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Prevalence, molecular identification and host preference of *Dactylogyrus*, an ectoparasite in Indian major carps

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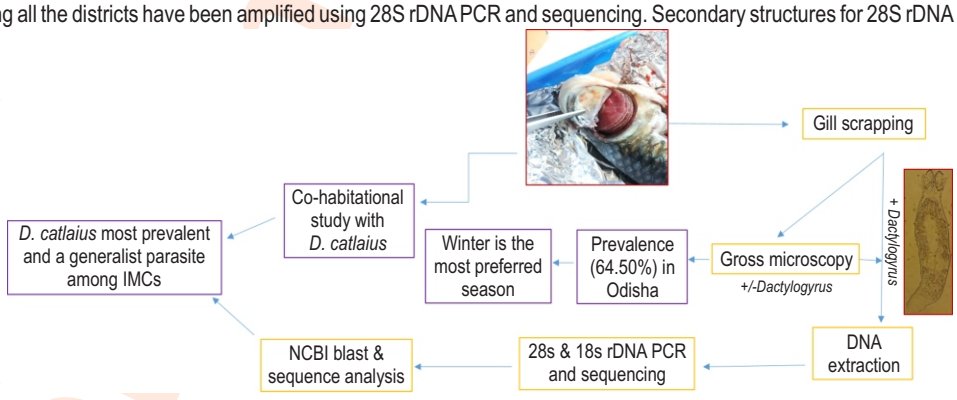
Abstract

Aim: The present study was carried out to ascertain the prevalence and species-spectrum of *Dactylogyrus* in Indian major carp farms of Odisha state, India. Further it is to establish host preference of *Dactylogyrus catlaius* among Indian major carps.

Methodology: The study was conducted from September 2018 to March 2020 in thirteen districts of Odisha. During that period, total 786 fish samples comprising of *Labeo rohita, Catla catla* and *Cirrhinus mrigala* were examined using Level II diagnosis. A total of 25 *Dactylogyrus* positive samples identified using microscopy representing all the districts have been amplified using 28S rDNA PCR and sequencing. Secondary structures for 28S rDNA fragments were predicted using MFold package (version 3.5) software. To ascertain host preference of *D. catlaius a* standard co-habitational challenge method was followed in which infected fishes were kept together with healthy fishes (all three Indian major carps).



Results: Total 786 fish samples comprising of *Labeo rohita*, *Catla catla* and *Cirrhinus mrigala*, all three Indian major carps were examined and overall prevalence of 64.50% for *Dactylogyrus* spp. was found. Further, winter season was the most favoured season whereas *L. rohita* was the most preferred host for this parasite. A total of 25 *Dactylogyrus* isolates, *i.e.*, 22 *Dactylogyrus catlaius*, two *D. vastator* and one *D. scorpius* were identified using 28S rDNA PCR and sequencing. Hence, *D. catlaius* has been identified as the most prevalent *Dactylogyrus* species in Odisha. Secondary structures for 28S rDNA fragments of three *Dactylogyrus* spp. were also predicted reflecting their diversity. Further in a cohabitational challenge study, *D. catlaius* was found to be a generalist parasite infecting all three Indian major carps with a higher preference for rohu *L. rohita*.

Interpretation: The present study provides a holistic information regarding *Dactylogyrus* prevalence in the state of Odisha. Further, the study illustrates host-parasite relation of *D. catlaius* among Indian major carps which would strengthen knowledge on developing any prophylactic measure in future.

Key words: *Dactylogyrus* spp., Indian major carps, Infection, Prevalence, 28S rDNA

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Introduction

Diseases are major limiting factor posing production and economic loss to fish farmers. Among different group of pathogens, parasites pose serious threat to the cultured fishes in freshwater aquaculture with an overwhelming incidence rate of 74.88% followed by bacterial, viral and fungal infections (Sahoo *et al.,* 2020). Monogeneans are vastly diverse obligate group of ectoparasites of freshwater and marine fishes, even to sharks, rays, amphibians, crustaceans and chelonian reptiles (Kearn, 2011). The direct lifecycle of monogeneans makes them more suitable to multiply rapidly in the aquatic environment and causes harmful impact on animal's inhabitant (Ilgová *et al.,* 2021). It is generally anticipated that each fish species harbour a unique species of monogeneans, thus the number of monogeneans is assumed to be 25,000 but very few has been described till date (Whittington, 1998).

