

**Research Article**

**MORPHOLOGICAL, MOLECULAR AND KARYOLOGICAL ANALYSIS OF  
CLINOSTOMUM SCHIZOTHORAXI KAW, 1950 (DIGENEA) FROM FISHES OF  
KASHMIR VALLEY, INDIA**

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**ABSTRACT**

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% G+C 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. *Clinostomum schizothoraxi* showed different chromosome number as well as chromosome morphology as compared to that of Monogeneans. It Possess 20 numbers of diploid chromosomes ( $2n=20$ ). First pair of chromosomes is metacentric; pairs 2 & 3 are submetacentric; pairs 4, 5 & 6 are subtelocentric and pairs 7, 8, 9 and 10 are acrocentric. The smallest chromosome measured 5.88  $\mu$ m and the largest 9.15  $\mu$ m. Number of chromosome arms (NF) are to be 26 in number; total haploid complement length (TCL) is 77.12  $\mu$ m. Arm ratio of the complement ranges between 1.51–18.31 and the centromeric index ranges from 5.18–39.88. The karyotype formula can be written as  $2n=20=1m+2sm+3t+4a$ . This species has been studied for the first time with respect to molecular and chromosome analysis.

**KEYWORDS:** *Clinostomum schizothoraxi*; Fish; Molecular; GenBank; Chromosome

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**INTRODUCTION**

Digenetic trematodes comprise an estimated 24000 species, many of which have yet to be described (Poulin and Morand 2004). 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 listed by Kanev et al. (2002) 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 in parallel to karyological study 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 (Blankenspoor 1974; Graczyk 1991; Perez-Ponce de Leon 1995). In this context, molecular markers and chromosomal study offer powerful and much-needed tools that have the potential to distinguish between morphologically similar species at any stage in their life cycle.

**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. The resultant DNA was examined on 1.5% agarose-TAE gels, stained with ethidium bromide and visualized under UV light.

## Extraction of DNA

Phenol-Chloroform Technique: The detailed protocol is as follows (Sambrook and Russell, 2001):

- Wash the parasite material in PBS.
- ↓
- Place in a 1.5 ml eppendorf tube (UV cross-linked), grind with a pestle (UV treated) and add 300 µl of NET buffer.
- ↓
- Add 15 µl SDS and 6 µl Protinase-K (10 mg/ml); store at -20°C.
- ↓
- Incubate homogenate at 37°C overnight.
- ↓
- Add 400 µl of 25:24:1 phenol/chloroform/isoamyl alcohol (stored at 4°C); mix the contents gently (~2 minutes).
- ↓
- Centrifuge at 12,000 rpm for 10 minutes.
- ↓
- Pipette out the upper phase (~280 µl) into a fresh 1.5 ml tube.
- ↓
- Add 300 µl of 24:1 chloroform/isoamyl alcohol (stored at room temp.) to the sample; mix gently (~2 minutes).
- ↓
- Centrifuge at 10,000 rpm for 10 minutes.
- ↓
- Remove upper aqueous phase to a fresh 1.5 ml tube.
- ↓
- Add 1 ml of ice-cold 100% ethanol (stored at -2°C) to the aqueous solution; mix gently; store at -2°C for 1 h.
- ↓
- Centrifuge at 10,000 rpm for 10 minutes (DNA will precipitate).
- ↓
- Remove supernatant.
- ↓
- Wash the pellet in 1 ml 70% ethanol at 10,000 rpm for 10 minutes.
- ↓
- Tip out the alcohol after the spin.
- ↓
- Dry DNA pellet; resuspended in mili-Q water, store at 4°C.

## Karyology

Trematode specimens of *Clinostomum* spp. were obtained from gills, gill cover, mouth cavity, eyes and fins of fish hosts collected from the water bodies of Kashmir. Middle part of each individual containing testes and ovary was used for spreading analysis according to Frydrychova and Marec (2002). Parasite tissue was placed into hypotonic solution (0.075 M KCl) for 15-20 minutes and then put into freshly prepared Carnoy fixative (ethanol/ chloroform/ acetic acid, 6:3:1) for 20-30 min. Fixed tissue pieces were transferred into a drop of 60% acetic acid on a slide and thorn by tungsten needles making cell suspension. It was overspread along the slide on a heating plate (45°C). Slides were dehydrated in an ethanol series (70%, 80%, 100%, 1 min each) and stored at -20°C until further use.

Measurements are based on all chromosomes from 10 best metaphase spreads of parasites. The terminology relating to centromere position follows that of Levan *et al.*, 1964. A chromosome is metacentric (m) if the ci falls in the range of 37.5–50.0, submetacentric (sm) if 25.0–37.5, subtelocentric (st) if 12.5–25.0; acrocentric (a) if <12.5 and telocentric if 0. When the centromere position was on the borderline between two categories, both are listed.

