and scanning electron microscopic studies. Molecular study was carried out to ascertain the findings of morphological characterization using the nuclear ribosomal DNA internal transcribed spacer (rDNA-ITS). The morphology and molecular based identification reveal that the larva is that of *Contracaecum osculatum*. The prevalence of infection was recorded highest in the monsoon season followed by pre-monsoon and post-monsoon season. The present study also showed that the environmental factors play an important role in the seasonality of helminth infections.

Keywords- *Anisakid; Zoonotic; Heteropneustes fossilis; rDNA-ITS; morphology; molecular characterization*

1. Introduction

Anisakids are the common parasites that infect fishes, mammals and birds (Adams *et al*., 1997; Shih, 2004; Farjallah *et al*., 2006). Anisakids are capable of parasitizing a wide range of marine fishes with high incidence and thus are found to have a global distribution (Koie et al., 1995; Wharton et al., 1999; Klimpel and Palm, 2011). Some larval anisakids infect humans and cause significant clinical diseases in a number of countries (Adams *et al*., 1997; McCarthy & Moore, 2000; Couture *et al*., 2003; Pellegrini *et al*., 2005). The larval ascaridoid nematodes of the genus *Contracaecum* have been associated with fish-borne zoonoses. The larval stages of *Contracaecum* usually occur in the body cavity and mesenteries of fish while the adults occur in the gut of the final hosts (Whitfield & Heeg, 1977). These parasites have been widely reported from cichlids and catfish from different parts of the world like Egypt (Amin, 1978), East Africa (Malvestuto & Ogambo-Ongoma, 1978; Aloo, 2001), and South Africa (Prudhoe & Hussey, 1977; Mashego & Saayman, 1981; Boomker, 1982, 1994; Van As & Basson, 1984). Humans are accidentally infected on consumption of raw, undercooked or improperly processed (e.g. marinated) parasitized fish harbouring the larvae. Across the world, there has been increase in the cases of human infection with increase in the consumption of raw and undercooked fishes (Audicana and Kennedy, 2008; Hochberg and Hamer, 2010).

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Due to the close resemblance in morphological traits among larvae of different *Contracaecum* species, at times specific identification becomes difficult and misleading. In such cases, application of molecular techniques, Polymerase Chain Reaction (PCR) and sequencing in particular, are able to provide simple and rapid alternative that can be and has been used for identifying closely related organism (Klimpel and Palm, 2011). In the present study, the nuclear ribosomal DNA Internal Transcribe Spacer (rDNA-ITS) region of the genome prove to be extremely valuable in differentiation and discrimination of closely related organism at species and generic level (Nadler et al., 2005; Sohn and Chai, 2011; Kuhn et al., 2013; Sharma et al., 2016). Since rDNA-ITS genes have relatively rapid rate of evolution, it is popularly used in resolving the taxonomic and phylogenetic issues among wide variety of organisms (Hwang and Kim, 1999; Sharma et al., 2016) including platyhelminthes (Goswami et al., 2009) and nematodes (Iniguez et al., 2009).

The objectives of the present study is to characterize the anisakid nematode larvae recovered from freshwater catfish *H. fossilis* using both morphological and molecular techniques supplemented with sequence analysis to identify the larval anisakid nematodes using rDNA gene sequences and to study the seasonality of infection, if any.

2. Materials and Methods

2.1 Specimen Collection:

The larvae of anisakid nematodes were removed from the body cavity of the freshwater catfish, *Heteropneustes fossilis*, washed several times in Phosphate Buffered Saline (PBS) and fixed in 10% formalin for stereomicroscopic study and fixed in 2.5% glutaraldehyde for SEM study.

2.2 Stereomicroscopic Preparation:

For stereomicroscopic study, after formalin fixation, specimens were washed several times in distilled water and dehydrated in an ascending series of graded glycerol for 10 minutes at each step and cleared twice with xylene for 10 minutes; all steps were performed at room temperature. The specimens were then viewed under a stereomicroscope and photographs were captured using Leica Phase Contrast Microscope (DM1000).

2.3 SEM Preparation:

To study the surface topography of the anisakid larvae by SEM, the specimen were fixed in 2.5% glutaraldehyde, followed by repeatedly washing with 0.1 M sodium cacodylate buffer (pH 7.2, at 4ºC). Specimens were further dehydrated in a series of graded acetone from 30% to 100% for 15 minutes at each grade at room temperature; dried in tetramethylsilane (TMS), mounted on a stub with double stick scotch tape, and sputter coated with gold and viewed under JSM-6360 (JEOL) Scanning Electron Microscope operated at 20kv.