Among monogeneans, those of genera *Dactylogyrus* (Diesing, 1850) and *Gyrodactylus* (von Nordmann, 1832) play significant role in causing infection in fish. *Dactylogyrus* is known as gill fluke as gills are the primary target organ. Clinical signs associated with dactylogyrosis are high mucus production on gills, hyperplasia, gill hemorrhages and asphyxia. The prevalence of dactylogyrosis is positively correlated with the stocking density and organic load of culture pond (Kumar *et al.,* 2017). The patterns and processes associated to host specificity of parasites represent one of the central themes in the study of host-parasite interactions (Mendlova and Simkova, 2014). Simkova *et al.* (2001) described dactylogyruses broadly into two groups: Specialists that only infect single host species and Generalists capable of infecting more than one host species. Hence, to understand the historical biogeography of parasite, host range of individual *Dactylogyrus* species needs to be ascertained (Simkova *et al.,* 2017).

Several reports are available on the sporadic prevalence of *Dactylogyrus* (Hossain *et al.,* 2008; King *et al.,* 2008; Borji *et al.,* 2012). Under a National Surveillance Programme for Aquatic Animal Diseases in India, incidences of *Dactylogyrus* sp. remained high as 18.90% of total parasitic diseases and greatly contributed to mixed parasitic (37.8%) infections (Sahoo *et al.,* 2020). Although gill fluke poses a major threat to Indian freshwater aquaculture, no recent information on its prevalence for any of the major aquaculture states of India have been reported. Further, the host preference information on many of the *Dactylogyrus* species present in India are lacking. The present study aims at exploring the prevalence of *Dactylogyrus* infection in three Indian major carps *L. rohita; C. catla* and *C. mrigala* farms in Odisha.

Materials and Methods

Site selection and collection of samples: The prevalence of *Dactylogyrus* spp. infection in different freshwater carp farms was carried out during September 2018 to March 2020 in thirteen districts of Odisha. The districts were selected in such a way that it covers maximum geographical distance, major culture areas in that area and represents data for the entire state of Odisha.

Selection of farms in each district was undertaken randomly. Samples (gill mucus) were collected on regular basis for two continuous years from 2018 to 2020. Sampling duration was spread over three major seasons, *i.e.,* winter (November-February), summer (March-June) and monsoon (July-October). In total 786 fish specimen mucus samples were collected from all the districts consisting of 415 numbers of *L. rohita*, 259, *C. catla* and 112 *C. mrigala*. Total samples collected during winter, summer and monsoon seasons were 320, 172 and 294, respectively. The fishes were screened for the presence of gill fluke (*Dactylogyrus* spp.) by collecting gill mucus in 4% neutral buffer formalin and 100% ethanol (EMSURE Merck, Germany), and brought to the laboratory for microscopic and molecular analysis. The microscopic analysis of gill mucus sample was done using a stereo microscope (Zeiss Stemi 508, Germany).

Microscopic examination of ectoparasite, *Dactylogyrus*: The mucus samples collected in 4% neutral buffer formalin (NBF) were analysed under light microscope in the laboratory. Briefly, the mucus collected in NBF was mixed thoroughly and a drop of it was placed on a grease free clean slide before examination under the microscope. Three such drops per sample were examined to find out the infection.

Molecular identification of parasite: Molecular identification of parasites was performed from the ethanol fixed samples that were found positive under microscopic analysis. DNA extraction was done using standard phenol-chloroform extraction method from ethanol-fixed mucus samples following Sambrook and Russell (2001). The extracted DNA was checked for its purity and quantity using NanoDrop (ND1000, Thermo Scientific, USA).

