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Re-characterization of Potential Zoonotic Trematode Parasite Parasitizing Intestine of Snakehead Fish, (*Channa striata*) Utilizing Multiple Sequence Alignment Tools (MSA)

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Abstract: Metacercariae of *Clinostomum* Leidy, 1856 are frequently encountered in freshwater fish. The species configuration and taxonomic antiquity of the genus *Clinostomum* has been very unsteady and under discussion. Two species, i.e. *Clinostomum complanatum* Rudolphi, 1814 and *Clinostomum marginatum* Rudolphi, 1819, have been specifically troublesome and their taxonomic validation has been debated. 19 human incidences of *Clinostomum complanatum* have been reported to cause pharyngitis and lacrimatitis from Japan, Thailand and Korea. *Clinostomum marginatum* is commonly known as yellow grub as it causes pinhead sized lumps in fishes that are white to yellow or black. In the present communication, we have made use of cytochrome oxidase c subunit 1 gene as DNA barcode for validating the taxonomy of *C. complanatum* and *C. marginatum* harboring phylogenetic studies and multiple sequence alignment tools. Currently, in India species of this group are distinguished mainly on the basis of morphological characters that underlie possible confusions. Therefore, we disinterred the species through molecular analysis. The current findings are salient to authenticate the phylogenetic place and relationship among two trematode spp. and bypass misidentification regarding their validation, as it is more necessary in that case when many species harbours the same host and are of zoonotic importance.

Keywords: Zoonotic, Helminth, *Clinostomum complanatum*, Taxonomic, Cox1, molecular, *Clinostomum marginatum*

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Introduction

The global aquaculture production has exceeded 91.4 million tons and generated an average income of more than US\$ 145.4 billion (Aohagi *et al.*, 1992; Sosa *et al.*, 2016). Aquaculture has

served as a prominent key factor in the economy of India. Increased parasitism and disease outbreaks have negative impacts not only on the fish production, but also leads to economic losses.

The parasitic diseases reduce the reproductive performance and have a negative impact on feed conversion efficiency leading to reduced growth and overall performance of the cultured fishes. Thus, the parasites fabricate productivity loss. In contrast the human population is increasing and meet out the aquatic food requirements become a challenge.

Digenetic trematodes constitute a major helminthic group that parasitize humans and animals, and are a major cause of morbidity and mortality. As compared with other parasitic diseases the diseases caused by trematodes have been neglected for years. The present communication deals with the report of incidence of two trematode helminth parasites, *Clinostomum complanatum* and *Clinostomum marginatum* parasitizing the intestine of fish *Channa striata* (Snakehead murrel). The very fact that *C. striata* has been known since long for its antiseptic and dermal wound healing properties has made this fish more consumable in its raw or partially cooked form. The biochemical composition of the flesh of snakehead murrel contains arachidonic acid that is precursor molecule for synthesis of collagen fibers at the wound site. Consuming raw or partially cooked fish leads to exposure of the parasites within the fish to enter the human host body and thus increases chances of parasitic infections. During this course of study, out of the two reported parasites, 19 human incidences of *Clinostomum complanatum* have been reported to cause pharyngitis and lacramalitis from Japan, Thailand and Korea. *Clinostomum marginatum* is commonly known as yellow grub as it causes pinhead sized lumps in fishes that are white to yellow or black.

The metacercariae of *Clinostomum* species are known to have similar morphology even though if there are some distinct features, it is not enough to discriminate up to the species level. The aim of the findings is to highlight the fact that still morphological basis of taxonomic validation for parasites have been extensively used till date in India, but morphological basis of identification is

not an authentic method for the same. In this study, cytochrome c oxidase (cox1) gene was used as a molecular marker as it is an evolutionary conserved segment and coding gene that will further provide insights into the biology of host parasite interaction of these parasites for disease control.

Materials and Methods

The metacercariae (n=15) were recovered from the intestine of the fish *Channa striata*, that were brought alive from different local fish markets of Meerut, India. They were thoroughly washed in phosphate buffered saline and further processed for molecular studies. We in this article are specifically giving a detailed outlet of molecular studies and MSA tools.