2.4 DNA Isolation, Amplification and Sequencing:

Total genomic DNA was extracted from a single parasite using the standard phenol-chloroform-isoamyl method (Sambrook & Russell, 2001). A region of the ribosomal DNA spanning the ITS1-5.8S-ITS2 was amplified using a set of PCR primers, namely, NC5 and NC2 (Hillis & Dixon, 1991). The PCR amplification of the chosen marker gene regions was performed following the standard protocol (White, 1993) with minor modifications in a 25 µl reaction volume, containing 50-100 ng of genomic DNA with 20 pmols of each primer. The following conditions were used for amplification - initial denaturation at 94°C for 5 min, then 35 cycles including denaturation at 94°C for 1 min, annealing at 56°C for 2 mins, extension at 72°C for 2 mins, followed by final extension at 72°C for 10 mins. The amplicons produced were then purified using HiPurA PCR Product Purification Kit as per the manufacturer's protocol and sequenced in both directions using the sequencing services provided by the MACROGEN (Seol, South Korea). The sequence obtained was edited and submitted manually to the National Centre for Biotechnology Information (NCBI) GenBank using the submission tool Bankit for validation and the accession number was obtained. (http://www.ncbi.nlm.nih.gov/genbank/).

2.5 Sequence and Phylogenetic Analysis:

The sequence generated in the study was combined with sequences of other anisakid and related taxa from GenBank and were aligned using MUSCLE program in MEGA6 (Tamura *et al*., 2013). The aligned sequences were also translated to sequence similarity index matrix using BioEdit v 7.2.5 (Hall, 1999) to see the extent of similarity among various species of *Contracaecum*. Phylogeny was inferred using MrByes by Bayesian Inference (BI) (Ronquist *et al*., 2012) taking *Ascaris lumbricoides* as out group. Branch support was given using the Metropolis-Coupled Markov Chain by Bayesian posterior probability (Bpp). The analysis was carried out for 5,00,000 generations and was sampled every 1,000 generations with first 25% being discarded as burn in phase.

2.6 Prevalence Studies:

The meteorological data was collected from the Indian Council of Agricultural Research for the period of 2012-15 (). Each year was divided into three seasons namely Pre-monsoon (March-June), Monsoon (July-October) and Post-monsoon (November-February). The data obtained were analyzed for the following parameters following Margolis *et al*.,(1982):

Prevalence (%) = number of hosts infected X 100 / total number of hosts examined
Abundance = number of parasites recovered / total number of hosts examined
Mean intensity = number of parasites recovered / total number of infected hosts

One-way Analysis of Variance (ANOVA) was applied to ascertain the significance of variations of the prevalence, abundance and mean intensity of infection between the three seasons during the study period. The ecological relationships between the abiotic and biotic parameters (prevalence, abundance and mean intensity) was determined by Pearson correlation coefficient (r) and p values were calculated *vide* http://faculty.vassar.edu/lowry/tabs.html and their significance were ascertained after the use of Bonferroni corrections.

3. Results

On studying the morphology of the recovered anisakid nematode from freshwater catfish *H. fossilis*, the parasite showed characteristic features of the genus *Contracaecum*. Further analysis carried out using the molecular and bioinformatics tools (rDNA-ITS sequence and BLAST) showed 91.3% resemblance with *C. osculatum* isolate from Denmark.

3.1 Stereomicroscopic Study:

Stereomicroscopic studies revealed that the worms were slender, elongated and cylindrical in shape with tapering head and tail ends. Body relatively thick; cuticle annulated, forming collar at the anterior end; lateral interruption at this area in some specimens. Excretory pore opening at anterior end (Fig. 1b). Two typical features of the *Contracaecum* species i.e. the presence of a prominent intestinal caecum extending from the ventriculus to the close proximity to the nerve ring and a ventricular appendix facing the posterior extremity were prominent in the larvae (Fig. 1a and Fig. 1c). The larvae also had lips with a cephalic tooth and a well-defined nerve ring near the cephalic end, characters that are also defined by other authors like Nadler & Hudspeth (1998) and Martins *et al*. (2005) (Fig. 1b). These larval nematodes were usually found in large numbers mainly on the intestinal mesenterium and body cavity. The reproductive system was not developed at all (Fig. 1d).