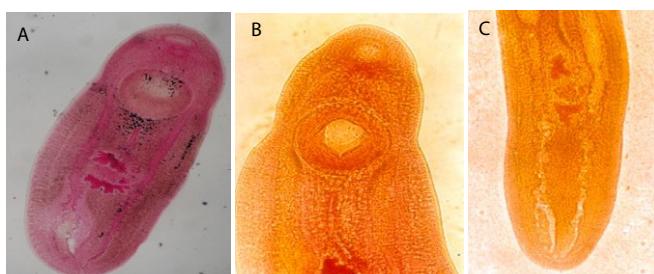
## Statistical Analysis

Parametric as well as the non-parametric tests were used for analyzing relative length of chromosomes. A computer program (Minitab for windows) was used for data analysis. The descriptive data was given as a mean ± standard deviation (SD). Chi-Square analysis was used to see the statistical significance in chromosome lengths. Pearson's correlation was used to find correlation between different species of helminths. Correlation analysis of data was carried out by using SPSS 16.5 package programme. Student's t-test was used to test the differences, which were considered to be significant when the p-value obtained was less than 0.05.

## RESULTS AND DISCUSSION

### Morphological analysis

***Clinostomum schizothoraxi* Kaw, 1950 (Figure 1).** Cysts enclosing metacercaria are spherical or broadly oval. Body of excysted worm elongated, linguiform, moderately fleshy with both ends rounded. Maximum width in or around region of gonads. Tegument aspinose. Oral sucker small, subterminal. Pharynx well developed,



**Figure 1:** Whole specimen, **b.** Anterior end showing oral & ventral suckers, **c.** Posterior end showing genital organs.

globular. Oesophagus practically absent. Intestinal caeca crenated on both sides behind level of acetabulum, inflated on one or both sides. Acetabulum large, muscular. Gonads immediately behind equatorial level of body. Shape of testes varies, being tandem, median, slightly lobed, more or less triangular. Cirrus sac small, ovoid; lies on right side of anterior testis and ovary; enclosing a coiled seminal vesicle, pars prostatica and long and coiled ductus ejaculatorius entire, intertesticular. Genital pore on right side at about equatorial level of an anterior testis. Ovary generally, more or less globular, sometimes oval and irregular. Uteroduct, commencing at ootype, extends round anterior testis along its left margin and opens into uterine sac at about beginning of its posterior third of its length. Uterine sac long. Metraterm well developed.

Genital atrium nearly circular in outline. Genital pore submedian, lying on right side of middle of anterior testis. Vitelline follicles indistinct. Excretory pore subterminal; excretory bladder extending to level of blind ends of caeca. Comparative characteristics of this species as given by different workers are summarized in Table 1.

### REMARKS

The distinguishing features of the present metacercarial form are; Body with narrow forebody and broad hindbody; length ratio of oral sucker and acetabulum 1:2.71; Genital pore on the left side at the level of anterior border of anterior testis; testes lobed to a varying degree; ovary irregular in shape. When compared with metacercarial forms of known species of the genus *Clinostomum*, the