DNA Isolation, Amplification and Sequencing:

The whole helminth worms were used for genomic DNA extraction using standard protocol (Hall, 1999). Partial Cox1 gene of both the trematodes were amplified by PCR using primers set 3S/A28 and Dice 1F/Dice 14R, respectively (Bowles *et al.*, 1995; Steenkiste *et al.*, 2015). The PCR product was purified using Genei Quick PCR purification kit. The generated sequences were submitted to NCBI and accession numbers of both the worms were obtained.

Multiple Sequence Alignment and Phylogenetic Analysis:

The generated sequences along with sequences of the other *Clinostomum* species retrieved from GenBank were used for analyses. They were aligned using the ClustalW program in MEGA6 (Hall, 1999). Multiple Sequence Alignment, taxonomy and protein level similarity was performed (Figs. 1-10).

Results

Data Availability:

The partial Cox 1 genes of both the trematodes were submitted to NCBI and accession numbers of *Clinostomum complanatum* and *Clinostomum*

marginatum were obtained to be ON796499 and ON803638, respectively.

Molecular Characterization:

Alignment description with top 10 matches, Multiple Sequence Alignment (MSA),

Phylogenetics study and Taxonomy was analyzed using molecular tools for both the trematode helminthes. Following were the results obtained:

Rediscription Using Molecular Tools:

(1) *Clinostomum complanatum*:

Taxonomy:

Organism	Blast Name	Score	Number of Hits	Description
Digenea	flatworms		121	
• Strigeidida	flatworms		5	
• Clinostomum complanatum	flatworms	444	2	Clinostomum complanatum hits
• Schistosomatidae sp. 2 W480	flatworms	217	1	Schistosomatidae sp. 2 W480 hits
• Schistosomatidae sp. 2 W402	flatworms	206	1	Schistosomatidae sp. 2 W402 hits
• Schistosomatidae sp. 2 W165	flatworms	206	1	Schistosomatidae sp. 2 W165 hits
• Paragonimus heterotremus	flatworms	222	73	Paragonimus heterotremus hits
• Plagiorchis sp. Lineage 9 OK-2021	flatworms	211	1	Plagiorchis sp. Lineage 9 OK-2021 hits
• Plagiorchis koreanus	flatworms	209	6	Plagiorchis koreanus hits
• Plagiorchis sp. B MN-2021	flatworms	204	4	Plagiorchis sp. B MN-2021 hits
• Paramphistomum cervi	flatworms	195	2	Paramphistomum cervi hits
• Cephalogonimus americanus	flatworms	195	2	Cephalogonimus americanus hits
• Plagiorchis sp. racIT	flatworms	193	1	Plagiorchis sp. racIT hits
• Paragonimus harinasutai	flatworms	191	13	Paragonimus harinasutai hits
• Paramphistomum sp. JAS-2013	flatworms	189	1	Paramphistomum sp. JAS-2013 hits
• Postharmostomum commutatum	flatworms	187	3	Postharmostomum commutatum hits
• Paragonimus bangkokensis	flatworms	187	4	Paragonimus bangkokensis hits
• Paragonimus sp. Sri Lanka type 3	flatworms	187	1	Paragonimus sp. Sri Lanka type 3 hits

Fig. 1: Taxonomy status of *Clinostomum complanatum* showing resemblance with digenetic trematodes.

Alignment description with top 10 matches:

Sequences producing significant alignments									
Download Select columns Show 10									
select all 10 sequences selected									
GenBank Graphics Distance tree of results MSA Viewer									
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession	
Clinostomum complanatum isolate 1 cytochrome c oxidase subunit I (COX1) gene, partial cds: mitochondrial	Clinostomum co...	444	444	100%	3e-120	100.00%	240	ON796499.1	
Clinostomum complanatum isolate CIFRI-NNCC cytochrome c oxidase subunit I (COX1) gene, partial cds: mit...	Clinostomum co...	444	444	100%	3e-120	100.00%	444	OL831193.1	
Paragonimus heterotremus isolate Sph-M4coi cytochrome c oxidase subunit 1 (cox1) gene, partial cds: mitocho...	Paragonimus he...	222	222	100%	1e-53	83.33%	380	EF446321.1	
Schistosomatidae sp. 2 W480 voucher MSB:Para:18695 cytochrome oxidase subunit 1 (cox1) gene, partial cd...	Schistosomatida...	217	217	78%	7e-52	87.30%	955	MF598176.1	
Paragonimus heterotremus mitochondrial CO1 gene for cytochrome oxidase subunit 1, partial cds: isolate: VNM1Paragonimus he...	Paragonimus he...	217	217	100%	7e-52	82.92%	396	AB827370.1	
Paragonimus heterotremus isolate 30.11sp2Liv2 cytochrome c oxidase subunit I (COX1) gene, partial cds: mit...	Paragonimus he...	217	217	100%	7e-52	82.92%	342	MT706110.1	
Paragonimus heterotremus isolate Psp2LVII cytochrome c oxidase subunit I (COX1) gene, partial cds: mitocho...	Paragonimus he...	217	217	100%	7e-52	82.92%	342	MT706104.1	
Paragonimus heterotremus cytochrome c oxidase subunit 1 (cox1) gene, partial cds: mitochondrial	Paragonimus he...	217	217	100%	7e-52	82.92%	387	AY618839.1	
Paragonimus heterotremus mitochondrion, complete genome	Paragonimus he...	211	211	100%	3e-50	82.50%	13927	NC_039430.1	
Paragonimus heterotremus isolate LC mitochondrion, partial genome	Paragonimus he...	211	211	100%	3e-50	82.50%	13526	KY952166.1	