Fig. 1. Light microscopic images of *C. osculatum* larvae. (A) anterior end of the worm showing the intestinal caecum (ic) and ventricular appendix (va), (B) enlarged view of thr anterior end showing cephalic tooth (ct), nerve ring (nr) and intestinal caecum (ic), (C) enlarged view of the middle region showing the ventricular appendix (va) and (D) posterior end of the parasites showing cloacal opening (co).

3.2 Scanning Electron Microscopic Study:

Scanning electron microscopy revealed the presence of a globular head. The larvae possessed a well-differentiated cephalic structure and a slit-like mouth opening (Fig. 2a and Fig. 2b). A small boring tooth was projecting dorsally at the anterior end (Fig. 2b). The cuticle showed somewhat irregularly spaced, continuous, transverse grooves with parallel, irregularly spaced longitudinal ridges (Fig. 2e). The tail end was conical in shape and showed the presence of cloacal opening (Fig. 2c and Fig. 2d).

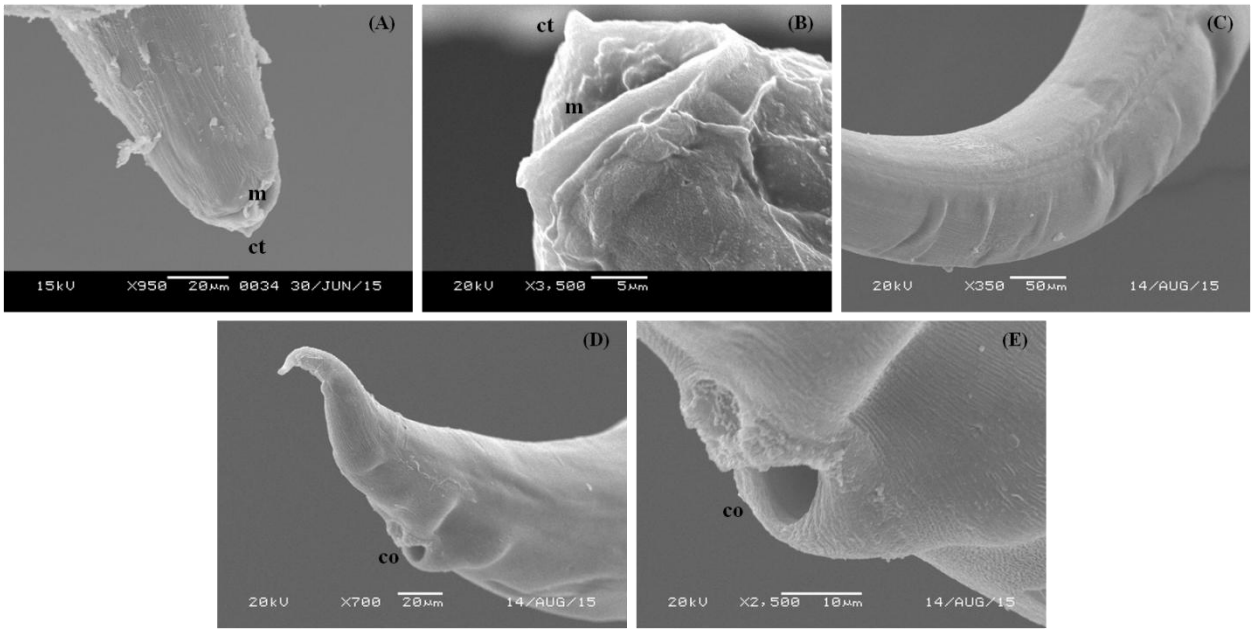

Fig. 2. Scanning electron micrographs of *C. osculatum* larvae. (A) anterior end showing mouth (m) and cephalic tooth (ct), (B) enlarged view of the anterior end showing slit-like mouth opening (m) and cephalic tooth (ct), (C) posterior end showing conical tail and cloacal opening (co) and (D) enlarged view of the cloacal opening (co).