**Table 1:** Comparative characteristics (Measurements in mm) of *Clinostomum schizophoraxi* Kaw, 1950.

Particulars	After Kaw, 1950	After Dhar & Kharoo, 1986	Fayaz & Chishti, 2000	Bakshi, 1999	Ara et al., 2002
Body length	3.5-4.75	3.72-4.35	3.718 (3.2-4.2)	4.2 (3.6-4.56)	3.5-4.1
Maximum width	1.0-1.66	1.28-1.5	1.264 (1.0-1.48)	0.956 (0.549-1.12)	1.01- 1.09
Oral sucker	0.2-0.26 x 0.31-0.38	0.15-0.22 x 0.25-0.32	0.233 x 0.307 (0.176-0.35 x 0.208-0.353)	0.224 x 0.356 (0.031-0.45 x 0.24-0.65)	0.23 -0.25 x 0 .24-0.25
Ventral sucker (Acetabulum)	0.58-0.68 x 0.6-0.68	-----	0.524 x 0.584 (0.466-0.598 x 0.58-0.626)	0.472 x 0.356 (0.031-0.45 x 0.24-0.65)	0.6 x 0 .7
Sucker length ratio	1:2.73	-----	1:2.37 (1:1.48-2.84)	1:2.105	-----
Acetabulum distance from anterior end	-----	0.42-0.50	0.565 (0.42-0.642)	-----	0.49-0 .69
Pharynx	-----	-----	0.17 x 0.177 (0.16-0.19 x 0.16-0.192)	0.59 x 0.43 (0.1-0.19 x 0.13-0.15)	0.17 x 0 .19
Anterior testis	0.18-0.3 x 0.19-0.3	0.26-0.46 x 0.2-0.35	0.205 x 0.243 (0.176-0.257 x 0.176-0.305)	0.284 x 0.223 (0.24-0.39 x 0.16-0.29)	-----
Posterior testis	0.2-0.22 x 0.18-0.33	0.28-0.46 x 0.25-0.40	0.19-0.24 (0.15-0.224 x 0.144-0.385)	0.234 x 0.302(0.21-0.29 x 0.019-0.17)	-----
Intertesticular distance	-----	-----	0.19 (0.16-0.224)	-----	-----
Cirrus sac	0.17-0.27 x 0.1-0.18	-----	0.22 x 0.088 (0.136-0.321 x 0.032-0.128)	0.222 x 0.111(0.121-0.29 x 0.019-0.17)	-----
Ovary	0.08-0.12 x 0.06-0.11	-----	0.076 x 0.085 (0.064-0.096 x 0.032-0.128)	0.220 x 0.292 (0.10-0.29 x 0.24-0.35)	0.141-0.152 x 0.72-0.079
Uterine sac length	0.5-0.6	-----	0.544 (0.433-0.691)	0.762 (0.65-1.01)	0.63-0.69
Caecal termination to hind end distance	-----	-----	0.11(0.032-0.2)	-----	0.11-0.13
Fish host	<i>Schizothorax niger</i> ; <i>S. esocinus</i> ; <i>Oreinus sinuatus</i>	<i>Cyprinus carpio spicularis</i> ; <i>Schizothorax niger</i> ; <i>S. esocinus</i> ; <i>S. curvifrons</i> ; <i>Nemachilus kashmirensis</i> ; <i>Oreinus plagiatus</i> and <i>Glyptothorax</i> sp.	<i>Crassius crassius</i>	<i>Carassius carassius</i> ; <i>Cyprinus carpio communis</i>	<i>Schizothorax esocinus</i> and <i>S. curvifrons</i>
Locations of cysts	Beneath skin, base of fins, tail and gill covers.	Beneath skin, on fins, gills and gill covers.	Gills, Gill covers and eye sockets	Gills, Fins and Operculum	On fins, operculum, mouth cavity, Gill lamellae
Locality	River Jehlum, Kashmir	-----	Anchar and Mansbal lake	Dal and Manasbal lake	River Jhelum

present form stands near *C. chrysichthys* Dubois, 1930; *C. gideoni* Bhalerao, 1942 ; *C. dasi* Bhalerao, 1942 and *C. piscidium* Southwell and Prashad, 1918 in general disposition of gonads and shape of testes. However, the present form is found to be distinct in the position of the opening of uteroduct into the uterine sac. From *C. dasi* it further differs in the position of genital pore.

## MOLECULAR ANALYSIS

### PCR Amplification

PCR amplification was carried out to amplify ITS

region of the Digenean parasite (Table 3). The size of the amplified product was found to be 544 bp long (Figure 2). In BLAST search of the sequences, it showed maximum similarity with *Clinostomum cutaneum*. 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 this parasite. Therefore, it can be assumed that the present form recovered from the fish host in Kashmir valley is *Clinostomum schizothoraxi* Kaw, 1950.