Fig. 2: Results showing top 10 sequences showing 100% similarity with *Clinostomum complanatum* and 82-83% similarity with other digenetic trematodes.

Phylogenetics study:

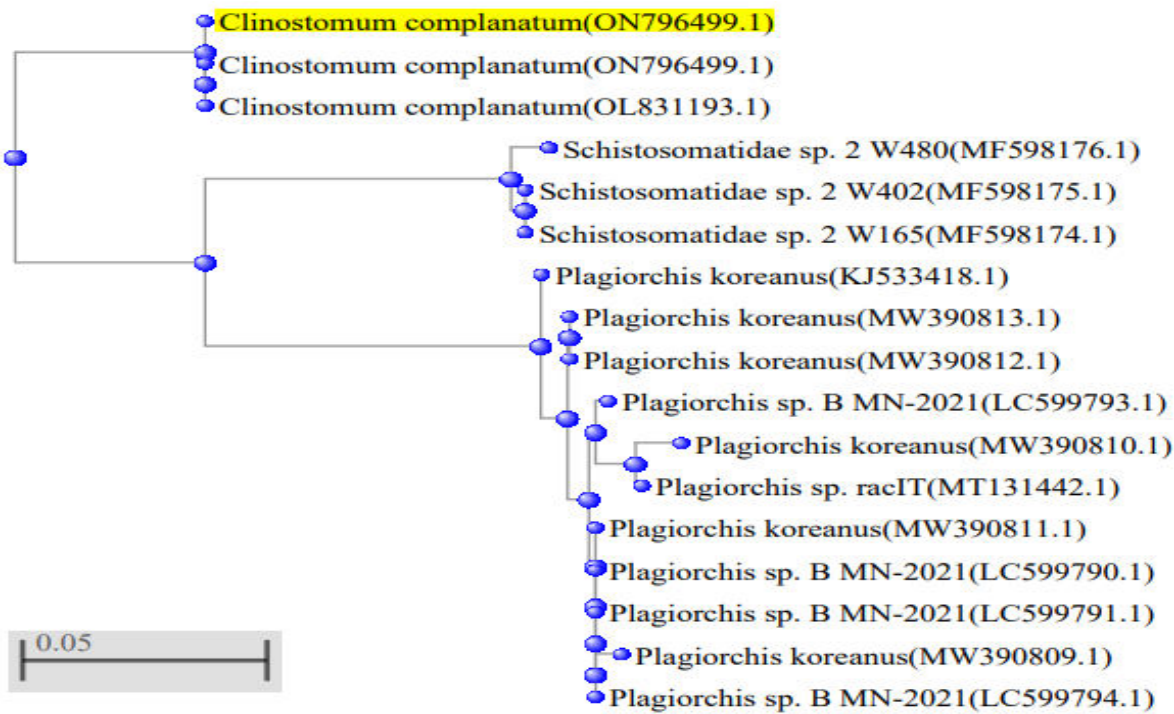


Fig. 3: Figure depicting the taxonomic position of the helminth showing close resemblance with *Clinostomum complanatum*, *Schistosomatidae* and *Plagiorchis* sp. using Neighbour- Joining method.

