

Morphological and Molecular Characterization of *Diplozoon kashmirensis*; *D. aegyptensis* and *D. guptai* Collected from Fishes of Kashmir Valley-India

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Abstract

The study reports the results of molecular characterization of the Internal Transcribed Spacer (ITS) of ribosomal DNA of 3 *Monogenean* species using polymerase chain reaction (PCR), nucleotide sequencing and construction of phylogenetic trees from different fish hosts of Kashmir. The present study shows that the size of the amplified product is 873bp long for *D. kashmirensis*, 1120bp long in *D. aegyptensis* and 687bp long in *D. guptai* revealing that there are intraspecific differences in their base pair lengths. Guanine and Cytosine (G+C) content of three *Diplozoon* species was found nearly constant for three species i.e., 47% (*D. kashmirensis*); 47% (*D. aegyptensis*) and 48% (*D. guptai*), this GC richness contributes to physical attributes of RNA structures, as there is correlation between GC content and optimal growth temperature. An important observation during the present study has been noticed that *Schizothorax niger* is infected by all the three species of *Diplozoidae*; *D. kashmirensis*; *D. aegyptensis* and *D. guptai*, but when all six fishes were collected simultaneously, parasitism by all the parasite species was never observed. Phylogenetic trees Maximum Parsimony (MP), Maximum Likelihood (ML) and Neighbor Joining (NJ) showed that *D. kashmirensis* and *D. aegyptensis* share a common host *Carassius carassius* and *S. niger*.

Keywords: *Diplozoon*; Ribosomal DNA; Schizothorax; Kashmir; Phylogenetic trees.

Introduction

Monogeneans belonging to the *Diplozoidae* are common parasites on the gills of cyprinid fish. The life cycle is direct, including free-swimming oncomiracidia, larval stage (diporpa) and adult. Two larvae (diporpa) permanently fuse into a pair to form the sexually matured adult. In the adult, the vitellaria and almost all the internal organs are situated in the anterior part of the body. The female and male reproductive organs and terminal part of the gut are situated in the posterior part. The attachment apparatus of adults consist of four pairs of clamps and a pair of small central hooks situated on the ventral side of the opisthaptor. Due to the complicated determination of several groups of *monogenean* parasites, molecular markers based on species-specific variability in the ribosomal DNA region (rDNA) their cytogenetics have been designed and shown to be useful for precise species identification [1-5]. The interspecific nucleic acid variability of Internal Transcribed Spacers of rDNA (ITS) has also been used to distinguish *diplozoid* parasites [6-11].

From the available data, it has been concluded that morphological and metrical differences in the clamp size, pharynx size, prohaptor length, opisthohaptor length, sucker distance, testis, ovary and egg size were the major criteria for species determination. Species determination of trematodes is difficult and demands great skill and experience. As the structures of taxonomic importance (central hooks, clamps etc.) grow gradually and the measurements of sclerotized structures are variable, species determination of trematodes in different developmental stages is not always clear. There are still some unclear descriptions of trematode species that differ only by host species, and some studies that did not employ recommended criteria [12,13]. Molecular biology techniques have been used as objective methods to distinguish between parasite species. The rDNA genes, particularly the 28S gene, have been found generally useful in molecular taxonomy and phylogeny of parasites [14-16]. However, there are no published

molecular studies of trematode genomes from the Kashmir valley. The present study reports the results of molecular analysis of the Internal Transcribed Spacer (ITS) of ribosomal DNA of 3 *Monogenean* species namely *Diplozoon kashmirensis* Kaw, *Diplozoon aegyptensis* Fischthal et Kuntz, *Diplozoon guptai* [17] using Polymerase Chain Reaction (PCR), nucleotide sequencing and construction of phylogenetic from different fish hosts of the Kashmir valley.

Materials and Methods

Parasite material

Parasite specimens of *Diplozoon* spp. were collected from the *Carassius carassius*; *Cyprinus carpio communis*; *C. c. specularis*; *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.

DNA isolation

Parasite specimens of three *Diplozoon* species were collected from fish hosts of *Carassius carassius*; *Cyprinus carpio communis*;

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Received August 18, 2015; Accepted August 24, 2015; Published October 27, 2015

Citation: Ahmad F, Fazili KM, Sofi TA, Sheikh BA, Waza AA (2015) Morphological and Molecular Characterization of *Diplozoon kashmirensis*; *D. aegyptensis* and *D. guptai* Collected from Fishes of Kashmir Valley-India. Fish Aquac J 6: 147. doi:10.4172/2150-3508.1000147

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Schizothorax curvifrons; *Schizothorax esocinus*; *Schizothorax niger* and *Schizothorax plagiostomus* from Wular lake, Anchar lake, Dal lake, Manasbal 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 by [18] and as such, these specimens were air dried to remove ethanol. The resultant DNA was examined on 1.5% agarose-TAE gels, stained with ethidium bromide (EtBr) and visualized under UV light.

Results

Morphological characterization

The present specimens having rectangular opisthaptor with four pairs of clamps and two individuals in form of a cross belongs to genus *Diplozoon* Nordmann, 1832. When compared *D. kashmirensis* with *D. aegyptensis*, they showed similarity in comparative size of clamps, size of eggs, form of oral suckers but differs in the proportion of body length to its breadth, shape of ovary, shape and position of testis. *D. kashmirensis* resembles *D. guptai* in egg size, absences of sticky glands in the anterior part, position of testis with respect to ovary, extent of vitellaria and in the arrangement of intestine in hind portion of the

worm but shows strong variations as regards total body length, size ratios, clamp size, the shape & size of testis and also prepharynx size. The variations of the three species can be regarded as intraspecific variations due to geographical isolation and are not sufficient for the creation of new species and thus the present specimens are described as *D. kashmirensis* Kaw, 1950; *D. aegyptensis* Fischthal et Kuntz, 1963 and *D. guptai* [17-24] (Table 1).