**Table 2:** Species of *Clinostomum* described from Indian region.

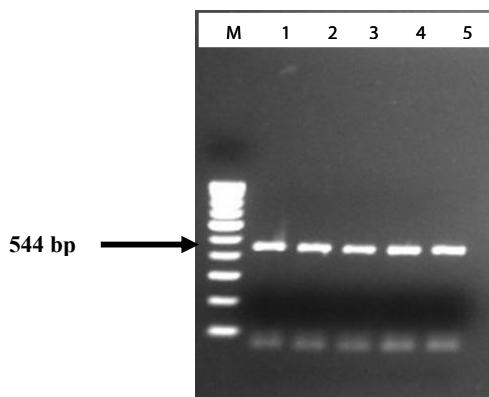
Species	Host of adult from (Avian Hosts)	Host of metacercarial from (Fish Hosts)	Synonym
<i>C. piscidium</i> , Southwell et Prashad, 1918	<i>Ardeola grayii</i> Exper. By Singh (1959)	<i>Trichogaster fasciatus</i> ; <i>Nandus nandus</i>	<i>C. complanatum</i> , Ukoli (1966) <i>C. marginatum</i> , Agarwal (1959)
<i>C. dasi</i> Bhalerao, 1942	<i>Bubulcus ibis</i> and <i>Ardea grayii</i> Exper. By Pandey (1966)	<i>Saccabranicus fossilis</i> ; <i>Heteropneustes fossilis</i>	<i>C. complanatum</i> , Ukoli (1966)
<i>C. gideoni</i> Bhalerao, 1942	-----	<i>Barbus sophare</i>	<i>C. complanatum</i> , Ukoli (1966)
<i>C. gideoni</i> Bhalerao, 1942	-----	Unidentified fish	-----
<i>C. anusi</i> Wesley, 1944	<i>Xenorthynchus asiaticus</i>	Unidentified fish	<i>C. complanatum</i> , Ukoli (1966)
<i>C. sp.</i> Srivastava, 1950	<i>Bubulcus cormandus</i> ; <i>Nycticorax nycticorax</i> Exper. By Srivastava (1950)	<i>Channa (Ophiocephala) punctatus</i>	-----
<i>C. schizothoraxi</i> , Kaw, 1950	-----	<i>Oreinus sinuatus</i> ; <i>Schizothorax esocinus</i> ; <i>S. niger</i>	-----
<i>C. deccanum</i> , Jaiswal, 1957	<i>Ardea cinerea</i>	-----	<i>C. complanatum</i> , (Rud. 1809); Ukoli (1966)
<i>C. demiegrattae</i> , Jaiswal, 1957	<i>Demiegretta asha</i>	-----	<i>C. complanatum</i> , Ukoli, (1966)
<i>C. hyderabadensis</i> , Jaiswal, 1957	<i>Ardea gravii</i>	-----	<i>C. complanatum</i> , Ukoli, (1966)
<i>C. macrostomum</i> , Jaiswal, 1957	<i>Tilapia nilotica</i> (Linn.)	<i>Channa (Ophiocephalus) striatus</i>	<i>C. mastacembeli</i> , Ukoli, (1966)
<i>C. mastacembeli</i> , Jaiswal, 1957	-----	<i>Mastacembelus armatus</i>	Syn. <i>C. macrostomum</i> , Ukoli, (1966)
<i>C. singhi</i> , Jaiswal, 1957	<i>Ardola gravii</i>	-----	<i>C. complanatum</i> , Ukoli, (1966)
<i>C. gignticum</i> , Agarwal, 1959a	<i>Bubulcus ibis</i> and <i>Ardeola gravii</i> Exper. By Agarwal (1959a)	<i>Channa (Ophiocephalus) Punctatus</i> ; <i>Barilius bendelisis</i>	<i>C. complanatum</i> , Ukoli, (1966)
<i>C. kalappah</i> , Bhalerao, 1947	(Mammalian Host) Accidentally – Cat Probably bird host in nature	-----	-----
<i>C. attenuatum</i> , Cort, 1913	<i>Ardea Herodias</i> , Jaiswal (1957) <i>Botaurus</i> , <i>Ardea</i> – Hunter et Hunter (1934)	(Amphebian Host) <i>Rana pipens</i> (U.S.A) <i>R. tigrina</i> – Cort (1913), Jaiswal (1957)	<i>C. marginatum</i> (Wright, 1879 nec. Rud., 1809) Beer (1933) Valid – Ukoli (1966)
<i>C. progonum</i> , Jaiswal, 1957	-----	<i>Rana cyanophlyctis</i>	<i>C. complanatum</i> , Ukoli (1966)

**Table 3:** PCR assay of Digenea which were carried out in a thermocycler (Eppendorf Mastercycler Personal) under different conditions.

Digenea	Initial Denaturation	Denaturation for 40 cycles	Annealing	Extension	Final extension
<i>Clinostomum schizothoraxi</i>	95°C for 10 minutes	40 cycles at 95°C for 30 seconds	52°C for 30 seconds	72°C for 75 seconds	72°C for 10 minutes

## NUCLEOTIDE SEQUENCES

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 4). Sequences were compared with other sequences of digenetic trematode species from GenBank. The consensus sequences



**Figure 2:** PCR product of *Clinostomum schizothoraxi* Kaw, 1950.

**Table 4:** Digenetic 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.	Digenetic Species	Host	Accession No	Family	Base pairs	Authors	Country	Year
1	<i>Clinostomum schizothoraxi</i> Kaw, 1950*	<i>Carassius carassius</i> ; <i>Schizothorax niger</i> ; <i>Schizothorax esocinus</i> ; <i>Schizothorax curvifrons</i>	AF973619	Clinostomatidae	544 bp	Present study	India	2015
2	<i>Clinostomum cutaneum</i>	Grey herons <i>Ardea cinerea</i> L. and Nile tilapia <i>Oreochromis niloticus</i> (L.)	FJ609421	Clinostomatidae	4614 bp	Caffara and Stanzani	Kenya	2010
3	<i>Clinostomum cutaneum</i>	<i>Ardea cinerea</i>	GQ339114	Clinostomatidae	4637 bp	Gustinelli et al.,	Kenya	2010

**Table 5:** 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 6:** Pairwise alignment of the 28S rDNA ITS consequences of *Clinostomum schizothoraxi* and *Clinostomum cutaneum*, numbering refers to ITS sequences.

