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Molecular Analysis, Based on 28s rDNA, of *Clinostomum schizothoraxi* Kaw, 1950 (Digenea) Parasitizing Fishes of Kashmir Valley, India

[1]Fayaz Ahmad, [1]Tanveer A. Sofi, [2]Omer Mohi ud Din Sofi, [3]Khalid Majid Fazili and [1]Bashir A. Sheikh

[1]Department of Zoology, University of Kashmir, Srinagar, India - 190006
[2]SK University of Agricultural Sciences and Technology, Shuhama, Aluestang, India
[3]Department of Biotechnology, University of Kashmir, Srinagar, India

Abstract: The aim of the present study is to observe the nucleotide sequence variation of ITS of *Clinostomum* spp. from different fish host species and different localities which was done by using DNA sequence analysis and construction of phylogenetic relationship of this parasite in fishes of Kashmir on the basis of (28S rDNA). Parasite specimens of *Clinostomum* spp. were collected from the *Carassius carassius*; *Schizothorax niger; S. esocinus; S. curvifrons* and *S. plagiostomus* of Kashmir and were used for DNA extraction. Samples were immediately fixed in 70% alcohol after collecting from the gills, gill cover, mouth cavity, eyes, & fins of host fish. These samples were remained in alcohol until the present study.The size of the amplified product of *Clinostomum schizothoraxi* was found 544 bp long and the sequence obtained was submitted to GenBank and their accession number acquired was AF973619. This species also showed 47% Gaunine+Cytocine content which reflects that they are also stable at high temperature. Specimens of *Clinostomum schizothoraxi* was found in fish hosts of *Carassius carassius, Schizothorax niger, S. esocinus, S. curvifrons* and *S. plagiostomus* as is evident from the NJ phylogenetic tree, which means that there is some correlation between Monogenean and Digenean parasites as far as their hosts are concerned. This species has been studied for the first time with respect to their molecular analysis.

Key words: *Clinostomum schizothoraxi* · Fish · Molecular · Digenean · Genbank

INTRODUCTION

Digenetic trematodes comprise an estimated 24000 species, many of which have yet to be described [1]. The Clinostomidae Luhe, 1901 is a family of digeneans the members of which, at the adult stage, live in the oral cavity, pharynx, or oesophagus of fish eating birds, reptiles and occasionally mammals, including man. Among the four subfamilies [2] is the Clinostominae Luhe, 1901, which comprises three genera infecting piscivorous birds, such as herons, cormorants and pelicans; *Clinostomum* Leidy, 1856 is the type genus. Due to the high degree of morphological variability within the same species, in the past, *Clinostomum* has been subjected to several taxonomic revisions. The application of a molecular approach may be particularly important for the completion of the life-cycle and identification of those *Clinostomum* species described in the past only on the basis of morphological features of the metacercarial stage without any subsequent description of the relative adult stages from the definitive host. Species-level identification of most trematodes is based exclusively on adult morphology. Difficulties arise because they are small, soft-bodied, have few stable morphological characters and are subject to host-induced phenotypic variation [3-5]. In this context, molecular markers study offer powerful and much-needed tool that have the potential to distinguish between morphologically similar species at any stage in their life cycle. The aim of the present study is to survey the different water bodies of Kashmir valley to collect *Clinostomum* species from fishes. Study the nucleotide sequence variation of Internal Transcribed Spacer (ITS) of *Clinostomum* species from different fish hosts and different localities which was done by using DNA sequence analysis. Construction of phylogenetic tree to show the relationship of *Clinostomum* species in fishes of Kashmir on the basis of molecular techniques (28S rDNA).

Corresponding Author: Tanveer A. Sofi, Department of Zoology, University of Kashmir, Srinagar – 190006,
Tel: +919797127214, E-mail: stanveer96@gmail.com.

MATERIALS AND METHODS

Parasite Material: Mature specimens of clinostomids were recovered from fish hosts of *Carassius carassius, Schizothorax niger, S. esocinus, S. curvifrons* and *S. plagiostomus* from Anchar, Manasbal, Wular, Dal lake, River Jhelum and River Sindh of Kashmir valley, preserved in 100% ethanol for genomic DNA extraction and stored at -20°C for good quality of DNA. For DNA extraction ethanol was removed from parasites as per the protocol given in methodology and as such specimens were air dried to remove ethanol [6]. The resultant DNA was examined on 1.5% agrose-TAE gels, stained with ethidium bromide and visualized under UV light.

