

# Molecular Analysis, Based on 28s rDNA, of Dactylogyroides Species, Parasitizing Puntius Species

Ritika Raj Shrivastava<sup>1</sup>, Nirupama Agrawal<sup>1</sup>, M.K.Upadhyay<sup>2,\*</sup>

<sup>1</sup>Department of Zoology, University of Lucknow, Lucknow, U.P. 226007

<sup>2</sup>Biotech Park, Kursi Road, Lucknow, U.P.

\*Corresponding Author: drmkupadhyay@gmail.com

Copyright © 2013 Horizon Research Publishing All rights reserved.

**Abstract** The interrelationship of the helminthes have attracted attention from both phylogeneticists restricting their attention to morphological characters and those relying solely on molecular data. The phylogenic relationships existing among four monogenoideans belonging to genus *Dactylogyroides* found on two species of freshwater cypriniformes *Puntius* as *P.sophore* and *P.chola* in the River Gomti, India. These relationships were investigated via the use of the partial 28S ribosomal DNA (rDNA).

**Keywords** *Dactylogyroides*, *Puntius*, 28s rDNA, Molecular Phylogeny

characters for the differentiation of closely related genera or species group are usually selected based on practical purposes, taking no account of the evolutionary values of the characters. It is really difficult to establish a criterion for genus erection for the diversified monogenoideans. Analyses of 28S region in this study revealed that this gene is a very good molecular marker for inferring a relationship between closely related species. The aim of this paper is to describe the phylogenetic relationships between the monogenoidean parasites (genus *Dactylogyroides*) infesting genus *Puntius*, using DNA sequence data from nuclear ribosomal clusters.

## 1. Introduction

Genus *Dactylogyroides* was proposed by Gusev [1], for *Dactylogyryus tripathii* [1,2], Gusev, [1] infecting gills of *Puntius stigma*, collected in Gomti River at Lucknow. Gusev differentiated the genus from *Dactylogyryus* by having anchors with their points directed towards each other and dorsal bar "V" shaped, breaking into two equal parts.

The sclerotized structures of the four *Dactylogyroides* species which were used in the present study show close similarity. Size and shape of the accessory pieces of copulatory complex and that of vagina in particular concomitant with smaller details of dorsal anchor and dorsal bar are sufficiently different to warrant elevation of to separate specific status according to previous morphological studies.

However, PCR technology and DNA sequencing techniques permit the identification of species, strains, and populations from any stage in their life history to distinguish among morphologically similar parasites [4-6]. So far, many nuclear and mitochondrial DNA (mtDNA) genes have come into a common use in intraspecies or interspecies molecular systematic on Platyhelminthes [7-8].

The evaluation of generic-level monophyly and morphological criteria for genus erections seems to be the most controversial area in phylogenetic and taxonomic studies of the Monogenoidea [9,10]. Morphological

## 2. Methodology

### 2.1. Parasites Collection and Identification

Fish were collected from River Gomti, Lucknow, India with the help of local fishermen, transferred to the laboratory and maintained in small glass aquaria. They were dissected as per requirement. Parasites were removed from the gills of freshly killed fish, placed in a drop of water on the slide, covered with a cover-slip and identified using a phase contrast microscope (Olympus CX 41 U-DA 4E 03365 Japan). The taxonomy of monogenoideans follows "An Encyclopaedia of Indian Monogenoidea" [11] and those of fish follow "Fishbase" [12]. A list of parasites recovered from their respective hosts and accession numbers of sequences (analyzed in present study and retrieved from NCBI) in Table 1.

**Table 1.** List of Sequences analysed in the present study

Name of Fish	Name of Parasites	GenBank Number
Puntius sophore (Hamilton,1822)	<i>Dactylogyroides tripathii</i>	JX960645
	<i>Dactylogyroides longicirrus</i>	GU903482
Puntius chola (Hamilton,1822)	<i>Dactylogyroides mahecoli</i>	JX960644
	<i>Dactylogyroides dorsali</i>	JX960643
Outgroup	<i>Ligophorus chabaudi</i>	JN996834
	<i>Ligophorus vanbenedenii</i>	JN996802