PCR amplification and sequencing: DNA sample was subjected to 28S rDNA PCR followed by sequencing to identify *Dactylogyrus* spp. 28S DG primers (DGR45 F/R) [forward primer (5' ACCCGCTGAATTTAAGCAT 3') and reverse primer- (5' CTCTTCAGAGTACTTTTCAAC 3')] were used in this study for the identification of *Dactylogyrus* spp. (Randrianambinintsoa *et al.,* 2014). PCR was performed using an automatic programmable thermal cycler (Applied Biosystem, California, United States) with following reaction conditions: initial denaturation (95ºC for 5 min), denaturation (95ºC for 45 sec), annealing (51ºC for 45 sec), extension (72 ºC for 45 sec) and final extension (72ºC for 5 min).

PCR reaction mixtures (25 µl) consisted of nuclease-free water (19.75 µl), PCR reaction buffer (2.50 µl, 10X), dNTPs (0.50 µl), forward primer (0.50 µl) and reverse primer (0.50 µl), *Taq* DNA polymerase (0.25 µl) and parasite DNA (1.00 µl of 50-100 ng μl^{-1}). PCR amplification was followed by agarose gel electrophoresis to visualize the amplified PCR product. Targeted PCR amplicons obtained were commercially sequenced by

Sanger's sequencing from Agri Genome Labs Pvt. Ltd., Kochi, India (three amplicons for each positive sample). The homology of 28S rDNA nucleotide sequences was analyzed using the Basic Local Alignment Search Tool (BLAST) of NCBI (http://www.ncbi.nlm.nih.gov/blast) for identification of parasite. Further the nucleotide sequences were submitted to NCBI to obtain accession numbers.

Secondary RNA structure prediction: Secondary structures for 28S rDNA fragments of *Dactylogyrus* spp. were predicted using minimal negative free energy state for isolated species via the online MFold package (version 3.5) following Verma *et al.* (2012).

Host specificity study of *D. catlaius*

Experimental fish: Apparently healthy fingerlings of *L. rohita* (22.30 ± 0.64 g), *C. catla* (20.45 ± 0.52 g), and *C. mrigala* (18.87 ± 0.34 g) were obtained from local fish farms. Representative fishes were examined for the absence of pathogens, particularly gill parasites. The fish were exposed to anti-parasitic treatment during the period of acclimatization. The fish were anaesthetized (clove oil: 50 µl l^{-1}) during any handling procedure. The fishes were acclimatized for 20 days in 500 l fibre-reinforced plastic tanks in wet-laboratory condition at 25 to 27°C under continuous aeration. Fishes were fed with commercial pellet feed at 3% of body weight. Water was exchanged (20-30%) regularly to remove excess feed and other faecal materials. *D. catlaius* infected (n=30 nos.) rohu juveniles were collected and left with fin-clipped healthy rohu fingerlings (30 nos.) to create a stock of freshly infected *L. rohita* in wet laboratory. Random screening of fin-clipped rohu followed by gill biopsy examination confirmed the heavy load of parasites (5-10 parasites/ microscope field) to create infective materials for co-habitational challenge study.

Co-habitational experiment: For this study, a standard co-habitational challenge method was followed where same tank infected fishes kept together with healthy fishes (Paul *et al.,* 2021). All three Indian major carps (6 nos. each of *L. rohita*, *C. catla* and *C. mrigala*) were kept in nine 500 l capacity FRP tanks under constant aeration. To each tank, two fin-clipped heavily infected rohu fingerlings were introduced to create experimental infection. At each sampling time point, fish from three tanks (5 fish per tank) were randomly sampled to obtain a representative of five from each fish species *i.e.* rohu, catla and mrigal. The cohabitation was carried out for 20 days and gill samples were collected on 0, 1, 3, 5, 10 and 20 days' post-infection (dpi). At each sampling time point, gills were collected from anaesthetized fish for microscopic observations and parasite counting. Only left gill arches were used for parasite counting purpose. Total fifteen fishes (five from each species) were examined at each time point.