PCR amplification

The PCR amplified products of ITS regions of rDNA were successfully obtained using the primers (Table 2). PCR amplification was carried out to amplify ITS region of *Diplozoon* species (Table 3). The size of the amplified product was found to be 873bp long for *D. kashmirensis*; 1120bp long in *D. aegyptensis* and 687bp long in case of *D. guptai* (Figure 1). In BLAST search of these sequences, they showed similarity with other *Diplozoon* spp. (Table 3). In bioinformatics analysis, the results tallied with those of the earlier study; hence, the same are not repeated here in. Based on morphological studies, these species were identified as belonging to three *Diplozoon* species. The present results of the molecular analysis corroborate the species identification of these forms. Therefore, it can be assumed that the present species recovered from the different fish hosts of water bodies

Species Particulars													
Particulars	Total Body Length	Forebody	Hind body	Length ratio between fore & hind body	Clamp size	Testis	Egg size	Anterior suckers	Prepharynx	Pharynx	Host	Site	Locality
<i>D. kashmirensis</i> Kaw, 1950	2.3-4.32	1.4-2.64 x 0.71-1.51	0.9 x 1.72 x 0.5-0.69	1:0.646	0.15 x 0.075 0.166 x 0.076 0.154 x 0.075 0.140 x 0.074	0.16 x 0.27-0.29	0.27 x 0.29 x 0.07-0.09	0.063-0.074 x 0.045-0.063	0.065	0.065-0.075	Schizothorax sp.	Gills	Dal Lake, Kashmir
<i>D. aegyptensis</i> Fischthal et Kuntz, 1963	4.529 (3.62-5.77)	2.665 x 0.558 (1.879-3.452 x 0.299-0.836)	1.128 x 0.178 (0.867-1.871 x 0.130-0.245)	----	0.070 x 0.097 0.065-0.079 x 0.092-0.102	0.136 x 0.08 (0.103-0.19 x 0.063-0.093)	0.292 x 0.107 (0.524-0.313 x 0.081-0.132)	0.038 x 0.07 (0.029-0.046 x 0.065-0.079)	0.027 (0.020-0.034)	0.062 x 0.044 (0.051-0.075 x 0.040-0.050)	Labeo forskalii	Gills	Giza Fish Market, Giza Fish Market, Egypt
<i>D. aegyptensis</i> Fayaz et Chishti 1993	4.2 (3.95-4.25)	----	----	----	0.114 (0.10-0.124) x 0.045 (0.04-0.048)	0.155 (0.14-0.17) x 0.105 (0.10-0.11)	0.25 (0.22-0.28) x 0.082 (0.076-0.088)	0.048 (0.032-0.064)	0.054 (0.041-0.068)	0.064 (0.056-0.072) x 0.047 (0.044-0.05)	Schizothorax niger	Gills	Dal lake, Kashmir
<i>Diplozoon guptai</i> Fayaz et Chishti 1999	1.873 (1.28-2.55)	1.66 x 0.604 (0.755-0.144 x 0.44-0.65)	0.603 x 0.395 (0.46-0.76 x 0.289-0.48)	1:0.523 (1:0.4-0.639)	0.102 x 0.045 0.106 x 0.032 0.093 x 0.038 0.081 x 0.035	0.813 x 0.0786 (0.076-0.12 x 0.052-0.128)	0.245-0.07 (0.228-0.268 x 0.06-0.08)	0.056 x 0.05 (0.04-0.08 x 0.032-0.072)	0.0356 (0.03-0.042)	0.057 x 0.025 (0.044-0.069 x 0.02-0.028)	Schizothorax niger; S. esocinus; Labeo sp. and Carasius carassius	Gills	Dal and Anchar Lake, Kashmir

Table 1: Comparative Morphological characteristics of Diplozoon species (measurements in mm).

Species	Primer Designed	GenBank Accession Number	Author and Year
<i>Diplozoon kashmirensis</i> Kaw, 1950	Forward	AF 369758 to AF 369761	Sicard et al., 2001
	Cer5.8S 2249:5GCTCACGTGACGATGAAGAG3'		
<i>Diplozoon aegyptensis</i> Fischthal et Kuntz, 1963	Reverse		
<i>Diplozoon guptai</i> Fayaz and Chishti, 1999	Cer28S 3116 :5TTCGCTATCGGACTCGTGCC3'		

Table 2: Primers used for Trematodes.

Monogenea	Initial Denaturation	Denaturation for 30 cycles	Annealing	Extension	Final extension
<i>Diplozoon kashmirensis</i> ; <i>D. aegyptensis</i> and <i>D. guptai</i>	95°C for 10 minutes	30 cycles at 95°C for 30 seconds	55°C for 30 seconds	72°C for 75 seconds	72°C for 10 minutes

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

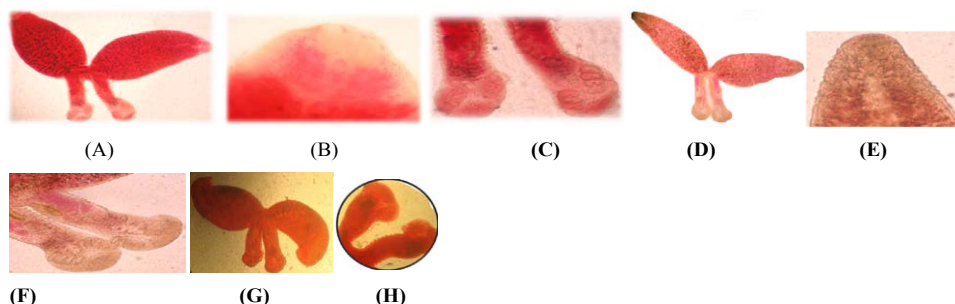


Figure 1: (A). Whole specimen of *D. kashmirensis*, (B). Anterior end showing prohaptor of *D. kashmirensis*, (C). Posterior end showing posthaptors of *D. kashmirensis*, (D). Whole specimen of *D. aegyptensis*, (E). Forebody showing suckers & pharynx of *D. aegyptensis*, (F). Hindbody showing clamps & eggs of *D. aegyptensis*, (G). Whole specimen of *D. guptai*, (H). Posterior body showing posthaptors of *D. guptai*.

S no	Monogenean Species	Host	GenBank Accession No.	Family	Base pair length	Authors	Country	Year
1.	<i>D. kashmirensis</i> Kaw, 1950*	Carassius Carassius, Cyprinus carpio cummunis, Schizothorax niger, S. esocinus, S. curvifrons	AF973616	Diplozoidea	873 bp	Present study	India	2015
2.	<i>D. aegyptensis</i> Fischthal et Kuntz, 1963*	Carassius Carassius, Schizothorax niger;	AF973617	Diplozoidea	1120 bp	Present study	India	2015
3.	<i>D. guptai</i> Fayaz and Chishti, 1999*	Schizothorax niger	AF973618	Diplozoidea	687 bp	Present study	India	2015
4.	<i>D. bliccae</i> (Glaser, 1965)	Blicca bjoerkna	AF369761	Diplozoidea	988 bp	Sicard et al.	France	2001
5.	<i>D. paradoxum</i> Nordmann, 1832	Abramis brama	AF369759 and AJ563372	Diplozoidea	769 bp	Matejusova	Czech Republic	2004
6.	<i>D. homoion</i> Bychowsky & Nagibina, 1959	Rutilus rutilus, Scardinius erythrophthalmus	AF369760	Diplozoidea	996 bp	Sicard et al.	France	2001

Table 4: Monogenean trematode species used for molecular comparison of ITS rDNA sequences along with their hosts, country and GenBank accession numbers for corresponding sequences (*Query sequence).

of Kashmir valley is *D. kashmirensis* Kaw, *Diplozoon aegyptensis* Fischthal et Kuntz, and *Diplozoon guptai* [24].