## 2.2. DNA Isolation, Purification and Amplification

Identified specimens of monogenoidean were fixed in 96 - 100% ethanol. The genomic DNA was isolated by using DNeasy Tissue Kit, Qiagen, Hilden, Germany with slight modifications as per requirement. The concentration of the isolated DNA was estimated at a wavelength of 260/280 nm using a Nanodrop spectrophotometer. For amplification, standard method was followed. The 28S region was amplified with the specifically designed primer Fwd Seq: 5'ACCCGCTGAATTTAAGCAT3', Rev Seq: 5'CTCTTCAGAGTACTTTTCAAC 3'. Amplification reaction was performed in a final volume of 25 µl, containing 1 unit of Taq polymerase, 10X PCR Buffer, 0.4 mM dNTP (10 pM), for each Polymerase Chain Reaction (PCR), 1 µl of MgCl<sub>2</sub>, 5 µl of genomic DNA and Milli Q water in a thermocycler (Eppendorf Mastercycler Personal set at 4°C) under following conditions:- 5 minutes at 95°C (Initial Denaturation), followed by 35 cycles of 30 secs at 95°C, 40 secs for annealing, 30 secs at 72°C (extension) and a final extension at 72°C for 7 minutes. PCR products were examined on 1.5% Agarose-TAE gels, stained with Ethidium Bromide and visualized in Gel Doc.

## 2.3. Amplified DNA Sequencing and Molecular Phylogeny

Sequencing was carried out by Xcelris Labs Limited, Ahmedabad using BigDye® Terminator v3.1 Cycle sequencing kit following manufacturer's instructions. The amplified DNA sequences were analyzed using NCBI BLAST programme and MEGA5 software [13]. The nucleotide sequences (28S of rDNA) of three species of *Dactylogyroides* were obtained and one was retrieved.

## 3. Observations

### 3.1. Nucleotide Sequence Analysis

Average of all the four *Dactylogyroides* species nucleotide sequences had total of 254 positions, in the final data set. It revealed the fewest Cytosine (22.8%). The degree of bias depends upon the codon composition i.e. 23.7% Cytosine in the first position, 23.1% in second position and 21.6% in third position. The first position rich in Adenine was 26.6%, the second position rich in Thymine was 28.0% and third position was also rich in Thymine 27% (Detailed species wise nucleotide compositions is given in Table-2).

Average Evolutionary Divergence over all Sequence Pairs is 9.51. Estimated results showed minimum 5.54 distance between *Dactylogyroides dorsally* and *Dactylogyroides longicirrus* where maximum distance 11.65 was shown between *Dactylogyroides mahecoli* and *Dactylogyroides longicirrus*.

Substitution matrix estimated between minimum 7.08 to maximum 38.25 and each entry shows the probability of substitution (r) from one base (row) to another base (column). The transition/transversion rate ratios are  $k_1 = 3.351$  (purines) and  $k_2 = 17.047$  (pyrimidines). The overall transition/transversion bias is  $R = 4.924$ , where  $R = [A * G * k_1 + T * C * k_2] / [(A + G) * (T + C)]$ .

In estimation of substitution pattern disparity, the probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Disparity Index test). A Monte Carlo test (1000 replicates) was used to estimate the *P*-values, which are shown below the diagonal. *P*-values smaller than 0.05 are considered significant.

**Table 2.** 6-Compute Nucleotide composition

Species name	Name of Nucleotides																			
	T(U)	C	A	G	Total	T-1	C-1	A-1	G-1	Pos #1	T-2	C-2	A-2	G-2	Pos #2	T-3	C-3	A-3	G-3	Pos #3
<i>Dactylogyroides tripathii</i>	24.7	22.6	26.1	26.6	368.0	22	19.5	30.1	28.5	123.0	25	26.0	21.1	27.6	123.0	27	22.1	27.0	23.8	122.0
<i>Dactylogyroides longicirrus</i>	30.7	20.2	22.6	26.5	257.0	31	24.1	21.8	23.0	87.0	34	17.4	22.1	26.7	86.0	27	19.0	23.8	29.8	84.0
<i>Dactylogyryrus mahecoli</i>	24.7	19.6	27.4	28.3	368.0	22	20.3	29.3	28.5	123.0	27	20.3	23.6	29.3	123.0	25	18.0	29.5	27.0	122.0
<i>Dactylogyroides dorsali</i>	27.1	28.2	22.2	22.5	365.0	25	31.1	23.8	19.7	122.0	26	27.0	20.5	26.2	122.0	30	26.4	22.3	21.5	121.0
Avg.	26.5	22.8	24.7	25.9	339.5	25	23.7	26.6	25.1	113.8	28	23.1	21.8	27.5	113.5	27	21.6	25.8	25.2	112.3