Counting of parasite: At each sampling time point (0, 1, 3, 5, 10 and 20 dpi), the intensity of *Dactylogyrus* on gills were quantified. Parasites were counted individually only from the left side gill arches (the number of parasites not doubled for further calculation). All the four gill arches of each fish were separated and kept in clean petri plates containing sterile water. The parasite count was performed immediately using a stereo microscope (Zeiss Stemi 508, Germany). The mean intensity and prevalence of parasite for each fish species were estimated (Margolis *et al.,* 1982).

Results and Discussion

Among different parasitic diseases, infection with *Dactylogurus* is one of the major threats noticed in freshwater fish farming sector worldwide (Paul *et al.,* 2021). The report on the prevalence of this particular parasite in aquaculture systems of India is scarce. Genus *Dactylogurus* has a high species diversity worldwide and the parasitic diversity in many regions is underexplored (Benovics *et al.,* 2021). Hence, it is worth to explore species predominance in different aquaculture regions of the country. The present study was undertaken to estimate the prevalence of *Dactylogyrus* spp. in Odisha, India with a specific emphasis on understanding species predominance in the region. Further host-parasite interaction in terms of host range was also

Table 1: Prevalence of *Dactylogyrus* spp. in different districts of Odisha

Districts	Total number of samples collected	Number of positive sample	Prevalence (%)
Puri	81	62	76.54
Cuttack	89	67	75.28
Balasore	72	52	72.22
Mayurbhanj	60	32	53.33
Ganjam	51	33	64.70
Rayagada	47	22	46.80
Kalahandi	55	41	74.54
Balangir	74	51	68.91
Boudh	65	43	66.15
Angul	57	34	59.64
Keonjhar	56	21	37.50
Sambalpur	61	41	67.21
Sundargarh	18	8	44.44

Table 2: Intensity of *Dactylogyrus catlaius* in different fish species

Fish species	1 dpi	3 dpi	5 dpi	10 dpi	20 dpi
L. rohita	2.5 ± 0.66	65.40 ± 9.45	73.33 ± 8.87	104.80 ± 9.23	110 ± 10.47
C. catla	Nil	Nil	12.56 ± 8.87	59.39 ± 10.28	90.11 ± 7.72
C. mrigala	Nil	Nil	4.2 ± 0.81	26.44 ± 7.19	32.33 ± 4.81

Values are mean±S.D.