Extraction of DNA: Mature specimens of clinostomids were recovered from fish hosts of *Carassius carassius, Schizothorax niger, S. esocinus, S. curvifrons* and *S. plagiostomus* from Anchar, Manasbal, Wular, Dal lake, River Jhelum and River Sindh of Kashmir valley, preserved in 100% ethanol for genomic DNA extraction and stored at -20°C for good quality of DNA. For DNA extraction ethanol was removed from parasites as per the protocol [6] and as such specimens were air dried to remove ethanol.

The thermal gradient of these marker regions started with an initial denaturation at 95°C (10 minutes), denaturation for 40 cycles (40 cycles at 95°C for 30 seconds), annealing, 28S at 52°C (30 seconds), extension 72°C (75 seconds) and final extension at 72°C (10 min). The amplified PCR products were separated by electrophoresis through 1.6 % (w/v) agarose gels in TAE buffer, stained with ethidium bromide, transilluminated under ultraviolet light and then photographed. PCR products were purified using Genei Quick PCR purification kit for DNA sequencing and sequenced in both directions using PCR primer sets utilizing Macrogen sequencing service, Korea.

Sequence Analysis: Similarity search was carried out using Basic Local Alignment Search Tool (BLAST) available at http://www.ncbi.nlm.nih.go v/blast. Since the full length of the 28S rDNA could not be retrieved from one direction sequencing, the contigs were created by assembling the forward and reversed sequences of the genes using DNA Baser v3.5.3 (http://www.dnabaser.com/). Multiple sequence alignments were done for each of the amplified markers from the studied metacercaria, with related sequences from family Clinostomidae retrieved from GenBank, using ClustalW of Bioedit software (http://www.ebi.ac.uk/clustalw). For sequence identities Bioedit software version 7.0.9.0 [7] was used.

Construction of Phylogenetic Tree: The phylogenetic tree is constructed by considering all initial clusters as leaf nodes in the tree and each time two clusters are joined, a node is added to the tree as the parent of the two chosen nodes. The branch lengths are set corresponding to the distance between clusters, which is calculated as the average distance between pairs of sequences in each cluster. The algorithm assumes that the distance data has the so-called molecular clock property, *i.e*., the divergence of sequences occurs at the same constant rate at all parts of the tree.

RESULTS

PCR amplification was carried out to amplify ITS region of the Digenean parasite (Table 1). The size of the amplified product was found to be 544 bp long (Fig. 1). In BLAST search of the sequences, it showed maximum similarity with the other families of *Clinostomum* spp.. Based on morphological characters, this species was identified as belonging to *Clinostomum schizothoraxi.* The present results of the molecular analysis confirmed the species identification of *Clinostomum schizothoraxi*. Therefore, it can be assumed that the present form recovered from the fish host in Kashmir valley is *Clinostomum schizothoraxi* Kaw, 1950.

The size of the nucleotide sequence was found to be 544 bp long. The sequence obtained were submitted to GenBank and their accession number acquired was AF973619 (Table 1). Sequences were compared with other sequences of digeneantrematode species from GenBank. The consensus sequences analysed by BLAST search gave a 93.25% identity with *C. cutaneum* (GQ339114.1) and 85% with *C. complanatum* (FJ609420.1). The alignment of 28S rDNA sequences of *Clinostomum schizothoraxi* showed few differences between the species of other digeneans.

The present observation shows that *Clinostomum schizothoraxi* has 544 base pair length with 171 amino acids. This species is more stable at higher temperature with 47% G+C content (Table 2).

Clinostomum schizothoraxi showed maximum similarity with those of *Clinostomum cutaneum* and *Clinostomum complanatum* as shown in Table 3.

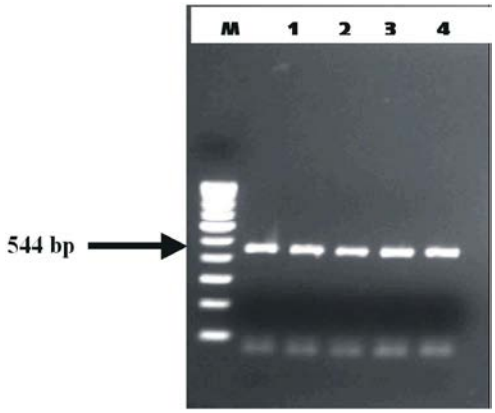

Fig. 1: PCR product of *Clinostomum schizothoraxi* Kaw, 1950 [M = marker; bp = base pairs (100 bp ladder); 1, 2, 3, 4, 5=DNA of *Clinostomum* spp.]