Multiple Sequence Alignment (MSA):



Fig. 4: Multiple Sequence Alignment results with top 10 helminth matches. Red region depicts the regions of similarity within the genome of the parasites.

(2) *Clinostomum marginatum*:

Taxonomy:

Reports	Lineage	Organism	Taxonomy
10 sequences selected			
Taxonomy	Number of hits	Number of Organisms	Description
Clinostomum marginatum	10	1	Clinostomum marginatum hits

Fig. 5: Taxonomy status of *Clinostomum marginatum*.

Alignment description with top 10 matches:

Sequences producing significant alignments									
Download Select columns Show 10									
select all 10 sequences selected									
Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession	
☒ Clinostomum marginatum isolate DC413 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Clinostomum ma...	536	536	100%	6e-148	100.00%	574	MK426662.1	
☒ Clinostomum marginatum isolate DC409 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Clinostomum ma...	536	536	100%	6e-148	100.00%	574	MK426661.1	
☒ Clinostomum marginatum isolate VT6088 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Clinostomum ma...	536	536	100%	6e-148	100.00%	574	MK426660.1	
☒ Clinostomum marginatum isolate VT6083 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Clinostomum ma...	536	536	100%	6e-148	100.00%	574	MK426658.1	
☒ Clinostomum marginatum isolate VT6064 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	Clinostomum ma...	536	536	100%	6e-148	100.00%	574	MK426657.1	
☒ Clinostomum marginatum isolate Cm_Lm_1 cytochrome c oxidase subunit 1 gene, partial cds; mitochondrial	Clinostomum ma...	536	536	100%	6e-148	100.00%	629	MH282538.1	
☒ Clinostomum marginatum voucher Cl.RM.D.S.18.2 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitocho...	Clinostomum ma...	536	536	100%	6e-148	100.00%	512	JF718619.1	
☒ Clinostomum marginatum voucher Cl.RM.1.R.8.1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitocho...	Clinostomum ma...	536	536	100%	6e-148	100.00%	511	JF718618.1	
☒ Clinostomum marginatum voucher Cl.LG.B.P.3.1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitocho...	Clinostomum ma...	536	536	100%	6e-148	100.00%	528	JF718616.1	
☒ Clinostomum marginatum voucher Cl.RM.D.P.18.1 cytochrome oxidase subunit 1 (COI) gene, partial cds; mitocho...	Clinostomum ma...	536	536	100%	6e-148	100.00%	588	JF718615.1	

Fig. 6: Results showing top 10 sequences showing 100% similarity with *Clinostomum marginatum*.

Phylogenetics study:

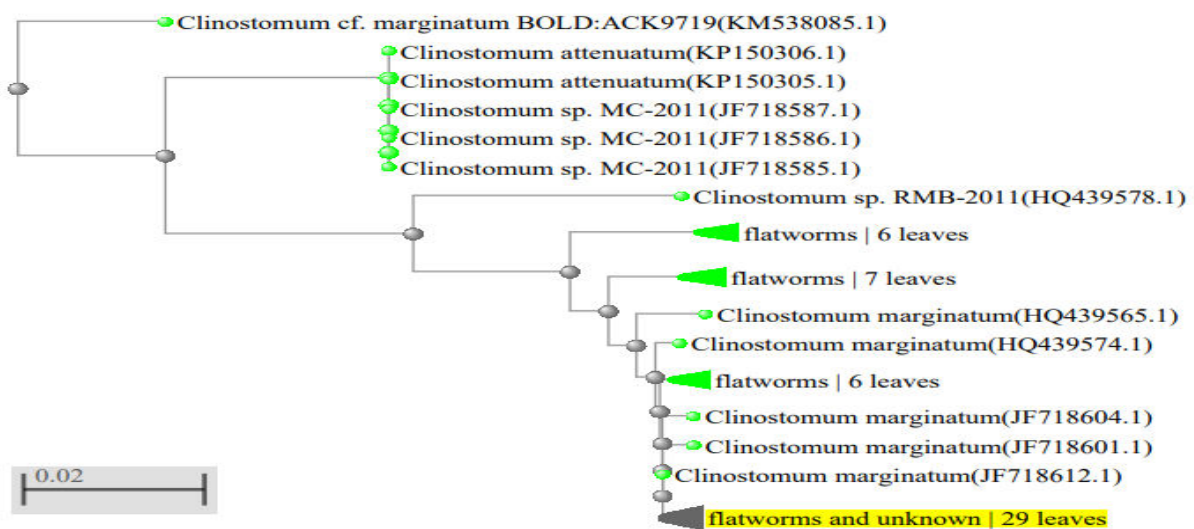


Fig.7: Figure depicting the taxonomic position of the *Clinostomum marginatum* in yellow showing close resemblance with other *Clinostomum* sp.