[Reagents for PCR: Taq DNA polymerase 3 U/μl, dNTP mixture 100 mM, primers 20 pmols, 10 × TaqDNA Polymerase buffer (Genei), PCR water (Sterile milli-Q)].

Sequences deposited in GenBank

GenBank: AF973616; *Diplozoon kashmirensis*, complete sequence.

GenBank: AF973617; *Diplozoon aegyptensis*, complete sequence.

GenBank: AF973618; *Diplozoon guptai*, complete sequence.

The three monogenean species of Trematodes viz., *Diplozoon kashmirensis* Kaw, 1950; *Diplozoon aegyptensis* Fischthal et Kuntz, 1963 and *Diplozoon guptai* [17] which were recovered during the present study are used for molecular study for the first time.

Nucleotide sequences

PCR products were visualized and documented, and the sizes of the sequences were estimated. The sequence obtained from three different *Diplozoon* species were submitted to GenBank and their accession number acquired were AF973616; AF973617 and AF973618 (Table 4). Sequences were compared with other sequences of monogenean species from GenBank. When the BLAST search was performed, the query sequence showed maximum similarity with 28S rDNA sequence of *Diplozoon* spp. The nucleotide sequences obtained and shown in (Figures 2-5) are as raw sequences (Table 5).

Pairwise alignment

Pairwise alignments of *Diplozoon* species were made by using

different softwares such as Gene Runner, DNA Dynamo, Chromas Pro. *D. kashmirensis* showed maximum similarity with those of *D. bliccae* where as *D. aegyptensis* showed maximum similarity with *D. paradoxum* and in case of *D. guptai* that showed maximum similarity to *D. homoion* (Tables 6-8).

Construction of phylogenetic tree

Phylogenetic trees were obtained by comparing the 28S rDNA sequences of the query parasite and other available sequences for related monogenean parasites. The E value was found to be zero up to the 100th sequence of BLAST search and the query coverage 95% and above. The species of *D. kashmirensis* and *D. aegyptensis* appeared to be the most closely related species, with well-supported clade by Neighbour joining and MP trees (Figures 6-8).

Above Table shows that *Diplozoon kashmirensis* having GenBank accession number AF973616 mostly resembles with *Diplozoon bliccae* with an accession number AF369761.1. Out of 867 base pairs of *Diplozoon kashmirensis*, 807bp match with that of *Diplozoon bliccae* i.e., 93.08% similarity with 15 gaps (1.73%).

From the Table 6 it is clear that *Diplozoon aegyptensis* having GenBank accession number AF973617 shows 94.13% similarity with *Diplozoon paradoxum* with an accession number AF369759.1. Out of 988 base pairs of *Diplozoon aegyptensis*, 930 bp match with that of *Diplozoon paradoxum* with 11 gaps (1.11%).

The present observation shows that *Diplozoon guptai* having GenBank accession number AF973618 shows 86.16% similarity with that of *Diplozoon homoion* having GeneBank accession number AF369760.1 (Table 7). 585 bp of *Diplozoon guptai* matches with *Diplozoon homoion* with 15 gaps, out of total 679 base pairs.

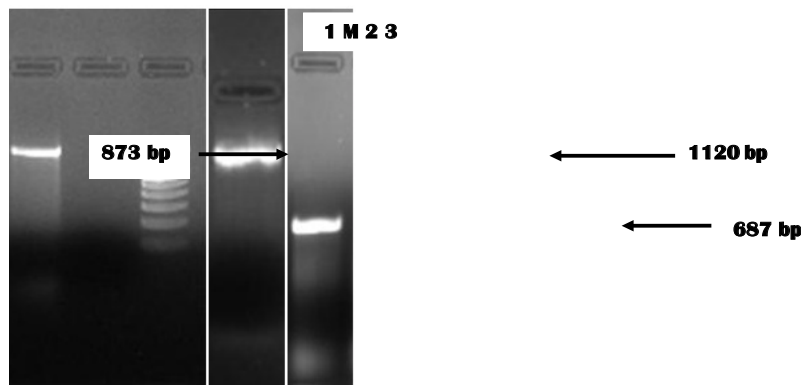


Figure 2: Polymerase Chain Reaction (PCR) products of Trematodes (Monogenea) M=marker; bp=base pairs (100 bp ladder), 1=*Diplozoon kashmirensis* Kaw, 1950, 2=*Diplozoon aegyptensis* Fischthal et Kuntz, 1963 and 3=*Diplozoon guptai* [24].

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1 TGCTTACTGA CTTGAGCATC GATTTCTTGA ACGTGAATTG CGGCATTACC CTCTAATGAT
61 GCCACGCCA GCGGAGTATC GGCATTAATTA CTAGCACGAC GCTTATTTGG TCCTGGCTTA
121 GAAAGTTGTC AGCCGTCGTG TTGTA CTTGG CAACGTGTTG TTCTGTTGTC AAGTCGGCGG
181 TATTATTGAC GCTTGCCAAA TGTAATGGAG AGTTTGATA TCGGAAATAT CTTCGGTAG
241 CCTGTTGGTG TTGGCTACGC TGCCCCGTGT ATTTTTTATT TGCATTTTGG TGCATACCGA
301 TGGGTGGTT AGCTTCTCGT CAGCAGTGCG TCCTTGCCGG TGGTGTCTG GAATGGGAAT
361 TTCAATAAGC ATTTCTGAAT CCTAATTGTG AAATTGTCAT TTTATGTGCT GTTCTCTTGA
421 GCCGCATGGC CCACTTGTG TCGATGACC AGTGACGCTT TGAATGCGAG TGCATGCATG
481 CCAGGTCTCA GCCTATTTGT GATCGCGACA GTGCTTTGCT TGTGTTCTGC GTTTAATTTT
541 TGTCACGTGT TCCCGCAAT GAGCGAGTCT GGCCCGAGAC GAGAGCATGT GCCCATGTGC
601 TGCTGTGCAG ACATTA CACTAC TCCATTCTTC GCTAAGTGTG TATCGGTGTC ACCCGTATTT
661 TACTGTACTT CTGTGGTGTG TGCACCTGAC CAAGGATTAG GCGTGATCAC CCGCTGAGCT
721 TAAGCATATC AATGGGCGGA GGAAAAGAAA CTAACCACTA TTCCCTTAGT AACGTCGAGT
781 GAACA CCGAT TAGCAAAGCA CCGAAGCTGC GGTCTTTTGG CCGTTCGGCA ATCCGGTGTG
841 TAGGTTATCA TACTCAGGCG ATGTA CTTGTG GTC
    
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Figure 3: Raw nucleotide sequences of *Diplozoon kashmirensis* Kaw, 1950.