Table 1: Digenean trematode species used for molecular comparison of ITS DNA sequences along with their hosts, country and GenBank accession numbers for corresponding sequences (*Query sequence)

S.No.	Digenean Species	Host	Accession No	Family	Base pairs	Authors	Country	Year
1	*Clinostomum schizothoraxi* Kaw, 1950*	*Carassiuscarassius; Schizothorax niger*; *Schizothorax esocinus*; *Schizothorax curvifrons*	AF973619	Clinostomatidae	544 bp	(Present study) Ahmad *et al*.,	India	2015
2	*Clinostomum cutaneum*	Grey herons *Ardea cinerea* L. and Nile tilapia *Oreochromisniloticusniloticus* (L.)	FJ609421	Clinostomatidae	4614 bp	Caffara and Stanzani [8]	Kenya	2010
3	*Clinostomum cutaneum*	*Ardeacinerea*	GQ339114	Clinostomatidae	4637 bp	Gustinelli *et al*. [9]	Kenya	2010

Table 2: Summary of base pairs and amino acids of *Clinostomum schizothoraxi* Kaw, 1950

Length	A	C	G	T	G+C
544 bp	127	112	148	157	47%
Total No. of Amino Acids	171				
Molecular Weight	19250 Da				

Table 3: Pairwise alignment of the 28S rDNA ITS consequences of *Clinostomum schizothoraxi* and *Clinostomum cutaneum*, numbering refers to ITS sequences

Digenean Species	ITS Sequences	DNA sequences	
C. schizothoraxi	89	CCTGACCTCGGATTAGGCGTGATTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAA	148
C. cutaneum	2935	CCTGACCTCGGATCAGACGTGATTACCCGCTGAACTTAAGCATATCACTAAGCGGAGGAA	2994
C. schizothoraxi	149	AAGAAACTAACCAGGATTCCCTTAGTAACGGCGAGTGAACAGGGATTAGCCCAGCACCGA	208
C. cutaneum	2995	AAGAAACTAACCAGGATTCCCT-AGT-ACTGCGAGTGAAGAGGGATTAGCCCAGCACCGA	3052
C. schizothoraxi	209	AG-CTGCGGTCATTTGGCCGTTCGGCAATGTGGTGTTTAGGTTGGCATGCTCAGGCGATG	367
C. cutaneum	3053	AGCCTGCGGTCATTTGACTGCTAGGCAATGTGGTGTTTAGGTTGGT-T-CTT-GGC-AT-	3107
C. schizothoraxi	368	TACTG-TGC----TAAGTCCATTCATGAATATGGNTAGCT--ATCTGGCCCAGAGAGGGT	427
C. cutaneum	3108	TACTGCTCCACCCTAAGTCCAGCAATGAGTACGGCTTACTGGAT-TGGCCCATTGAGGGT	3166
C. schizothoraxi	428	GAAA--CCCGTG	439
C. cutaneum	3167	GAAAGGCCCGTG	3178

gb>AF973619 of *Clinostomum schizothoraxi* and gb>GQ339114.1 of *Clinostomum cutaneum*. *Clinostomum schizothoraxi* Length=544

Score 544 bits, E value = 6e-60

Identities = 235/252 (93.25%), Gaps = 16/252 (6.35%)