3.3 Sequence and Phylogenetic Analysis:

The nematode larvae isolated from the freshwater catfish, *H, fossilis* corresponded by morphological evaluation to the genus *Contracaecum.* The primers NC5 and NC2 successfully amplified approximately 950bp regions of the rDNA-ITS. The sequence was deposited in GenBank under accession no. KX673182. The preliminary analysis of the anisakid larva rDNA sequence using Nucleotide BLAST showed maximum similarity of 91.3% with the Denmark isolate of *C. osculatum.* Sequence identity matrix also corroborated highest genetic relatedness of our specimen's sequence with other geographical isolates of *C. osculatum* (Table 1 and 2).

The consensus tree inferred from ITS shows that *Contracaecum* is monophyletic when the sequences of *Contracaecum* available in GenBank are included, with a posterior probability of 100%. The phylogenetic trees, with overall good nodal support, showed the presence of three clades labelled as clade-I, clade- II and clade-II (Fig. 3). Isolates of *C. osculatum* and *C. rudolphi* emerged to form a common cluster, and appeared on a single clade (clade-III). *C. multipapillaum* is seen consisting the clade- I while *C. microcephalum* represented the clade-II. In both the tree, *Ascaris lumbricoides*, the out group, formed a separate clade.

Table 1. ITS2 sequences with their GenBank accession numbers of *Contracaecum* sp. and related taxa used in analysis.

Sl. No.	Name of species	Accession number	Locality
1.	*Contracaecum osculatum**	KX673182	India
2.	”	KU306718	Denmark
3.	”	KM273050	Denmark
9.	*C. rudolphii*	AF411204	Poland
10.	”	EU678869	Italy
11.	*C. multipapillatum*	KC437338	Australia
12.	”	KC437337	Australia

13.	*C. microcephalum*	FM177525	Australia
14.	”	FM210410	Australia
15.	*Ascaris lumbricoides*	AB571298	Japan

* Query sequence

Table 2. Sequence identity matrix with numerical values indicating % identities (PI) and % differences (PD) among the species (inter-specific variations ranging from 0.3-35.0%)

	Cosc_ India*	Cosc_ Denmark 1	Cosc_ Denmark 2	Crud_ Poland	Crud_ Italy	Cmul_ Australia 1	Cmul_ Australia 2	Cmic_ Australia 1	Cmic_ Australia 2
Cosc_India*	ID								
Cosc_Denmark 1	91.3/8.7	ID							
Cosc_Denmark 2	91.3/8.7	100	ID						
Crud_Poland	83.2/16.8	85.8/14.2	85.8/14.2	ID					
Crud_Italy	76.7/23.3	79.3/20.7	79.3/20.7	93.4/6.6	ID				
Cmul_Australia 1	64.2/35.8	68.9/31.1	68.9/31.1	70.2/29.8	73.5/26.5	ID			
Cmul_Australia 2	64.5/35.5	69.2/30.8	69.2/30.8	70.4/29.6	73.7/26.3	99.7/0.3	ID		
Cmic_Australia 1	77.0/23.0	80.2/19.8	80.2/19.8	85.2/14.8	89.4/10.6	73.6/26.4	73.8/26.2	ID	
Cmic_Australia 2	77.0/23.0	80.2/19.8	80.2/19.8	85.2/14.8	89.4/10.6	73.6/26.4	73.8/26.2	100	ID

* Sequence generated in the study; Cosc- *C. osculatum*; Crud- *C. rudolphii*; Cmul- *C. multipapillatum*; Cmic- *C. microcephalum*.