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1 AA CTGCAAA C TGCCTTGAGC AAATTAGTTG TGAAGTAAA TTACGGCAGG AGGCTCCCC
61 TGATAACACG CCTAGCCCCG TGTGGCATT AAATCGATCA CGACGCTTAA TTGGTTGTGG
121 CTTAGTTTGT TGTACCCGT CGTGTGTAC TTGGCAACGT GTTGTTCAGT TGTCAAGTAG
181 ACGGTATTAT TGACGCTTGC CAAATGTAAT GGAGAGTTAG NDATGCGAAA TATCCGCTGG
241 TAGCCITGTTG GTGTTGGCAA CGCTGGCCCC TGTATGGTTT ACTTGTTTTT TTGTGCATAC
301 TCATGGGGGC GGTTAACTTC GCGTCATCAG AGCGTGTTG CCGGAAGTGT ATTGCAGTGG
361 CGTGGGAAAT TCAATGAGCA TTTGTGAATG GTAATTGTTA AATTGCCATT TTATGTGCTG
421 TTCTCTGAG CCTTTTGCC CACGGGTTGT GCGGTGACCA GTGTTGCTTT GAATGCGTGC
481 GCATGCATGC CAGGTCGCAG CCTATTGTGA TCGCGACAGT GCTTTGCTTG TGTCTGCGT
541 TTAATTTTTG TCACTCCCGC ACTGGTCTGT AAGTGCATGT CCCGAGATGA GATTGTGTGC
601 CCATGTCATG CTGGGCTGAC ATTA CTA CACTGGTCCG TAAAGTCATG TCGGTGTCAT
661 CAGTATTCTA CTGTA CTTGTG TGCACCTGAC CTCGGATTAG GCGTGATTAC
721 CCGCTGAACT TAAGCATATC AATAAGCGGA GGATTAGAAA CTAACCAAGGA TTCCCTTAGT
781 AACGGCGAGT GAACAGGGAT TAGCCCAGTT CCGAAGCTGC GGTCTTTTGG CCGTTCGGCA
841 ATGTTATGTT TAGGTTGGCA TACTCAGGCG ATGTA CTTGTG CTAAGTCCAT TCATGAATAT
901 GGCTAGCTAT CTGTCCAGA GAGGGTGAAG GGCCCGTGA CATAGTACGT TGTCTGTCT
961 TAGCCAA CCG TTGAGTCGGG GTTTTACTTG AGGCAGCCCA AAAAGTAGAC GGTATTATTG
1021 ACGCTTGCCA AATGTAATGG AGTTAGTGTG ACCCGAGATG AGATTGGTTG GCATACCGAG
1081 GCGATGTA CTTGTGCTAAGTC CAGGTGTTG CATTATTAGT
    
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Figure 4: Raw nucleotide sequences of *Diplozoon aegyptensis* Fischthal et Kuntz, 1963.

1 TGCTGCAAACTGCCTTGAAAATCTTCTTCTGAAACGCGAA TCGCGGTATTAGGTACTGCC
 61 TGATGCCACG CCTAGCCGAG TGTGGCATT ATATCTATCA CGACGCTTAA TTGGTCGTGG
 121 CTTAGGCGGT TGTCCTCCGT CGTGTTTTAC TTTGCAACGT GTTGCTCAGT TGTACTGTGC
 181 ACGGTATTAT TGACGCTTGC CAAATGTAAT GGAGAGTGTG TATATGCGAA ATTTCTGCCG
 241 GTAGCCTGTT GGCTGCGGCG ACGCTGCCCC GTGGCCGGTT TACTTGCATT TTTGTATCTA
 301 CCGATTGGGG CGGTTAGCTT GTATTCATCA GCCCCGTGTT GCCGGTGGTG ACTCGTGGTG
 361 GCGTGGGAAT TTCAATAAGC ATTACTGAAT GGTAATTAAT AAATTGCCAT TATATATGCT
 421 GTGCGCTTGA GCCTTTTGGC CCACGGGTTG TATTGTGACC AGTGTTCCT TGAATGCGCT
 481 CGCAAGCATG CCAGGTCTCA GCCTATGGTG ATCGAGACAG TTCTTTGCTT GTGTTATGCG
 541 TTTAGGTGTT GTCACCTCTA CTTGCATATG TGCTAGTGTG TACGCGGAAT GAGCTTTTGT
 601 GCCCATGTCA TGCTGTGCTG ACGTACTTC TCCACTGGTC CAGAAGTGCA TGTCGGGGTC
 661 ACCATAACTT TGCTGTATTG TGGGTGC

Figure 5: Raw nucleotide sequences of *Diplozoon guptai* [24].

	<i>Diplozoon kashmirensis</i>	<i>Diplozoon aegyptensis</i>	<i>Diplozoon guptai</i>
Length	873 bp	1120 bp	687 bp
A	177	237	123
C	191	224	148
G	226	312	188
T	279	345	228
G+C	47%	47%	48%
Total No. of Amino Acids	280	353	219
Molecular Weight	30827 Da	38825 Da	24323 Da

Table 5: Summary of base pairs and amino acids of *Diplozoon kashmirensis* Kaw, 1950, *Diplozoon aegyptensis* Kuntz, 1963 and *Diplozoon guptai* Fayaz et Chishti, 1999.