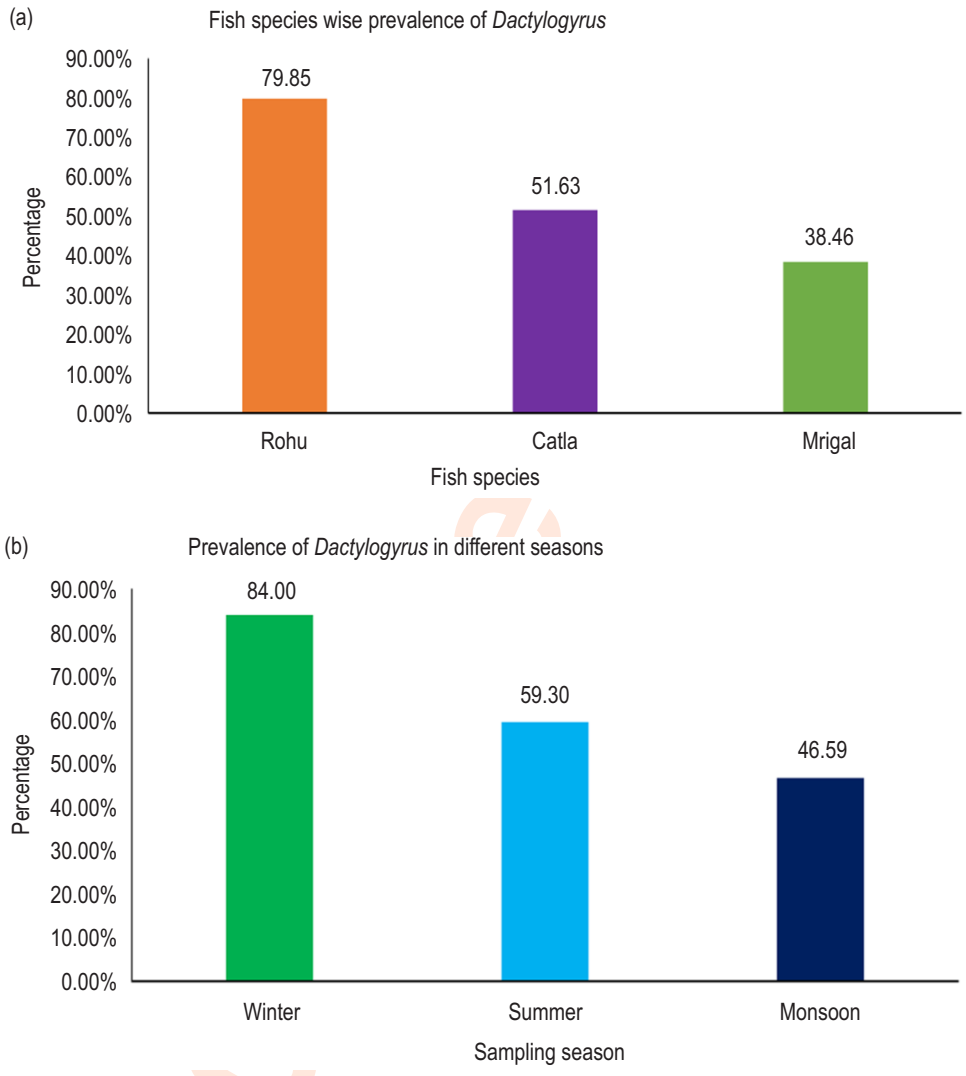

Fig. 1: (a) Prevalence of *Dactylogyrus* spp. in Indian major carps; (b) Season-wise prevalence of *Dactylogyrus* spp. in Indian major carps.

ascertained for better understanding and future prophylactic measure development. The overall geographical distribution of *Dactylogyrus* spp. is same as with the area of natural distribution of cyprinids, which includes Asia, Africa, North Europe and America. Almost 95% report of genus *Dactylogyrus* pertain to infection in cyprinids fishes, although few sporadic reports on the presence of *Dactylogyrus* on non-cyprinid fishes *i.e.* Japanese sea perch (*Lateolabrax japonicas*) and ruffe (*Gymnocephalus cernuus*) (Gusev 1985; Cone *et al.,* 1994; Simkova *et al.,* 2006). *L. rohita, C. catla* and *C. mrigala* are widely cultured carp species in Indian aquaculture. In the present study, three Indian major carps in carp culture system have been examined for two consecutive