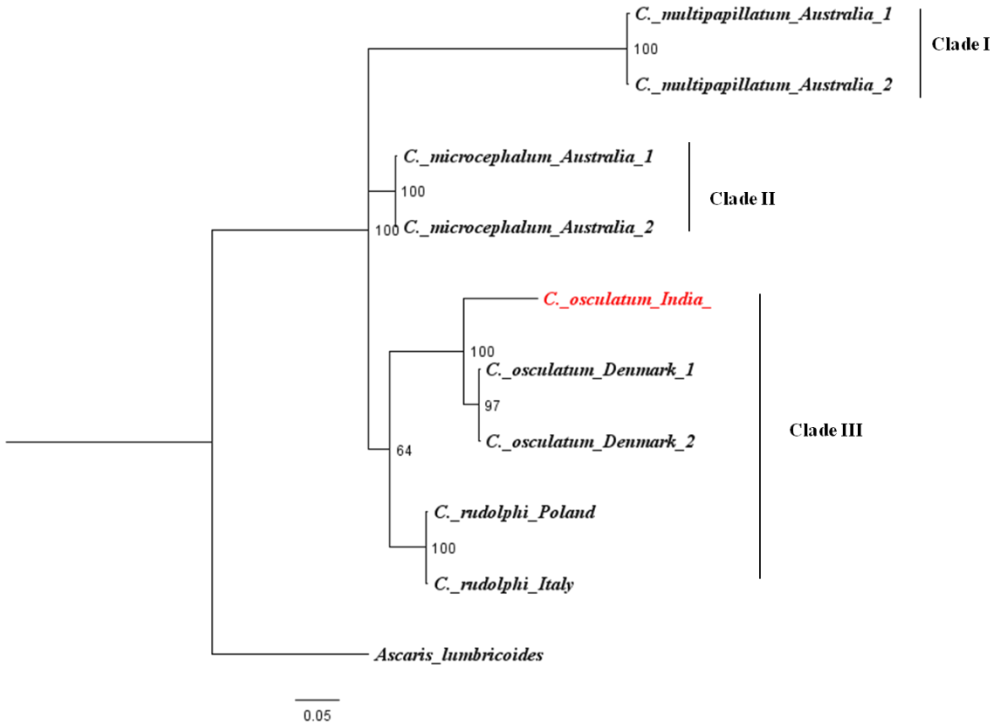

Fig. 3. Phylogenetic trees inferred from the ITS sequence data of various *Contracaecum* species using MrBayes. Query sequence highlighted.

3.4 Prevalence Studies:

A total of 207 numbers of *H. fossilis* were dissected, of which 88 were found to be infected infected with the larval *C. osculatum*. The prevalence of infection was found to be highest during monsoon season (53.13±4.77) followed by pre-monsoon (47.38±1.36) and post-monsoon seasons (35.33±0.21). The abundance and mean intensity values however decreased from 3.00±0.58 and 6.32±1.22 in the pre-monsoon season to 1.67±0.33 and 3.16±0.66 in monsoon and 1.00±0.00 and 2.83±0.02 in the post-monsoon season respectively (Fig. 4). The atmospheric factors (temperature, humidity and rainfall) showed a positive correlation with the prevalence (+0.98, +0.97 and +0.89), abundance (+0.66, +0.29 and +0.84) and mean intensity (+0.46, +0.04 and +0.68) of parasitic infections (Table 3 and 4).

Table 3. Meteorological factors (Average Temperature, Average Rainfall and Average Relative Humidity) in the state of Tripura during the study period.

Year	Seasons	Temp. (°C)	Rainfall (in mm)	Humidity (%)
2012-13	Pre-monsoon	28.08	235.55	70.00
	Monsoon	28.35	253.05	78.38
	Post-monsoon	19.59	12.55	64.00
2013-14	Pre-monsoon	27.75	317.98	71.13
	Monsoon	28.29	189.03	77.00
	Post-monsoon	19.95	8.35	63.63
2014-15	Pre-monsoon	26.5	255.03	73.38
	Monsoon	29.28	247.35	79.38
	Post-monsoon	21.69	4.65	69.63

Table 4. Correlation coefficient (r) of prevalence, abundance and mean intensity of *C. osculatum* infection with meteorological parameters '+' indicates significant positive correlation (*p≤0.05 and ***p≤0.001).

	Temperature	Humidity	Rainfall
Prevalence	+0.98***	+0.97***	+0.89***
Abundance	+0.66*	+0.29	+0.84***
Mean Intensity	+0.46	+0.04	+0.68*