<i>C. schizothoraxi</i>	89	CCTGACCTCGGATTAGGCCGTGATTACCCGCTGAACCTAACGCATATCAATAAGCGGAGGAA	148
<i>C. cutaneum</i>	2935	CCTGACCTCGGATCAGACGTGATTACCCGCTGAACCTAACGCATATCAACTAACGGAGGAA	2994
<i>C. schizothoraxi</i>	149	AAGAAACTAACCGAGGATTCCCCTTAGTAACGGCGAGTGAACAGGGATTAGCCAGCACCGA	208
<i>C. cutaneum</i>	2995	AAGAAACTAACCGAGGATTCCCT-AGT-ACCTGGAGCTGAAGAGGGATTAGCCAGCACCGA	3052
<i>C. schizothoraxi</i>	209	AG-CTGCGGTCAATTGGCCGTCGGCAATGTGGTTAGGTTGGCATGCTCAGGCCAGATG	367
<i>C. cutaneum</i>	3053	AGCCTGCGGTCAATTGACTGCTAGGCAATGTGGTTAGGTTGGT-T-CTT-GGC-AT-	3107
<i>C. schizothoraxi</i>	368	TACTG-TGC---TAAGTCCATTCATGAATATGGNTAGCT--ATCTGGCCCAGAGAGGGT	427
<i>C. cutaneum</i>	3108	TACTGCTCCACCCTAAGTCCAGCAATGAGTACGGCTTACTGGAT-TGGCCCATTGAGGGT	3166
<i>C. schizothoraxi</i>	428	GAAA--CCCGTG	439
<i>C. cutaneum</i>	3167		3178
<i>C. cutaneum</i>	3167	GAAAGGCCCGTG	

**Table 7:** Pairwise alignments of the 28S rDNA ITS consequences of *Clinostomum schizothoraxi* and *Clinostomum complanatum*, numbering refers to ITS sequences.

<i>C. schizothoraxi</i>	89	CCTGACCTCGGATTAGGCCGTGATTACCCGCTGAACCTTAAGCATATCAATAACCGGAGGAA	148
<i>C. complanatum</i>	2932	CCTGACCTCGGATCAGACGGTATTACCCGCTGAACCTTAAGCATATCAACTAACCGGAGGAA	2991
<i>C. schizothoraxi</i>	149	AAGAAACTAACCAAGGATTCCCTTAGTACGGCGAGTGAAACAGGGATTAGCCACCCGA	208
<i>C. complanatum</i>	2992	AAGAAACTAACCAAGGATTCCCT-AGT-ACTGCGAGTGAAGAGGGATTAGCCCAGCACCGA	3049
<i>C. schizothoraxi</i>	209	AG-CTGCGGTCAATTGGCCGTTCGGCAATGTGGTGTAGGTTGGCATGCTCAGGCATG	367
<i>C. complanatum</i>	3050	AGCCTGCGGTCAATTGACTGCTAGGCAATGTGGTGTAGGTTGG-T-CTT-GGC-AT-	3104
<i>C. schizothoraxi</i>	368	TACTG-TGC---TAAGTCATTGATGAATATGGNTAGCT--ATCTGGCCCAGACAGGGT	427
<i>C. complanatum</i>	3105	TACTGCTCCACCCTAAGTCCAGCAATGAGTACGGCTTACTGGAT-TGGCCCATTGAGGGT	3163
<i>C. schizothoraxi</i>	428	GAAAGGCCCCGTG	439
<i>C. complanatum</i>	3164	GAAAGGCCCCGTG	3175

gb>AF973619 of *Clinostomum schizothoraxi* and gb>[FJ609421.1](#) of *Clinostomum complanatum*. Length=544  
Score = 544 bits,  
E Value = 6e-60  
Identities = 232/252 (92.06%),  
Gaps = 16/252 (6.35%); Strand=Plus/Plus

(ML); Maximum Parsimony (MP) and Neighbour Joining (NJ) trees were constructed (Figurtes 4 and 5). 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 Tables 6 and 7 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.

## DISCUSSION

*Clinostomum schizothoraxi* was first described for the first time by Kaw (1950) from *Schizothorax niger*, *S. esocinus* and *Oreinus sinuatus* from the River Jhelum and then by Dhar (1978) and Dhar and Kharoo (1986) from *Cyprinus carpio specularis*, *S. curvifrons*, *Nemachilus kashmirensis*, *Oreinus plagiostomus* and *Glyptothonax* sp. In order to contribute to the known molecular data on metacercarial form of *Clinostomum* species, we sequenced almost all of the 28S rDNA of *Clinostomum schizothoraxi* 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%. As described by Hills and Dixon

(1991), 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 (Hills & Dixon, 1991; Hershkovitz & Lewis, 1996; Coleman, 2003) 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 (Troitsky & Bobrova, 1986; Troitsky et al., 1991; Suh et al., 1992). 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 (Hillis & Dixon, 1991) and to explore species boundaries in digenetic families (Nolan & Cribb, 2005). During the present study it was observed that ITS1 of *C. schizothoraxi* is characterized by the presence of tandem repeat units at the 5' 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 Telorchidae (Nolan & Cribb, 2005) and support 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, such as van Herwerden et al. (1998) for *Schistosoma* spp. and Dvorak et al. (2002) for *Trichobilharzia* spp. The different lengths of ITS1 are due to the presence of the repeat elements (Luton et al., 1992; Kane & Rollinson, 1994; Kane et al., 1996), 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

Morgan & Blair (1995) found repeat units of ITS2 in the Echinostomatidae. It is also characterised by differences in length within and between families. In our sequences, we found different length i.e., 544 bp in *C. schizothoraxi* moreover, we did not observe intraspecific variations as described by some authors for other digeneans (Luton *et al.*, 1992; Galazzo *et al.*, 2002; Jousson & Bartoli, 2002). 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 (Olson *et al.*, 2003). 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, as suggested by Nolan & Cribb (2005), 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, we described for the first time the metacercarial form of *C. schizothoraxi* 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 our parasite, we were also able to link the adult stage to the metacercarial stage found in Kashmir valley from the same environment, confirming the morphological observations. Following the suggestion of Matthews & Cribb (1998), who 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.