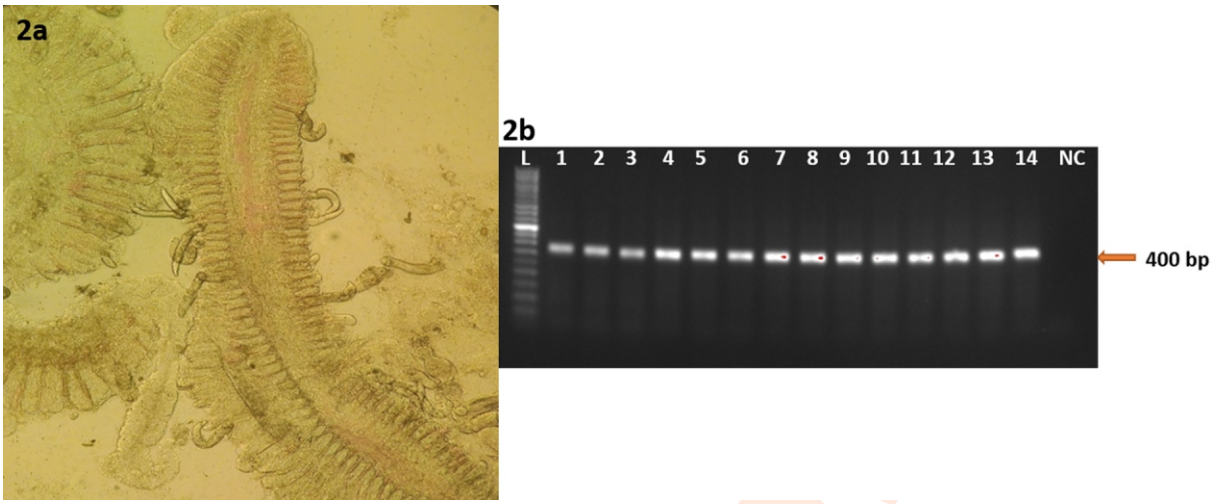

Fig. 2: (a). *Dactylogyrus* spp. in infected gill samples under microscope; (b). Representative image of *Dactylogyrus* 28S rDNA amplicons with the primers DGR45 F/R; L. 50 bp ladder; Lanes (1-14): Samples amplified using DGR45 F/R from districts Puri, Cuttack, Balasore, Mayurbhanj, Ganjam, Rayagada, Kalahandi, Balangir, Boudh, Angul, Keonjhar, Bargarh, Sambalpur and Sundargarh, respectively; NC: Negative control.

years in three different seasons to know the prevalence of *Dactylogyrus* in Odisha state. A total of 786 fish samples comprising 407 numbers of *L. rohita*, 275 numbers of *C. catla* and 104 numbers of *C. mrigala* were screened, and among them 507 samples were found to be positive for *Dactylogyrus* (64.50%). Earlier reports have also described high prevalence of *Dactylogyrus* in different parts of the world. Borji *et al.* (2012) reported *Dactylogyrus* infection in common carp as the most prevalent one followed by *I. multifiliis* in north-east of Iran. Two different *Dactylogyrus* species, *D. extensus* and *D. anchoratus* revealed prevalence of 29% and 2%, respectively. Hossain *et al.* (2008) mentioned monogenean parasites, *i.e.*, *Dactylogyrus* as one of the most prevalent parasites present in Bangladesh. *D. eucalius* was found to be 100% prevalent in a lake of Central Ontario in adults of *Culaea inconstans* surveyed with mean intensity of 8.8 (King *et al.,* 2008). There have been some sporadic reports on *Dactylogyrus* prevalence in India. A survey conducted in three districts of West Bengal, India have revealed that ectoparasitic infections are the major problem in culture condition and contributes about 37% among parasitic disease incidence (Chanda *et al.,* 2011).

Sahoo *et al.,* (2020) reported the incidence of dactylogyrosis of about 18.90% in the states of Odisha and Andhra Pradesh, besides incidences of mixed parasite infections wherein *Dactylogyrus* recorded to be a major contributor in a passive surveillance-based study. The present study also supported *Dactylogyrus* infection as one of the most prevalent infection of Odisha in aquaculture system. A higher incidence (64.50%) noticed in Indian major carps, as compared to earlier reports from India, might be due to the targeted survey of this particular genus taking only Indian major carps as the study material. The prevalence of *Dactylogyrus* spp. was noticed to be different for different districts with the highest being observed for Puri district (76.54%) and the lowest in the district of Keonjhar (37.50%). Prevalence for other districts have been described in Table 1. The current study also supported cyprinids being suitable host for gill flukes.

The prevalence of *Dactylogyrus* is highly correlated with several other predisposing environmental factors. Water temperature fluctuations at different seasons play a significant role in the prevalence of dactylogyrosis. Hossain *et al.* (2008) found winter as the most favourable season for the occurrence of ecto-parasitic diseases in fishes with a prevalence of different monogenean and protozoan parasites amounting 37.16% in rainy season, 40.08% in winter and 30.25% in summer. In the same line, Yang *et al.* (2016) found the mean intensity of *D. lamellatus* at peak during late winter and spring, and then sudden drop in number during summer. During the present study, prevalence of *Dactylogyrus* was found to be 84% during winter followed by summer (59.30%) and monsoon (46.59%). Sudden decrease in water temperature during winter may weaken the fish immunity which in turn helps the parasites to propagate faster. Among three Indian major carps surveyed, *L. rohita* (79.85%) was found to be the most susceptible host species, followed by *C. catla* (51.63%) and *C. mrigala* (38.46%) (Fig. 1).