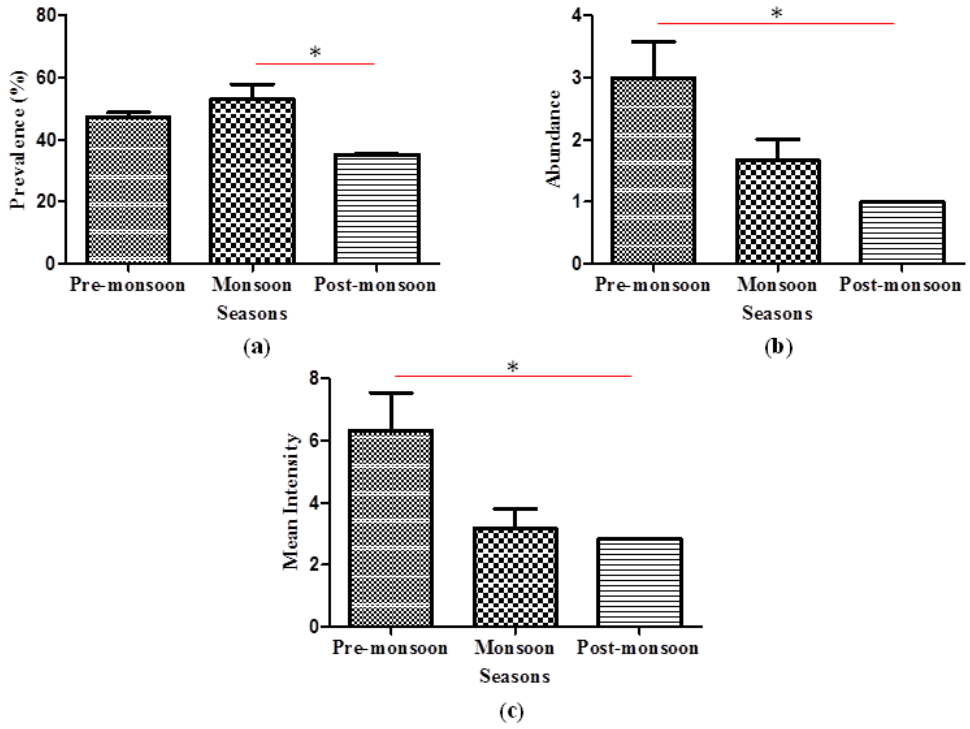

Fig. 4. Seasonal fluctuations of *C. osculatum* in *H. fossilis*: (a) Prevalence, (b) Abundance and (c) Mean Intensity. Values are expressed as Mean ± SEM (N=12). * p≤0.05. One-way ANOVA, Tukey Test.

4. Discussion

The ability to identify and distinguish between the species of different anisakid nematodes accurately in different hosts and at any developmental stage has an important implication in studying their life cycles, epidemiology, taxonomy, population biology and the controlling measures for the diseases they cause. The present study demonstrates the identification of the zoonotic anisakid larvae, *C. osculatum* depending on the morphological as well as molecular data.

The larval forms of the genus *Contracaecum* have been recorded from catfish and other fish species by authors like Whitfield and Heeg (1977), Mashego and Saayman (1981), Boomker (1982, 1994), Saayman *et al.* (1991), Chishawa (1991), Douëllou (1992), Barson (2004), Malvestuto and Ogambo-Ongoma (1978), and Aloo (2001). They are the cosmopolitan parasites of fish-eating birds and mammals (Hartwich, 1974; Anderson, 1992). They can reach alarming intensities without affecting the condition of the host (Mashego and Saayman, 1981; Boomker, 1982; Paperna, 1996), which is probably an adaptation to ensure that the larvae survive to reach the final host without killing the intermediate host.

It is difficult to differentiate the *Contracaecum* larvae into species except when using molecular analysis or alternatively infecting experimental hosts in order to obtain the adult worms. Authors like, Saayam *et al*. (1991), Mokgalong (1996) and Marshall (2004) recorded adult *Contracaecum* species from fish-eating birds.

Koyama *et al.* (1969), Kikuchi *et al*. (1970), Shiraki (1974) and Koyama (1974) described the larval forms of *C. osculatum* using specimens from Japanese gadid fishes. The present specimen shows morphological characters similar to that of the observations made by Berland (1961), Koyama *et al.* (1969) and Kikuchi *et al.* (1970) in possessing a relatively small boring tooth and a tapering tail without mucron, a short ventriculus and venticular

appendix longer than the intestinal caecum and the under-developed reproductive system. Based on the Scanning electron microscopic studies, Weerasooriya *et al*. (1986) characterized the *C. osculatum* larvae from Pacific cod by a well-differentiated cephalic structure and a slit-like transverse mouth opening. The parasite under this study showed similar characters on exposure to Scanning electron microscopy as described by Ishii *et al*. (1991).

Being fast, safe and easy, the PCR technique is one of the convenient tools used for parasite identification. It allows examining small amounts of material and repeating the experiment many times. In addition, the sample materials can be stored in a frozen state as well as can also be conserved in alcohol, which is important in case of transportation to the laboratory.