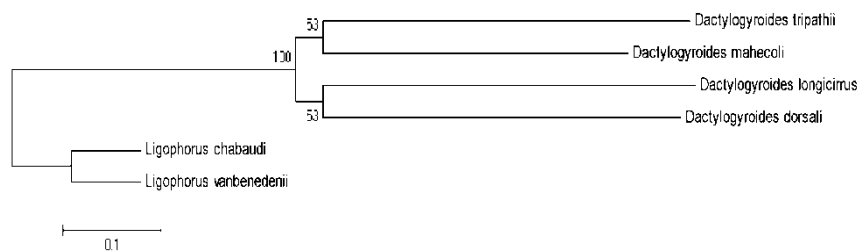
In Codon Based Z- Test of Selection, the values of  $P$  less than 0.05 were considered significant at 5% level (Table 3). The variance of the difference was computed using bootstrap method (1000 replicates). In Tajima Test of Neutrality, number of segregating sites ( $p_s$ ) was 0.984252, nucleotide diversity 0.677656 and the Tajima Test statistic calculated (D) 4.269047.

**Table 3.** Codon Based Z- Test Of Selection

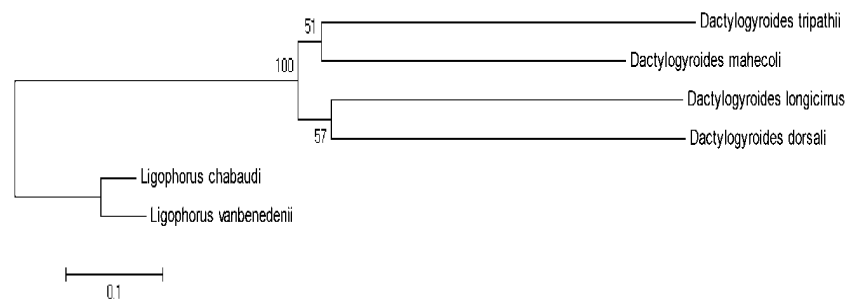
<i>Dactylogyroides tripathii</i>		-0.60	0.99	-1.25
<i>Dactylogyroides longicirrus</i>	0.55		0.57	-0.21
<i>Dactylogyrus mahecoli</i>	0.33	0.57		-1.82
<i>Dactylogyroides dorsali</i>	0.21	0.83	0.07	

### 3.2. Phylogeny

The phylogenetic reconstructions (Figs.1 & 2), inferred from analysis of 28S rDNA sequences showed great resolution for the species of the monogenoideans. 28S rDNA sequences were aligned using Clustal W [14]. All four *Dactylogyroides* species under study and two *Ligophorus chabaudi* and *Ligophorus vanbenedenii* (Ancyrocephaline monogenoideans), recovered from GENBANK for phylogenetic reconstruction, revealed clear differences in nucleotide sequences among different species. Sequence analysis was conducted using the nucleotide BLAST program in the NCBI database (National Center for Biotechnology Information, NIH, Bethesda, Maryland, USA) for similarity and nucleotide length. We also calculated the fractional GC contents of the nucleic acid sequences. The length of 28S partial rDNA sequence of *D.tripathii*, *D.longicirrus*, *D.mahecoli* and *D.dorsali* were 368 , 301, 381 and 364 bases respectively. They had 92% (minimum) - 93% (maximum) similarity for *D.tripathii*, 91%- 93% for *D.longicirrus*, 90%- 91% for *D.mahecoli*, 89%- 91% for *D.dorsali*, with the sequences of other available at NCBI in BLAST search. GC content of these four sequences were between 49.2 % - 50.8%.