The microscopic analysis of gill samples of fish collected from different polyculture ponds revealed the presence of *Dactylogyrus* spp. (Fig. 2a). The amplification of PCR product using 28S rDNA primers of positive samples generated amplicons of 400 bp (Fig. 2b). In the recent past, 28S rDNA PCR-sequencing have been used as important tool in identifying several parasites including *Dactylogyrus* sp. (Jovelin and Justine, 2001; Singh and Chaudhary, 2010; Paul *et al.,* 2018; Roohi *et al.,* 2019). Total twenty-five *Dactylogyrus* sp. have been identified using 28S rDNA PCR and sequencing from different districts of

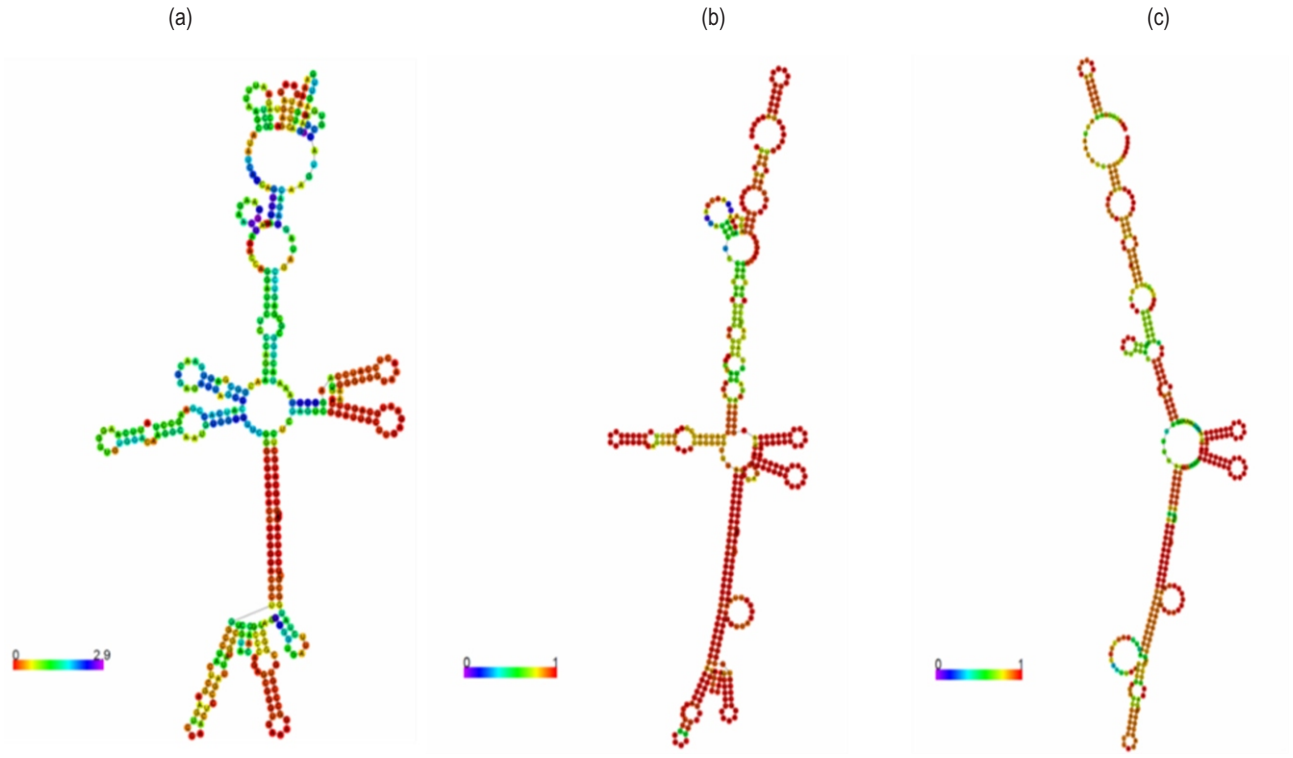

Fig. 3: Predicted secondary 28S rDNA structure orientation with the different loops types for all three isolated *Dactylogyrus* species. **a:** *D. catlaius*, **b:** *D. vastator* and **c:** *D. scorpius*) using MFold software package based on the minimal free energy state.