<i>D. kashmirensis</i>	6	ACTGCCTTGAGCATCGACTTCTTGAACGTAATTGCGGCATTAGGCTCTGCTGATGCCAC	65
<i>D. bliccae</i>	6	GCTGACTTGAGCATCGATTTCTTGAACGTAATTGCGGCATTACCCCTCTAATGATGCCAC	65
<i>D. kashmirensis</i>	66	GCCTAGCCGAGTGTCCGCATTAATCTATCACGACGCTTAATTGGTCTGGCTTAGTTG	125
<i>D. bliccae</i>	66	GCCTAGCCGAGTATCCGCATTAATCTAGCACGACGCTTAATTGGTCTGGCTTAGAAAG	125
<i>D. kashmirensis</i>	126	TTGTCAGCCGTCGTGTTGTACT---CAACGTTGTTTCAGTTGTCAAGTCGACGGTATTA	185
<i>D. bliccae</i>	126	TTGTCAGCCGTCGTGTTGTACTTGGCAACGTTGTTCTTTTGTCAAGTCGCGGTATTA	185
<i>D. kashmirensis</i>	186	TTGACGCTTCCCAAATGTAATGGAGAGTTGTATATGC--AATATCTGCCGCTAGCCTGT	245
<i>D. bliccae</i>	186	TTGACGCTTCCCAAATGTAATGGAGAGTTGTATATGCCAAATCTTCCGGTAGCCTGT	245
<i>D. kashmirensis</i>	246	TGGTGTGGCTACGCTGCCCGTGTATGGTTATTTGCATTTTGTGCATACCGATGGGG	305
<i>D. bliccae</i>	246	TGGTGTGGCTACGCTGCCCGTGTATTTTTATTTGCATTTTGTGCATACCGATGGGG	305
<i>D. kashmirensis</i>	306	TGGTTAGCTTCTCGTCATCAGTGCCTGTTTCCGGTGG-GTCGTGGCGTGGGAATTTCAA	365
<i>D. bliccae</i>	306	TGGTTAGCTTCTCGTCAGCAGTGCCTGTTTCCGGTGGTGTCTGGAATGGGAATTTCAA	365
<i>D. kashmirensis</i>	366	TAAGCATTCTGAATGGTAATTGTGAAATTGTGCAT---ATGTGCTGTTCTCTTGAGCCTT	425
<i>D. bliccae</i>	366	TAAGCATTCTGAATCCTAATTGTGAAATTGTCAATTTATGTGCTGTTCTCTTGAGCCGC	425
<i>D. kashmirensis</i>	426	TTGGCCCACGGGTTGTGCGGTGACCAAGTGTGCTTTGAATGCGAGCGCATGCATGCCAGG	485
<i>D. bliccae</i>	426	ACGGCCCACTTATTGTGCGATGACCAGTGACGCTTGAATGCGAGTGCATGCATGCCAGG	485
<i>D. kashmirensis</i>	486	TCCGACGCTATTTGTGATCGCGAC-GTGCTTTGCTTGTGTTCTGCGTTAATTTTTGTCA	545
<i>D. bliccae</i>	486	TCTCAGCCTATTTGTGATCGCGACAGTGTGTTGCTTGTGTTCTGCGTTAATTTTTGTCA	545
<i>D. kashmirensis</i>	546	CTGTTTCTTGCGAATGAGCGAGTCTGGCCCGAGACGAGATTATGTCATGTCGTGCTG	605
<i>D. bliccae</i>	546	CTGTTTCCC CGAATGAGCGAGTCTGGCCCGAGACGAGCATGTGCCATGTCTGTGCTG	605
<i>D. kashmirensis</i>	606	TGCAGACATTACTACTCCATTGGTCGCTAAGTGCATATCGGTGTC--CCGATTCTACTG	665
<i>D. bliccae</i>	606	TGCAGACATTACTACTCCATTCTTCGCTAAGTGTGATCGGTGTCACCCGTAATTTACTG	665
<i>D. kashmirensis</i>	666	TACTGCTGTGGTGTGTGCACCTGACCTCGGATTAGGCGTGATTACCCGCTGAACCTAAGC	725
<i>D. bliccae</i>	666	TACTTCTGTGGTGTATGCACCTGACCAAGGATTAGGCGTGATCACCCGCTGAGCTTAAGC	725

<i>D. kashmirensis</i>	726	ATATCAAT AAGCGGAGGAAAAGAACTAACCAGGATTCCCTT-GTAACGGCGAGTGAACA	785
<i>D. bliccae</i>	726	ATATCAATGGGCGGAGGAAAAGAACTAACCCTATTCCCTTAGTAACGTCGAGTGAACA	785
<i>D. kashmirensis</i>	786	GGGATTAGCCCAGCACCGAAGCTCGGGTC--TTGGCCGTTCCGC AATGTGGTGTTAGGT	845
<i>D. bliccae</i>	786	CCGATTAGCAAAGCACCGAAGCTCGGGTCTTTTGGCCGTTCCGC AATCCGGTGTTAGGT	845
<i>D. kashmirensis</i>	846	TGGCATACTCAGGCGATGTACTGTGTAG	873
<i>D. bliccae</i>	846	TATCATACTCAGGCGATGTACTGTGCC	873

Table 6: Pairwise alignments of the 28S rDNA ITS consequences of *Diplozoon kashmirensis* and *Diplozoon bliccae*, numbering refers to ITS sequences.