**Figure 1.** Neighbor Joining



**Figure 2.** Minimum evolution

For phylogenetic analysis, the phylogenetic trees were computed with Neighbor- Joining (NJ) method and Minimum Evolution (ME) method. The evolutionary distances were computed using p-distance method and are in the units of the number of base differences per site. Codon position included were 1<sup>st</sup>+2<sup>nd</sup>+3<sup>rd</sup>+Noncoding. Gaps and missing data were eliminated. Minimum Evolution was also based on p-distance method. The ME tree was constructed using close-neighbor- interchange (CNI) algorithm. Branch lengths are generally not obtained for each topology; the sequences at each node are inferred to be those that require the least number of changes to give each of two immediately descendant sequences [15]. Bootstrap values were included to test the reliability of inferred trees and the estimation of evolutionary divergence between sequences was computed. The phylogenies were tested with 1000 bootstrap replicates. Bootstrap values, indicating the robustness of the internal nodes were set at 1000 replications. Above mentioned two methods- Neighbor- Joining (NJ) and Minimum Evolution (ME) gave trees with similar topology and approximately similar bootstrapped values. These trees showed two monophyletic groups of parasites and forming two subclade, first subclade included two species *Dactylogyroides tripathii* and *Dactylogyroides mahecoli* and having bootstrap value 51-53%, second also included two species *Dactylogyroides longicirrus* and *Dactylogyroides dorsali* having bootstrap value between 53% to 57% .

#### 4. Discussion

Traditional study was based, to a large extent, on the morphology of the sclerotized components of the haptor parts. In recent times, molecular techniques are being increasingly used in taxonomy and phylogenetic species and have emerged as valuable supplementary tools in providing authentic and unambiguous identification of species. 28S rDNA markers have been used to detect species boundaries [16].

Phylogenetic relationships based on morphological characters and molecules are mostly concordant [17,18]. From our data, we observed genetic variation among species in parasitic Platyhelminthes. The each variation is very variable based on each gene among species. We think that these differences in the nucleotide length, GC percentage, nucleotide differences, and number of gaps of gene can be attributed largely to varying numbers of repeat, copy numbers, deletions, alignment gaps, and base substitutions and additions. Base substitutions and additions are characterized by very high C content which can, at time represent pure poly C structures. In addition this may be a consequence of mutations in the lineage.

Genus *Dactylogyroides* showed close similarity with the genus *Dactylogyrus* of the same subfamily Dactylogyryinae. Only difference lies with the structure of bar. *Dactylogyroides* possess 'V' shaped bar in two parts. Only five species of this genus are so far reported in India four on

two species of fish *Puntius* and one on fish *Osteobrama cotio* (not included in the present study).

Two subclade were formed in this study. Those *Dactylogyroides* species which were found in same subclade do not parasitize the same fish species. The result from the phylogenetic analyses did not indicate that *Dactylogyroides* species coexisting on the same host evolve by intra-host speciation, which was inferred to be an important process of parasite diversification in cyprinid fish species [19]. We compared gene sequences by NJ and ME analysis for phylogenetic analysis, and then we acquired the same tree pattern.

The molecular phylogenetic analyses showed that *Dactylogyroides* from different *Puntius* species clustered together. The two species *D. tripathii* and *D.mahecoli*, in the first cluster showed that on the basis of nucleotide composition shows similarity at an average of T position and T-1 and G-1 position. Molecular phylogeny shows similarity between *D. tripathii* and *D. mahecoli* having bootstrap value 51-53% in ME and NJ methods. These observations are further supported by similarity in the structure of taxonomically important sclerotised structures like haptor armature and copulatory complex, although they infect two different species of the host [20]. It seems most likely that the two species co-specified, infecting two different host species of the same host genus. Similarly, the second cluster of *D. longicirrus* and *D. dorsali*, have different nucleotide composition, as supported by ME tree (bootstrap value 57). It is worthwhile to mention here that these species are morphologically quite distinct as well [1,20].

#### Acknowledgements

We are grateful to Prof. K.C.Pandey Department of Zoology, University of Lucknow, Lucknow for helpful suggestions and Dr. N. S. Nagpure N.B.F.G.R. Lucknow for providing necessary literature. Facilities developed under UGC-SAP (DRS-I) of the, Department of Zoology, University of Lucknow, Lucknow were utilized in the present study.