## KARYOLOGICAL ANALYSIS

The Clinostomidae Luhe, 1901 is a family of digeneans, the members of which live in the oral cavity, gills, gill covers, eye sockets, operculum, fins, and gill lamellae of fishes. Due to the high degree of morphological variability within the same species, *Clinostomum* has been subjected to several taxonomic revisions (Gustinelli *et al.*, 2010). The application of a karyology in parallel to morphological study may be particularly important for the identification of *Clinostomum* species described in the past only on the basis of morphological features.

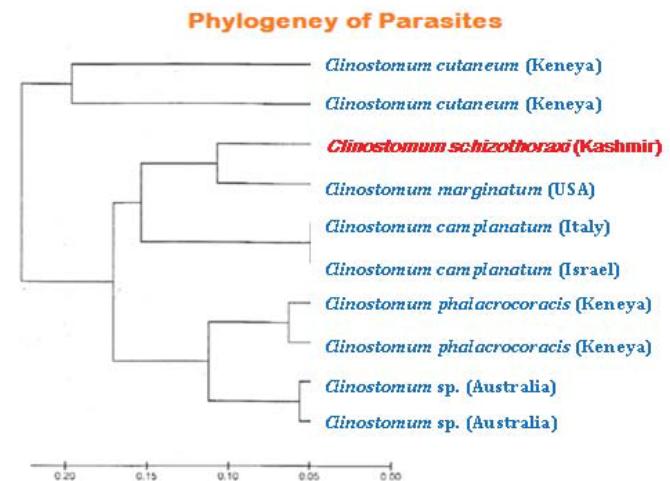
Colchicine treatment allowed us to examine enough number of metaphase plates with well-contrasted mitotic chromosomes. In this way, the number and morphology was determined with high accuracy. A diploid complement of  $2n=20$  was found in 43 dividing cells of *Clinostomum schizothoraxi* (Figure 6) collected from *Schizothorax* and *Carassius* spp. The chromosomes are large; the smallest measured 5.88  $\mu\text{m}$  and the largest 9.15  $\mu\text{m}$  (Table 9).

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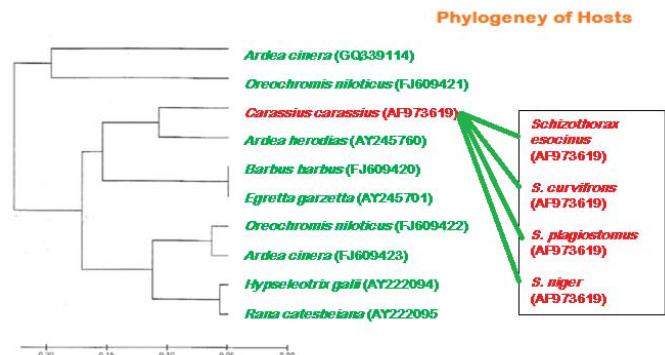
1 TATTCTGTGTT GCTGGAGTTA AAAATCTGT AGGGGGATCT GGGGGGTCG TTCTCGCCCC
61 TTTACCGCCTT CACCAAGATTG GGTATGACCC GGTAACGGGT TCAAGTGGGT GTAGTGATCG
121 TGCCCTCCCTT CAGCCGTGTC TGCTCAAACG GGAAAGACT GTTTTGATAA GCCTGCTTT
181 TAAACCCCTTT GGATGCTTC TAGATGCCCT TTACATAGTG TCGGGACGGG ATCTCCATC
241 TACTTTGAC AAAACTGGGT GCTCAAGACA GGCTATGGG CCTGAAATT ACCTCCATC
301 AGAAATGTTAT AGGACATTCGG TTCTATCTTA TTGGGTTTG GGGCTACGAA ATGTTTAAG
361 AGGGCACAGC GGGGCATTT GTATGGCGGT GTGCTAGGT AAATTCTGGG ATCGCCCCCA
421 GACAAACATC ATGAAAGCA TTGCCAAGA ATGTTTCAT TGATCAGGAG CGAAAGTCAG
481 ATGTTCTGAAG ACCATCTGTG ACCCTCTGTG CTCTGACCAT AATTGATGCC AACTGTGGAT
541 GTGC

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**Figure 3:** Raw nucleotide sequences of *Clinostomum schizothoraxi* Kaw, 1950.