Studies on ascaridoid and strongylid nematodes have shown that there exists significant differences in the ITS regions between the species (Hoste *et al*., 1995; Stevenson *et al*., 1995; Chilton *et al*., 1997; Jacobs *et al*., 1997; Zhu *et al*., 1998). Hence, the analyzed region of the ribosomal DNA (ITS2) is a good amplification target, allowing molecular identification of the anisakid larvae examined. The PCR-based technique using the universal primers allowed the identification of the larval nematodes under the study as the larval forms of *C. osculatum*. On comparison of the nucleotide sequences obtained from GenBank showed that the present parasites formed a clade along with the other isolates of the same species reported from different locations.

The larval *C. osculatum* showed a peak in the pre-monsoon season, suggesting a seasonal fluctuation in infection. The continuous recruitment and development of the parasite may occur, although the parasite reproduction may be at its peak in the pre-monsoon season. This result agrees with the observations made on nematodes infecting *Mugil cephalus* in Saloum and Senegal rivers (Dione et al. 2014). However, Skorping (1980) is on the opinion that non-seasonal patterns in infection levels result from an overlap in the seasonal rates of nematode mortality and recruitment, and concluded that small shifts in these rates would lead to more pronounced seasonal patterns. This hypothesis may explain differences in the seasonal changes of infection as observed in the present study.

In natural conditions, the rate of infection is expected to be low as the parasites are normally in equilibrium with their hosts (Paperna, 1996), which ensures that the parasite does not kill the intermediate host and reaches its final host to complete the life cycle. In case of *C. osculatum*, however, even very heavy infections of fish have not much affect on the health condition of host (Mashego and Saayman, 1981; Aloo, 1999 and Aloo, 2001) but may render the fish unsightly and unsuitable for human consumption especially if the larva encysts in the muscle.

Changes in the prevalence and mean intensity of parasitic infection might be influenced by various factors such as water pollution, parasite biology, host hormonal status, host immunological response, host migration, changes in the feeding habits of the host and the availability of infected intermediate hosts (Chubb, 1963; Kennedy, 1969; Pennycuick, 1971; Hanzelova and Zitnan, 1985 and Simkova et al., 2005). However, the influence of these factors is difficult to distinguish because they are most likely interrelated and influence each other.

Authors like, Kennedy (1969a), Niyogi et al. (1982), Gupta et al. (1984), Amin (1987), De (1993), Bush et al. (1997), Choudhury and Dick (2000), Farhaduzzaman et al. (2010), Pandey et al. (2012), Ranibala et al. (2013) and Aydogdu et al. (2015) have studied the seasonality in helminth infections in different fish hosts across the world and concluded that factors like the contemporary and past geography of the various regions, ecology of both host and parasite, feeding behaviour and specializations of various freshwater fish, as well as localized ecological conditions influence the helminth fauna with different characteristics, irrespective of latitude and longitude. In the present study, the prevalence of infection was highest during pre-monsoon which can be due to the fact that water temperature acts directly on the larval stage of the parasites or indirectly through the fish behaviour, especially feeding behaviour and its metabolic activity. Factors other than temperature also have a casual effect on the seasonal occurrence of the parasites. However, the influence of these factors is difficult to distinguish because they are most likely to be

interrelated. In the present study, the rate of infection increased with increase in temperature. Thus, the temperature range of 26-28°C revealed to be favourable for the propagation of fish parasites in the study area.

5. Conclusion

Anisakid nematodes are common parasites of biological, medical and economic importance worldwide. The larval anisakid worms are not host specific and hence they have high probability of transmission (Smith, 1983; Mattiucci *et al*., 1997). Based on morphology, PCR and sequencing, all the examined larvae were identified as *Contracaecum osculatum*. Sequencing and phylogenetic tree analyses of the *C. osculatum* larvae in ITS1-5.8S-ITS2 region showed that the present sample was in the same cluster as *C. osculatum* published in the GenBank. This is the first report of *C. osculatum* reported from the freshwater catfish, *H. fossilis*. In the present study, the rate of infection increased with increase in temperature. Hence, a temperature range of 26-28°C revealed to be favourable for propagation. Therefore, treatments provided during the pre-monsoon season will prove to be effective in controlling the helminth infections. At the same time it will help the scientific community and also the pisciculturists to know about the parasite species found to be infecting different fish hosts.

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