<i>D. aegyptensis</i>	1	TGCAAAGCTGCTTGGAGCATCGACTTCTTGAACGTA AATTGCGGCATTAGGCTCTG-CTGA	59
<i>D. paradoxum</i>	4	TGCAAAGCTGCTTGGAGCCTCGACTTCCCAGACGTA AATTACGGCATTAGGCTCTGCTCGA	63
<i>D. aegyptensis</i>	60	TGCCACGCTAGCCGAGTGTCCGCAATAAATCTATCAGCAGCCTAATTTGGTCGTGGCTT	119
<i>D. paradoxum</i>	64	TGCCCAGCCTAGCCGAGTGTCCGCAATAAATCTATCAGCAGCATAATTTGGTCGTGGCTT	123
<i>D. aegyptensis</i>	120	AGTTTGTGTCAGCCCTCGTGTGTA CTTGGCAACGTGTTGTT CAGTTGTC AAGTCGACG	179
<i>D. paradoxum</i>	124	AGTTTGTAAAAGCCGTGTGTA CTTAACAA CGTGTGTT CAGTTGTC AAGTAGACG	183
<i>D. aegyptensis</i>	180	GTATTATTGACGCTTCCAAATGTAATGGAGAGTTGTATATGCGAAATATCTGCCGGTA	239
<i>D. paradoxum</i>	184	GTATTATTGACGCTTCCAAATGTAATGGAGAGTTAG-NDATGCGAAATATCCGCTGGTA	242
<i>D. aegyptensis</i>	240	GCGTGTGGTGTGGCTACGCTGCCCGTGATGGTTATTTCATT TTTGTGCATACCG	299
<i>D. paradoxum</i>	243	GCGTGTGGTGTGGCAACGCTGTC CCGTGATGGTTACTTGCATT TTTGTGCATACCG	302
<i>D. aegyptensis</i>	300	AT-GGGGTGGTAGCTTCTCGTCATCAGTGC GTGTTTCCGGTGGTG---C-GTGGCG	353
<i>D. paradoxum</i>	303	ATGGGGGGCGTTAGCTTCCGCTCAGAGCGTGT TTGCCGTTGGTGTATTGCAGTGGCG	362
<i>D. aegyptensis</i>	354	TGGGAATTC AATAAGCATTCTGAATGGTA AATTGTAAATTGTCA TTTATGTGCTGTT	413
<i>D. paradoxum</i>	363	TGGGAATTC AATGAGCA TTTGTGAATGGTA AATTGTAAATTGCCAT TTTATGTGCTGTT	422
<i>D. aegyptensis</i>	414	CTCTTGAGCC TTTTGGCCACG GGTTGTGCGGTGACCAGTGTGCTT TGAATGCCGACGC	473
<i>D. paradoxum</i>	423	CTCTTGAGCC TTTTGGCTTTC GGGTGTGCGGTGACCAGTGTGCTT TGAATGCCGACGC	482
<i>D. aegyptensis</i>	474	ATGCATGCCAGGTGCAGCC TATTTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGTT	533
<i>D. paradoxum</i>	483	ATGCATGCCAGGTGCAGCC TA-TTGTGATCGCGACAGTGCTTTGCTTGTGTTCTGCGTT	541
<i>D. aegyptensis</i>	534	TAA TTTTGTCACTGTTTCTTCCGAATGAGCGAGTCTGGCCCGAGACGAGATTATGTGCC	593
<i>D. paradoxum</i>	542	TAA TTTTGTCACTGCCGCTTGCCTATGTGCGAGTGTACCCGAGATGAGATTGTGTGCC	601
<i>D. aegyptensis</i>	594	CATGTCGTGCTGTCAGACCTA CTACTCCATTGGTCGTAAGTG CATATCGGTGTACC	653
<i>D. paradoxum</i>	602	CATGTCATGCTGCTGACCTA CTACTCCACTGGTCGTAAGTG CATATCGGTGTACC	661
<i>D. aegyptensis</i>	654	CGTATTCTACTGTACTGCTGTG--GTGTGTGCA CCGCTGACCTCGGATTAGCGTGATTACC	711
<i>D. paradoxum</i>	662	AGTATTCTACTGTACTGCTGTGTTGTGTGTGCAC CCGTACCGTGGATTAGCGTGATTACC	721
<i>D. aegyptensis</i>	712	CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAACTAACCAGGATTCCCTTAGTA	771
<i>D. paradoxum</i>	722	CGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAACTAACCAGGATTCCCTTAGTA	781
<i>D. aegyptensis</i>	772	ACGGCGAGTGAACAGGGATTAGCCAGCACCGAAGCTCGGGTCTTTTGGCCGTTCCGGCAA	831
<i>D. paradoxum</i>	782	ACGGCGAG---CAGGGATTAGCCAGCACCGAAGCTCGGGTCTTTTGGCCGTTCCGGCAA	841
<i>D. aegyptensis</i>	832	TGTGGTGTTAGGTTGGCATACTCAGGCGATGTACTGTGCTAAGTCCATTCATGAATATG	891
<i>D. paradoxum</i>	842	TGTGGTGTTAGGTTGGCATACTCAGGCGATGTACTGTGCTAAGTCCATTCATGAATATG	901
<i>D. aegyptensis</i>	892	GCTAGCTATCTGGCCAGAGAGGGTGA AAGGCCCGTGAGC ATAGTGCCTCGTTCTGTCTT	951
<i>D. paradoxum</i>	902	GCTAGCTATCTGGCCAGAGAGGGTGA AAGGCCCGTGAGC ATAGTGCCTCGTTCTGTCTT	961
<i>D. aegyptensis</i>	952	AGTCAAACCGTTGAGTCCGGTTGTTTAGGAATGCAGCC	988
<i>D. paradoxum</i>	962	AGCAAACCGTTGAGTCCGGTTGTTTAGTAATGCAGCA	998

Table 7: Pairwise alignment of the 28S rDNA ITS consequences of *Diplozoon aegyptensis* and *Diplozoon paradoxum*, numbering refers to ITS sequences.

<i>D. guptai</i>	9	ACTGCCTTGAGCATCGACTTCT--AACGTAATCGCGGTATTAGGCTCTGCCTGATGCCA	68
<i>D. homoion</i>	6	ACTGACTTGAGCATCGATTTCTTGAAAGTGAATTGCGGCATTACCCCTCT-AATGATGCCA	64
<i>D. guptai</i>	69	CGCCTAGCCGAGTGTTGGCATTATCTATCACGACGCTTAATTGGTCGTGGCTTAGTTT	128
<i>D. homoion</i>	65	CGCCTAGCCGAGTATCGGCATTAATCTAGCACGACGCTTATTTGGTCCTGGCTTAGAAA	124
<i>D. guptai</i>	129	GTTGTGACCCGTCGTGTTTTACTTTGCAACGTTGCTCAGTGTAAAGTCGACGGTATT	188
<i>D. homoion</i>	125	GTTGTGACCCGTCGTGTTGACTTGGCAACGTTGTTCTGTGTCAAGTCGGCGGTATT	184
<i>D. guptai</i>	189	ATTGACGCTTGCCAAATGTAATGGAGAGTGTATATGCGAAATTTCTGCCGG-AGCCTG	248
<i>D. homoion</i>	185	ATTGACGCTTGCCAAATGTAATGGAGAGTGTATATGCGAAATATCTTCGGTAGCCTG	244
<i>D. guptai</i>	249	TTGGCGTTGGCAGCGCTGCCCGTGTATGGTTACTTGCATTTTTGTCATACCGATTGG	308
<i>D. homoion</i>	245	TTGGGTGTTGGCTACGCTGCCCGTGTATTTTTTATTTGCATTTTTTGTCATACCGA-TGG	303
<i>D. guptai</i>	309	GGCGGTAGCTTTGTCGTATCAGTGCCTGTTGCCGTTGGTATTGTGGTGCGGTGGGA	368
<i>D. homoion</i>	304	GGTGGTLAGCTTCTCCTGACGAGTCGTCCTTGCCGGTGG----TGCTGGAATGGGA	358
<i>D. guptai</i>	369	ATTTCAATAAGCATTACTGAATGGTAATTAATAAATTTGCCATTATATGCTGTTCTCTT	428
<i>D. homoion</i>	359	ATTTCAATAAGCATTTCTGAATCTAATTGTGAAATTTGCATTTTTATGCTGTTCTCTT	418
<i>D. guptai</i>	429	GAGCCTTTTGGCCACGGGTTGTGCGGTGACAGTGTGCTTTGAATGCGTGCGCATGCA	488
<i>D. homoion</i>	419	GAGCCGCATGGCCCACTTGTGTCGATGACAGTGTGCTTTGAATGCGAGTGCATGCA	478
<i>D. guptai</i>	489	TGCCAGGTCGCAGCCTA-TTGTGATCGCGACAGTGTCTTTGCTTGTGTTCTGCGTTTATTT	547
<i>D. homoion</i>	479	TGCCAGGTCGCAGCCTATTGTGATCGCGACAGTGTCTTTGCTTGTGTTCTGCGT--AATT	538
<i>D. guptai</i>	548	GTTGTCACTGCTACTTGCATATGTCGAGTGTGTACCCGGAATGAGATTTTGTGCCCATG	607
<i>D. homoion</i>	539	TTTGTCACTGTTTCCCGCAATGAGCGAGTCTGG-CCCGAGACGAGAGCATGTGCCCATG	597
<i>D. guptai</i>	608	TCATGCTGTGCTGACATTACTTCTCCACTGGTCGATAAGTGATGTCGGTGTCCACAGTA	667
<i>D. homoion</i>	598	TCGTGCTGTGCAGACATTACTACTCCATTCTTCGCTAAGTGTGATCGG-GTCCCGTA	657
<i>D. guptai</i>	668	CTTTGCTGTA-TT--GTG-T	687
<i>D. homoion</i>	658	TTTACTGTACTTCTGTGGT	677