#### REFERENCES

- [1] Gusev AV. 1976. Freshwater Indian Monogenoidea. Principles of systematic, analysis of world faunas and their evolution. Indian J. Helminth 25-26:1-124.
- [2] Tripathi YR. 1959. Monogenetic trematodes from fishes of India. Indian J. Helminthol. 9:1-149.
- [3] Yamaguti S. 1963. Systema Helminthum. IV. Monogenea and Aspidocotylea. Intersc. Publ., N.Y 699 (1961).
- [4] Olson PD, Littlewood DTJ, Bray RA, Mariaux J. 2001. Interrelationships and evolution of the tapeworms (Platyhelminthes: Cestoda). Mol. Phylogenet. Evol. 19:

- 443–67.
- [5] Olson PD, Cribb TH, Tkach VV, Bray RA & Littlewood DTJ. 2003. Phylogeny and classification of the digenea (Platyhelminthes: Trematoda). *International Journal of Parasitology* 33: 733–755.
- [6] Lockyer AE, Olson PD, Littlewood DTJ, 2003. Utility of complete large and small subunit rRNA genes in resolving the phylogeny of the Neodermata (Platyhelminthes): implications and a review of the cercomer theory. *Biol. J. Linn. Soc.* 78:155–171.
- [7] Blaxter ML, De Ley P, Garey JR, Liu LX, Scheldeman P, Vierstraete JR, Vanfleteren JR, Mackey LY, Dorris M, Frisse LM, Vida JT and Thomas WK. 1998. A molecular evolutionary framework for the phylum Nematoda. *Nature* 392:71–75.
- [8] Vilas R, Criscione CD & Blouin MS. 2005. A comparison between mitochondrial DNA and the ribosomal internal transcribed regions in prospecting for cryptic species of platyhelminth parasites. *Parasitology* 131: 839–846.
- [9] Desdevises Y, Morand S, Jousson O and Legendre P. 2002. Co evolution between *Lamellodiscus* (Monogenea: Diplectanidae) and Sparidae (Teleostei): the study of a complex host-parasite system. *Evolution* 56:2459–2471.
- [10] Wu XY, Zhu XQ, Xie MQ, Li AX. 2007. The evaluation for generic-level monophyly of Ancyrocephalinae (Monogenea, Dactylogyridae) using ribosomal DNA data. *Mol. Phylogenet. Evol.* 44:530-544.
- [11] Pandey KC and Agrawal N. 2008. *An Encyclopaedia of Indian Monogenoidea*. New Delhi, Vitasta Publishing Pvt. Ltd.
- [12] Froese R and Pauly D. 2007. *Neolamprologus lelupi* [Internet], FishBase, World Wide Web Electronic Publication. Available from: <[www.fishbase.org](http://www.fishbase.org)>
- [13] Tamura K, Peterson D, Peterson N, Stecher G, Nei M and Kumar S. 2011. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. *Molecular Biology and Evolution* 28:2731-2739.
- [14] Thompson JD, Higgins DG, Gibson TJ. 1994. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-80.
- [15] Dopazo, 1994 Estimating errors and confidence intervals for branch lengths in phylogenetic trees by a bootstrap approach. *Journal of Molecular Evolution* 38:300-304.
- [16] Kaukas A and Rollinson D. 1997. Interspecific variation within the hypervariable region of the 18S ribosomal RNA gene among species of *Schistosoma* Weinland, 1858 (Digenea). *Systematic Parasitology* 36:157-160.
- [17] Bernardi G and Crane NL. 2005. Molecular phylogeny of the humbug damselfishes inferred from mtDNA sequences. *J. Fish Biol* 54:1210-1217.
- [18] Ward RD, Zemlak TS, Innes BH, Last PR and Hebert PDN. 2005. DNA Barcoding Australia's fish species. *Philos. Trans.R.Soc.B. Biol.Sci.* 360: 1847-1857.
- [19] Simkova A, Plaisance L, Matejusova I, Morand S and Verneau O. 2003. Phylogenetic relationships of the Dactylogyridae Bychowsky, 1933 (Monogenea: Dactylogyridae): The need for the systematic revision of the Ancyrocephalinae Bychowsky, 1937. *Systematic Parasitology* 54:1-11.
- [20] Agrawal N, Pandey KC and Tripathii A. 2002. Remarks on Indian species of *Dactylogyroides* Gusev, 1976, with description of a new species on freshwater cyprinids of Lucknow. *Indian J. Helminth.* (N.S.) 20:15-18.