Multiple Sequence Alignment (MSA):

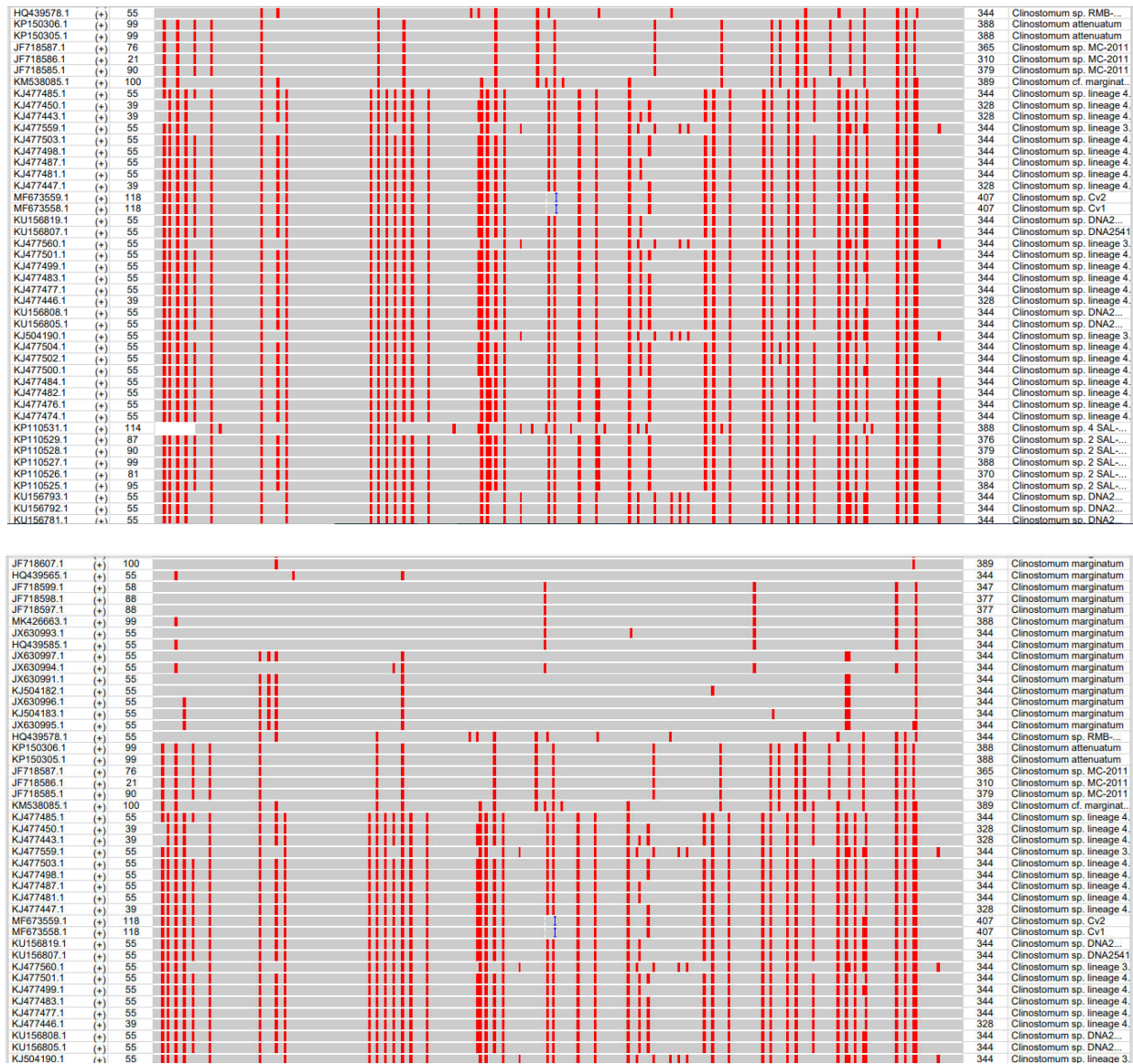


Fig. 8: Multiple Sequence Alignment results with top 10 helminth matches. Red region depicts the regions of similarity within the genome of the parasites.