Strand=Plus/Plus

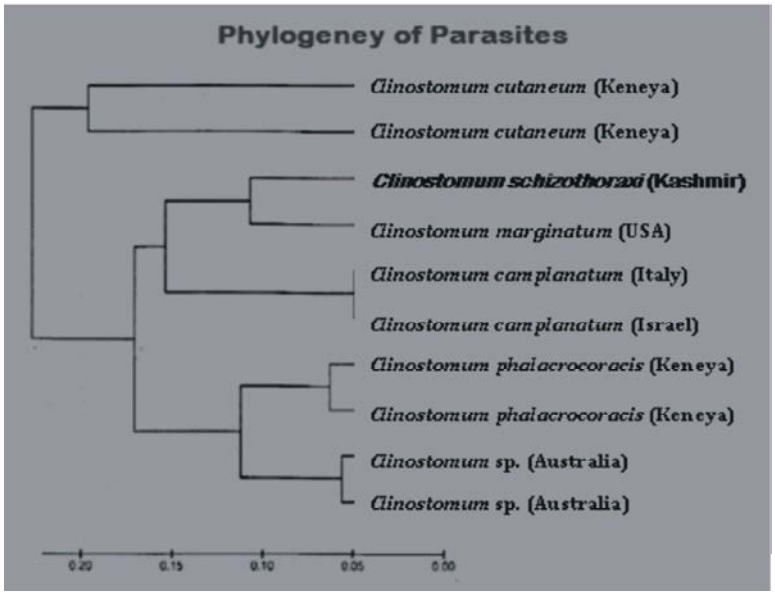

Fig. 2: Neighbour Joining (NJ) phenogram of Kimura 2-parameter distances in sequences of 28S rDNA ITS sequence of *Clinostomum schizothoraxi* Kaw, 1950

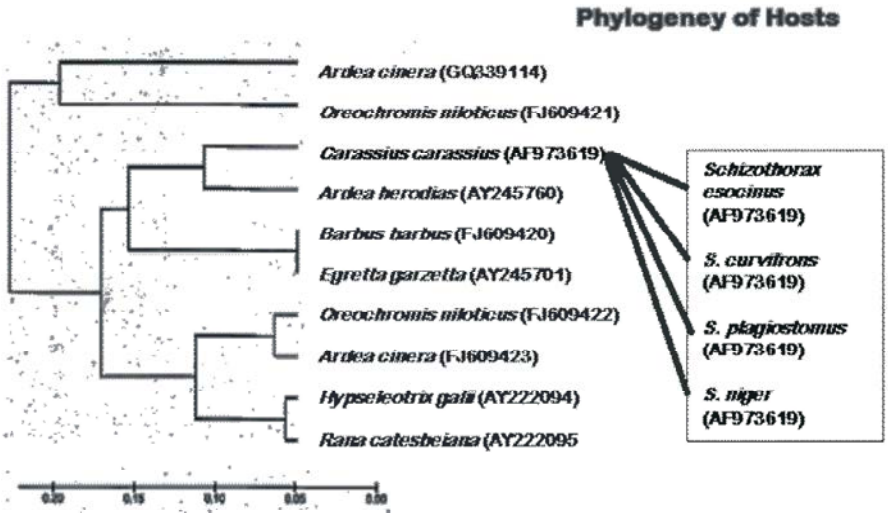

Fig. 3: Neighbour Joining (NJ) phylogenetic tree of hosts of *Clinostomum* species with their GenBank accession numbers of *Clinostomum schizothoraxi* Kaw, 1950

For phylogenetic analysis, Maximum likelihood (ML); Maximum Parsimony (MP) and Neighbour Joining (NJ) trees were constructed (Figs. 2&3. The ITS sequences of *Clinostomum schizothoraxi* were compared with the sequences available for other Digenean parasites in the GeneBank database. The trees showed that the query ITS of *Clinostomum schizothoraxi* sequences stand close to of *C. cutaneum.*

It is clear from the Table 4 that *Clinostomum schizothoraxi* with GeneBank accession number shows AF973619 shows 93.25% and 92.06% resemblance with that of *Clinostomum cutaneum* (GQ339114.1) and *Clinostomum complanatum* (FJ609421.1) with 16 gaps respectively. Out of 252 base pairs *Clinostomum schizothoraxi*, 235 & 232 base pairs match with that of *Clinostomum cutaneum* & *Clinostomum complanatum* respectively.

Table 4: Sequences producing significant alignments in *Clinostomum*species

Description	Max score	Total score	Query cover	E value	Identity	Accession No.
Clinostomum cutaneum voucher 129/09 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	241	241	24%	2e-59	93.25%	GQ339114.1
Clinostomum cutaneum voucher 118/07 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	241	241	24%	2e-59	92.06%	FJ609421.1
Clinostomum complanatum voucher 297/02 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	241	241	24%	2e-59	85%	FJ609420.1
Clinostomum phalacrocoracis voucher 10/08 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	241	241	24%	2e-59	82%	FJ609422.1
Clinostomum phalacrocoracis voucher 16/08-2TA 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	233	233	24%	4e-57	78%	FJ609423.1