Table 8: Pairwise alignments of the 28s rDNA ITS consequences of *Diplozoon guptai* and *Diplozoon homoion*, numbering refers to ITS sequences.

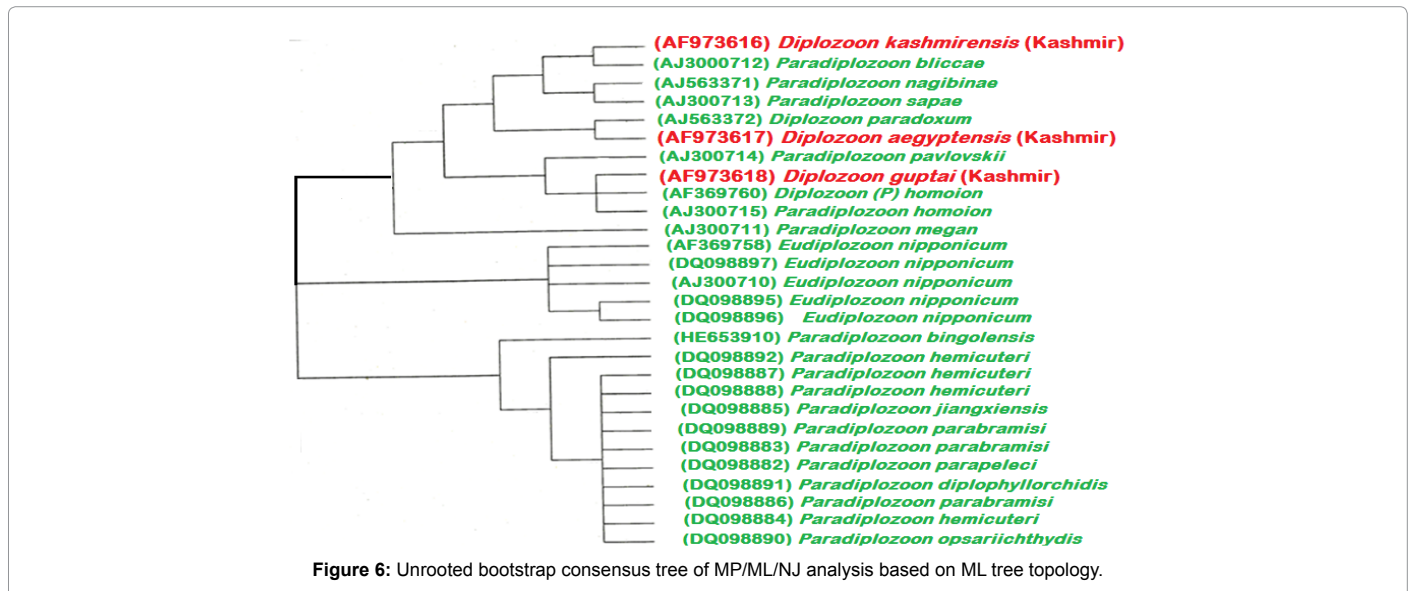


Figure 6: Unrooted bootstrap consensus tree of MP/ML/NJ analysis based on ML tree topology.

Discussion

The rDNA second Internal Transcribed Spacer (ITS2) was amplified using primers Cer5.8S2249 and Cer28S3116 [7] for 3 species of *diplozoids*. Analysis of the ITS2 region following sequencing clearly allowed us discrimination at the species level and produced the

same results as species identification made by using morphological structures. During the present study it was observed that the alignment of nucleotide sequences with those of other *Diplozoon* species of *D. bliccae*; *D. paradoxum* and *D. homoion* [2,6,7], clearly revealed the boundaries of the 5.8S and 28S rDNA genes, as the sequences in these species closely resembles to those of *D. kashmirensis*, *D. aegyptensis* and

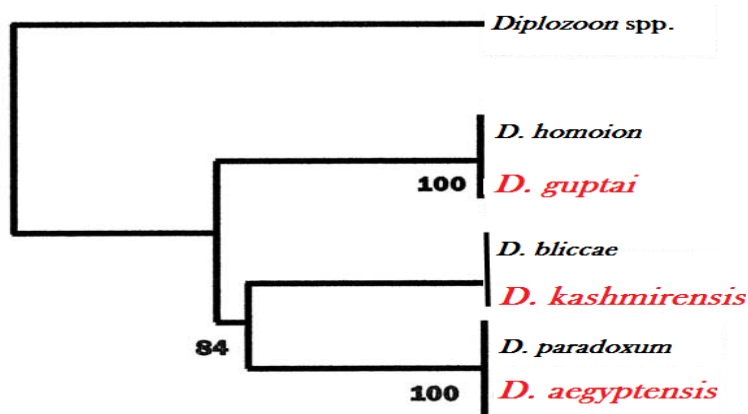


Figure 7: Phylogenetic tree depicting the genetic relationship among three of *Diplozoid* species by Neighbouring Joining (NJ).