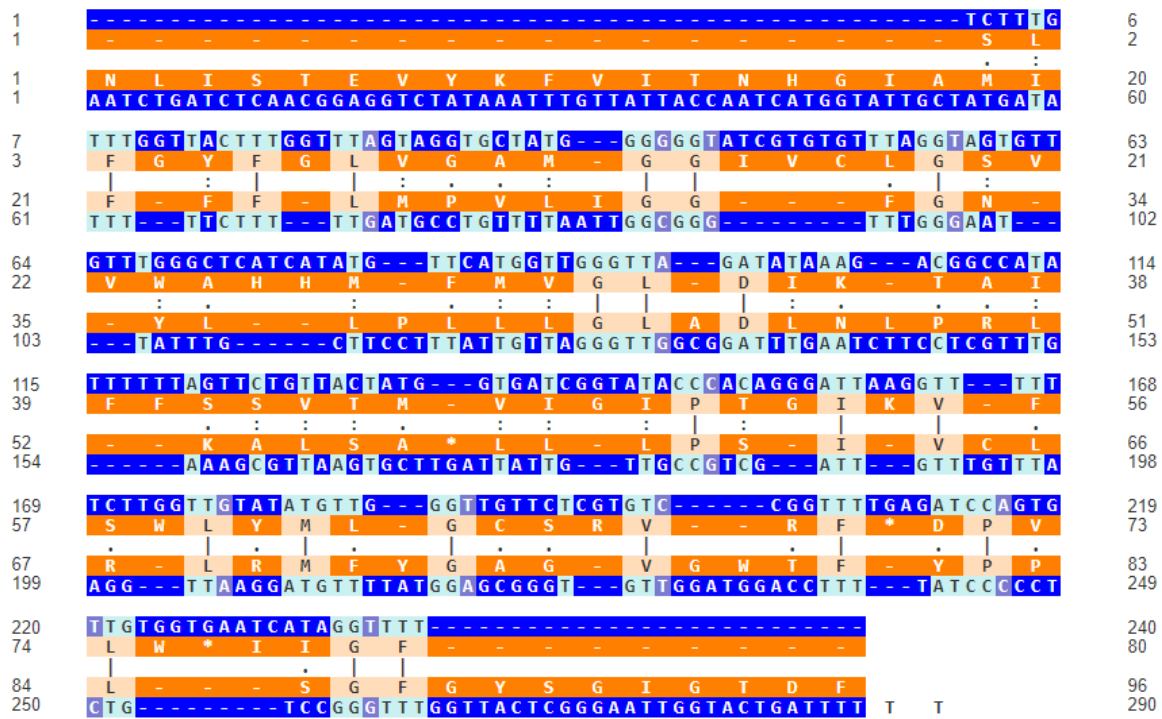


Fig. 9: DNA alignment of cox 1 gene of *Clinostomum complanatum* and *Clinostomum marginatum* based upon the translated proteins. Alignment length: 116, Identity: 21/116 (18.10%) , Similarity: 39/116 (33.62%) .