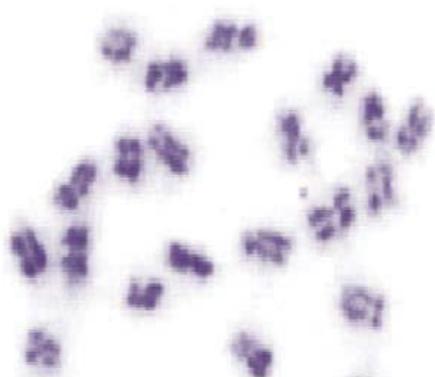


**Figure 4:** Neighbour Joining (NJ) phenogram of Kimura 2-parameter distances in sequences of 28S rDNA ITS sequence of *Clinostomum schizothoraxi* Kaw, 1950.

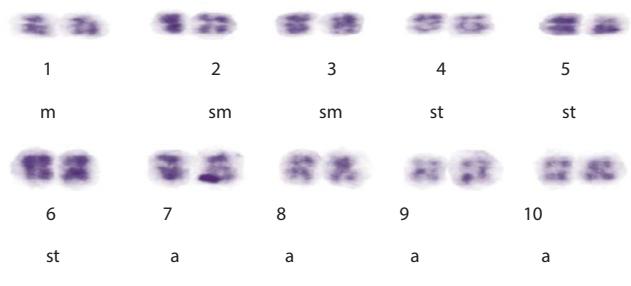


**Figure 5:** Neighbour Joining (NJ) phylogenetic tree of hosts of *Clinostomum* species with their GenBank accession numbers of *Clinostomum schizothoraxi* Kaw, 1950.

Karyotype (Figures 3 and 7) included one metacentric (no. 1); two submetacentric (no.s 2 and 3); three subtelocentric (no.s 4; 5 and 6) and four acrocentric (no.s 7, 8; 9 and 10) chromosome pair; the karyotype formula may be summarized as  $2n=20=1m+2sm+3t+4a$ . Number of chromosome arms (NF) was 26; total haploid complement length (TCL) was 77.12  $\mu\text{m}$ . Arm ratio of the complement ranges between 1.51–18.31 and the centromeric index ranges from 5.18–39.88 (Table 4). There is less chromosome length difference between 2 & 3; 4 & 5, 6, 7 & 8 and 9 & 10 which are statistically less significant ( $P<0.05$ ; Students T-test). On the basis of absolute length and centromeric position the chromosomes have been arranged in order of decreasing length in an ideogram (Figures 8 and 9) and the karyotype formula is: (K)  $2n=20=1m+2sm+3t+4a$ .



**Figure 6:** Chromosome preparation of *Clinostomum schizothoraxi*.



**Figure 7:** Karyotype constructed from mitotic cells of *Clinostomum schizothoraxi* stained with Giemsa (m=Metacentric; sm= Submetacentric; st= Subtelocentric; a= Acrocentric).

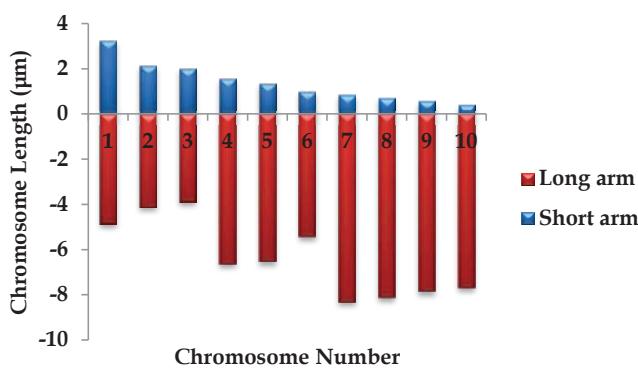
Scale Bar 10μm

**Table 8:** Sequences producing significant alignments in *Clinostomum* species.

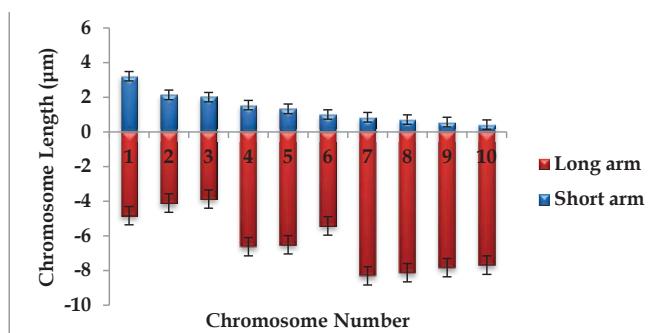
Description	Max score	Total score	Query cover	E value	Identity	Accession No.
<i>Clinostomum cutaneum</i> voucher 129/09 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and 28S ribosomal RNA gene, partial sequence	241	241	24%	2e-59	93.25%	GQ339114.1
<i>Clinostomum cutaneum</i> 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
<i>Clinostomum complanatum</i> 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
<i>Clinostomum phalacrocoracis</i> 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
<i>Clinostomum phalacrocoracis</i> 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