DISCUSSION

Clinostomum schizothoraxi was first described for the first time [10] from *Schizothorax niger*, *S. esocinus* and *Oreinus sinuatus* in the River Jehlum [11, 12] from *Cyprinus carpio specularis*, *S. curvifrons, Nemachilus kashmirensis, Oreinus plagiostomus* and *Glyptothorax* sp. In order to contribute to the known molecular data on metacercarial form of *Clinostomum* species, the 28S rDNA of *Clinostomum schizothoraxi* was sequenced from *Scizothorax* and *Carassius* spp. collected from Kashmir valley. Comparison of the alignments obtained from sequences of the 28S rDNA genes and the internal transcribed spacer regions ITS1 and ITS2 of *Clinostomum schizothoraxi* with the other Digenean species sequences available in GenBank produced interesting results. The present observation showed that the alignment of the 28S rDNA exhibited few differences between the *Clinostomum schizothoraxi* and *C. cutaneum*, with distances of 6.75% [13] this region is highly conserved and is characterized by a slow evolutionary rate, useful for evaluating ancient evolutionary events however; it may be useless in some cases for discriminating organisms at the species level but in the case of the present study, the alignment showed little differences between the species. Several authors [14-15] described this gene as characterized by a high level of conservation similar to that of 18S rRNA gene, so it is used in phylogenetic studies [16-18]. In addition, this coding region is too short 160 bp in *Clinostomum* species, to produce phylogenies across large time scale.

The internal transcribed spacers, ITS1 and ITS2, are relatively conserved regions within species or genera and have been used as markers in population genetic studies [13] and to explore species boundaries in digenean families [19]. During the present study it was observed that ITS1 of *C. schizothoraxi* is characterized by the presence of tandem repeat units at the 50 end that provide the variability in the sequence composition at both the interspecific and intraspecific level. These repeats are also known in some digenetic trematode families, such as the Haematoloechidae, Mesometridae, Opecoelidae, Schistosomatidae, Strigeidae and Telorchiidae [19] and supports the present observation. The ITS1 sequences of *C. schizothoraxi* were 544 bp long and interspecific variation in the length of the ITS1 was observed, like the observations of others authors, Van Herwerden *et al.* [20] for *Schistosoma* spp. [21] *Trichobilharzia* spp. The different lengths of ITS1 are due to the presence of the repeat elements [22-24] which supports our results. The ITS2 spacer is characterized by a lower degree of variation with a high degree of conservation at the species level. Usually, it does not contain repeat units, although [25] found repeat units of ITS2 in the Echinostomatidae. It is also characterised by differences in length within and between families. In this study, it was found different length *i.e.,* 544 bp in *C. schizothoraxi* moreover, intraspecific variations were not observed as described by some authors for other digeneans [22, 26, 27]. The 28S rDNA gene is longer and has more variations in the rate of evolution than the 18S rRNA. This gene has been used in phylogenetic studies [28]. The alignment of

the 28S rDNA sequences obtained showed very low differences between the sequences compared, particularly between *C. schizothoraxi* and *C. cutaneum* with only 17 nucleotide differences.

Even though sequencing DNA represents the primary approach of modern systematics, the combination with traditional techniques, such as the use of morphological parameters, is essential for describing parasites at the species level. In fact, Nolanand Cribb [19] the best way to approach species identification is to perform morphological descriptions on half the specimens and molecular analysis on the other half.

In the present study, the metacercarial form of *C. schizothoraxi* was described for the first time from cyprinid fishes of Kashmir valley, the definitive host of the parasite, combining a traditional morphological approach with molecular analyses. Besides sequencing the DNA of parasite, the present study also able to link the adult stage to the metacercarial stage found in Kashmir valley from the same environment, confirming the morphological observations. Matthews and Cribb [29] suggested the need for a reorganization of the genus *Clinostomum* using both morphological and molecular approaches, this study hopefully represents the first input towards a systematic revision of this complex group of parasites.

CONCLUSION

It was concluded that the *Clinostomum schizothoraxi* Kaw, 1950 which was previously identified on the basis of morphology in various size parameters *viz*., body length, body width, clamp size, pharynx size, sucker distance, prohaptoral length *etc* are hereby supported on the basis of molecular characterization which was confirmed during the present study. Morphological findings are hereby supported using molecular tools like DNA extraction, nucleotide sequencing and base pair length. Thus, this study and its implications would definetly help in justifying the taxonomical studies of *Clinostomum* spp. and digeans as a whole.

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