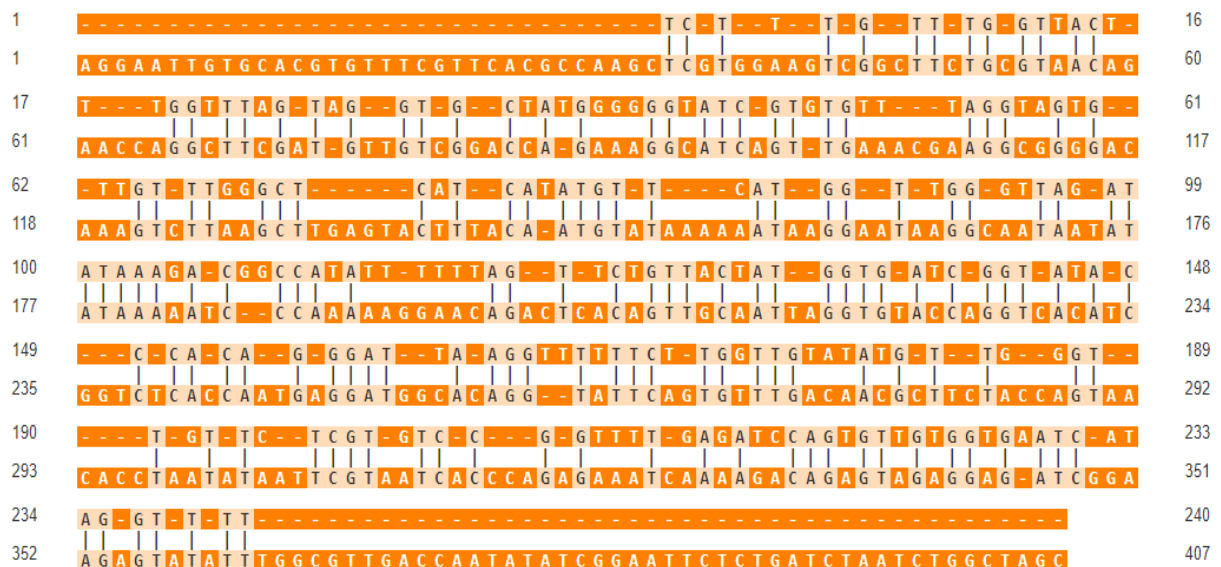


Fig. 10: Protein alignment results within genome of zoonotic trematode parasitic worms of *Clinostomum complanatum* and Schistosomatidae. Level of similarity was analyzed to be 50-52%.

Discussion

Clinostomum species are common fish parasites throughout the world. The final hosts of this fluke are generally piscivorous birds, including herons and egrets (Aohagi *et al.*, 1992). Till date, many

human infections have been reported resulting from eating raw freshwater fish. The present communication confirms the taxonomic validity of *Clinostomum complanatum* and *Clinostomum marginatum* worms which were found infecting the intestine of *Channa striata* (snakeheaded

murrel). *Channa striata* is one of the only widely consumed raw fish and its raw consumption reason lies behind in its raw flesh that has certain vital biochemical components that helps in wound healing. Apart from its antiseptic properties it is also commonly edible and cheap that makes it more pocket friendly.

In the current findings, both the parasites not only causes significant loss in aquaculture by fabricating damages in fishes but also many cases of zoonosis of *Clinostomum complanatum* have been reported worldwide, causing pharyngitis and lacramalitis in humans. The authors confirm the taxonomic validity of the worms via phylogenetic tree construction using Neighbour joining method employing cytochrome oxidase 1(cox 1) gene. Cox 1 gene has been widely accepted as DNA barcode revealing the hidden evolutionary relationships among different worms. Multiple Sequence Alignment (MSA) of *Clinostomum complanatum* and *Clinostomum marginatum* was performed that showed 100-82% similarity at respective locations in the gene. MSA is biologically good and accurate method that shows homology and relationships to deduce meaningful information at evolutionary level. Multiple sequence alignments provide more information than pair-wise alignments since they show conserved regions within a protein family which are of structural and functional importance.

Thus, upon analysis *Clinostomum complanatum* cox 1 gene showed 82-88% similarity with Schistosomatidae and 82-85% with *Plagiorchis* sp. and *Clinostomum marginatum* showing close resemblance with other *Clinostomum* sp. Upon analysis of phylogenetic of *Clinostomum complanatum* it was noted that it shares common ancestary with Schistosomatidae and *Plagiorchis* sp. Schistosomatidae are digenetic zoonotic trematodes commonly known as blood-flukes, are responsible for a highly significant group of infections in humans termed schistosomiasis. To understand the similarities in the mitochondrial genome of both zoonotic trematodes i.e. *Clinostomum complanatum*

(Accession no. ON796499) and Schistosomatidae (Accession no. QMK001002786), further multiple sequence alignment of both was performed using VectorBuilder Tool to explore the similarity at level of gene expression i.e at proteins level and it was analyzed that there genome had 50-52% similarity with good alignment at respective segments (Fig. 10). This highlights that both the zoonotic parasites has appropriate percentage of similarity within their genomes. Thus, present communication emphasizes on the usage of multiple sequence alignment as a method of study of evolutionary relationship and genome comparison of parasites revealing their taxonomy and similarity within the genomes that will further help in disease control caused by the parasites using bioinformatics tools.

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