**Table 9:** Measurements and classification of chromosomes of *Clinostomum schizothoraxi* Kaw, 1950.

Chromosome pair number	Length of short arm (μm) 'S'	Length of long arm (μm) 'L'	Total Length/Absolute Length (μm) L+S	Arm Ratio (L/S)	Relative Length (%)	Centromeric Index (ci)	Classification	T-Value = -32.64 P-Value = 0.020 P<0.05
1	3.21	4.84	8.05	1.51	10.44	39.88	Metacentric	
2	2.13	4.11	6.24	1.93	8.09	34.13	Submetacentric	T-Value = -32.64 P-Value = 0.020 P<0.05
3	2.00	3.88	5.88	1.94	7.62	34.01	Submetacentric	
4	1.54	6.63	8.17	4.31	10.59	18.85	Subtelocentric	T-Value = -52.78 P-Value = 0.012; P<0.05
5	1.33	6.52	7.85	4.90	10.18	16.94	Subtelocentric	
6	1.00	5.43	6.43	5.43	8.34	15.55	Subtelocentric	T-Value = -9.54
7	0.84	8.31	9.15	9.89	11.86	9.18	Acrocentric	P-Value = 0.011 P<0.05
8	0.71	8.13	8.84	11.45	11.46	8.03	Acrocentric	
9	0.56	7.84	8.40	14.00	10.89	6.67	Acrocentric	T-Value = -61.25 P-Value = 0.010 P<0.001
10	0.42	7.69	8.11	18.31	10.52	5.18	Acrocentric	



**Figure 8:** Haploid idiogram of *Clinostomum schizothoraxi* Kaw, 1950.



**Figure 9:** Error bars with standard error of *Clinostomum schizothoraxi* Kaw, 1950.

## DISCUSSION

Digenean Trematodes are karyotypically conservative, and their karyotypes tend to have the same number and closely related gross chromosome morphology of the genus and family (or even higher) taxonomic level. Most of the karyologically studied members of Digenean families are Troglotrematidae, Plagiorchiidae, Telorchiidae, Prosthogonimidae, and Lecithodendriidae. Information on the cytogenetics of Clinostomidae trematodes is limited largely to the description of the chromosome numbers. *Clinostomum schizothoraxi* possess 10 haploid chromosomes in which first pair is metacentric; pairs 2 & 3 are submetacentric while pairs 4; 5 & 6 are subtelocentric and pairs 7; 8; 9 and 10 are acrocentric which were observed during the present investigation. Britt (1947) also reported 10 haploid chromosomes in various species of the family Clinostomidae. According to Britt (1947), the largest chromosome were observed in the family Allocereadiidae, where some pairs measures up to 8 μm in length and smallest in the families Clinostomidae and Plagiorchiidae, in case of the present study the chromosomes are medium sized; the smallest measured 5.88 μm and the largest 9.15 μm. His work was mainly concerned with collecting cytological evidences to find an evolutionary mechanism in Platyhelminthes. However, Short and Menzel (1959) concluded that morphological changes accompanying separation of the genera seem to have been the results of translocations, inversions, deletions, and changes in the number of smaller chromosomes of Digeneans. The

present results on karyotype of *Clinostomum schizothoraxi*, 2n=20, are in agreement with Britt (1947) who described 20 chromosomes of *Clinostomum marginatum*. Despite limitations of the method used, Britt (1947) established that small chromosomes are characteristic of Clinostomidae species and our data confirm these findings. Mitotic chromosomes of *Clinostomum schizothoraxi* are medium sized, up to 9.15 μm. Notable workers like Cable (1931, 1974); Anderson (1935); Chen (1937); Rees (1937); Pennypacker (1936, 1940); Markell (1943); Ciordia (1949; 1950, 1956); Willmott (1950); Willey and Godman (1950); John (1956); Dhingra (1954a-c, 1955a-c); Guildford (1955, 1961); Short, (1957) and Short and Menzel (1957, 1959); Sanderson (1959); Barton (1960); Greson (1958, 1964); Sharma et al. (1968); Saksena (1969); Reddy and Subramanyam (1973, 1975); Sharma & Nakhasi (1974) and Jha (1975) have observed interesting cytological variations regarding the chromosome number and morphology in the order Digeneans.

Changes in chromosome form in digeneans trematodes were believed most commonly from centric fusion, pericentric inversions, changes in the amount of heterochromatin and euchromatin, and through other chromosomal rearrangements (Grossman & Cain, 1981). Often a likely route for the evolution of chromosomes within a family or genus can be visualised; key indicators include: karyotypic variation between related species whose diploid complements differ (Barsiene & Grabda-Kazubska, 1988); atypically large chromosomes (Grossman & Cain, 1981); the relative lengths of chromosomes comprising the haploid genome (White, 1973); and the chromosome arm number (Mutafova, 1994). One of the most commonly observed evolutionary pathways occurring in the digenean genome results from a Robertsonian translocation.

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