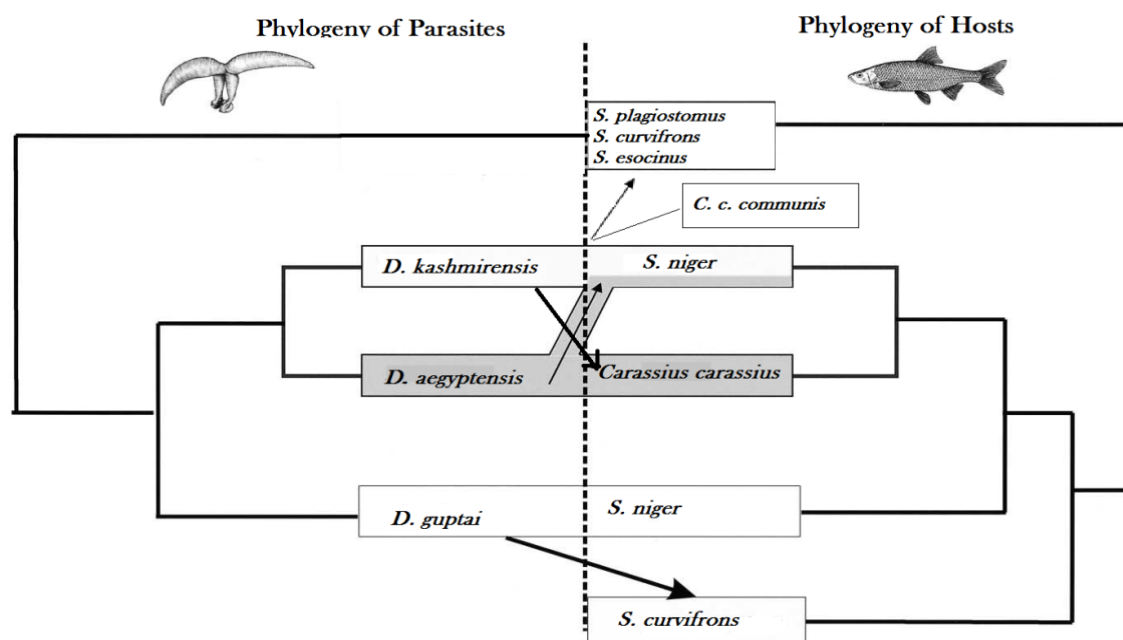


Figure 8: Construction of phylogenetic tree of parasites and hosts showing host parasite relationship of three *Diplozoon* species in Kashmir.

D. guptai. As noted in comparison of ITS2 sequences of *Monogenean* species, the first part of the ITS2 is also highly conserved, with only 6 variable sites in the first 65 nucleotides of the *diplozoid* sequences.

Species discrimination of *diplozoids* based on the shape of clamp sclerites and the length of the central hook can be difficult because of similarities in the shape of certain sclerites and overlapping ranges of central hook measurements. The PCR product of 3 species of *diplozoids*: *D. kashmirensis*; *D. aegyptensis* and *D. guptai* were clearly discriminated on the basis of nucleotide sequences which were different in their length of base pairs. The length of the PCR product could be useful to distinguish *Diplozoids* from the genus *Eu Diplozoon* and *Para Diplozoon* from other *Diplozoids* [2,6]. Length differences in the ITS2 have also been recorded in the genus *Gyrodactylus* [2,6] but are not generally as large as those found in the ITS1 region of *Lamellodiscus* and *Gyrodactylus* [2,6,19,20]. During the present study there are length difference of PCR products of three *Diplozoon* species i.e., *D.*

kashmirensis contains 873bp; *D. aegyptensis* contains 1120bp and *D. guptai* contains 687bp of 28S rDNA genes, so on the basis of length of base pairs the three *diplozoid* species can be discriminated. ITS region have been found to be useful species markers for *monogenean* parasites [1,2-6] so, this method was performed to distinguish the *diplozoid* species. During the present study, the intraspecific variations within *diplozoid* species were studied and differences were detected in the ITS regions, but [2,6] studied that ITS region lacks intraspecific variation in groups of *Monogenea* which is due to the same species recovered from different hosts.

Diplozoids are generally considered parasites of Cyprinid species but the host specificity differs and relates to geographical origin. In Eurasia, *diplozoid* occurrence is restricted to host fishes from the Cyprinidae and Perciformes families [2,6,8,10,21,22]. However, in Africa they also parasitize members of the Characidae [20,22]. All *diplozoid* species described in the present study are also host specific.

ML, MP and NJ trees showed that *D. kashmirensis*; *D. aegyptensis* and *D. guptai* are closely related species, and this mirrors the close relationship of their hosts, thus all of these species are found in cyprinids from the same genus *Schizothorax*. These species have been described morphologically based on clamp shape, total body length, sucker, and pharynx length [17,24]. The present observations on molecular characterization demonstrate sufficient genetic variations between parasites from different hosts to confirm the validity of these species and that they appear to be host specific, as are many monogenean parasites. It may be speculated that the similarity of these species is a result of a relatively recent divergence of one from the other following a host-switching event. An important observation during the present study has been noticed that *Schizothorax niger* is infected by all the three species of *Diplozoidae*: *D. kashmirensis*; *D. aegyptensis* and *D. guptai*, but on all six fishes collected, simultaneous parasitism by all the parasite species was never observed. Two types of factors can be involved in the constitution of such a host-parasite system.

(a) Competition hypothesis: the installation of a first *Diplozoon* species prevents any other species from settling on the same gill. (b) Since natural hybridization has been reported between the two fishes, the introgression of genes from *Carassius carassius* into the genome of *S. niger* allows a host capture of the latter by *D. aegyptensis* and *D. guptai* but excludes the infestation by its natural parasite *D. kashmirensis*.

Conclusion

The present study has confirmed the existence of 3 species of *diplozoids* from 6 species of *cyprinid* fishes from the water bodies of Kashmir valley. All the species were clearly distinguished by differences in nucleic acid sequences within the second ribosomal DNA Internal Transcribed Spacer region (ITS2). Analysis of additional specimens from different cyprinid hosts by molecular methods may be helpful to clarify the systematics of this fascinating family *Diplozoidae*.

Acknowledgment

The authors extend their thanks to the authorities of the Department of Zoology and Biotechnology, University of Kashmir for providing laboratory facilities. TAS is also highly thankful to Prof. Fayaz Ahmad for giving valuable suggestions while compiling this paper.

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Citation: Ahmad F, Fazili KM, Sofi TA, Sheikh BA, Waza AA (2015) Morphological and Molecular Characterization of *Diplozoon kashmirensis*; *D. aegyptensis* and *D. guptai* Collected from Fishes of Kashmir Valley-India. Fish Aquac J 6: 147. doi:10.4172/2150-3508.1000147