Odisha, among which twenty-two were found to be *D. catlaius* (GenBank accession numbers: MW479163, MW479164, MW479165, MW479166, MW479167, MW479168, MW479169, MW479170, MW479171, MW479172, MW479173, MW479174, MW479175, MW479176, MW479177, MW479178, MW479179, MW479180, MW479181, MW479182, MW479183, MW479184), two as *D. vastator* (GenBank accession numbers: MW479394, MW479395) and one as *D. scorpius* (Paul *et al.,* 2021) (GenBank accession number: Mt509586).

Further predicted secondary 28S rDNA structure orientation with different loops types for all three isolated *Dactylogyrus* species suggested their potential use for species identification/confirmation of monogenean parasites as reported earlier (Koyee *et al.,* 2016). Koyee *et al.* (2019) predicted secondary RNA structure using 28S rDNA sequence of *D. andalouensis, D. vistulae, D. squamous, D. omenti, D. malleus, D. mascomai, D. lenkoranoides, D. minutus, D. inexpectatus, D. anchoratus* and *D. extensus*. All the species showed unique secondary structure which helps in further distinction for species identification. In the present study unique RNA secondary structures of *D. catlaius, D. vastator* and *D. scorpius* were predicted and found to be exclusive (Fig. 3). Loop diversity was found to be the highest for *D. catlaius* (78.83) followed by *D. scorpius* (49.15) and *D. vastator* (34.66). Negative free energy of thermodynamic was 131.11 kcal mol^{-1}, 139.04 kcal mol^{-1} and 124.05 kcal mol^{-1} for *D. catlaius, D. vastator* and *D. scorpius*, respectively. Frequency of MFE structure was 0.01%, 0.09% and 0.03% for *D. catlaius, D. vastator* and *D. scorpius,* respectively. Hence, rDNA sequencing along with RNA secondary structure analysis may play potential role in species identification of gill flukes in the advent of large number of taxonomically different species within the genus and lack of qualified classical taxonomists. The study also predicted the RNA secondary structure of these three reported species for the first time. Host specificity of *D. catlaius* (most prevalent parasite found in Odisha) has been ascertained among three Indian major carps for better understanding of host-parasite interaction.

Many species *Dactylogyrus* have been found to infect only a particular fish, and are termed as specialist whereas some can infect an array of fish species and are called as generalist (Simkova *et al.,* 2017). Recently, Paul *et al.* (2021) reported a monogenean parasite, *D. scorpius* as a specialist parasite to rohu among three Indian major carps in India. In most cases, the information on host-range of an isolate is insufficient. *D. catlaius* was found to be a generalist *Dactylogyrus* infecting all three species of Indian major carps both in natural and experimental conditions. In co-habitational challenge rohu was found to be the most preferred species for *D. catlaius*, although it infects all three Indian major carps. Simkova *et al.* (2004) also emphasized the importance of studying the host-parasite interaction and their

phylogenetic analysis to know about the biogeographical source and distribution. The host-specificity might be linked with the morphological likeness of ever evolving host organisms (Mendlova and Simkova, 2014). Along with host-specificity, the gill samples of all three Indian major carps were also subjected to parasitic prevalence and intensity analysis. During co-habitational challenge, experiment with *D. catlaius*, prevalence of parasite was 100% in rohu from 1 dpi throughout the experimental period. Infection with the parasite in *C. catla* and *C. mrigala* was observed from 5 dpi. The intensity of parasite was found to increase gradually in all three Indian major carps (Table 2). Similarly, Kaur *et al.* (2018) also found 100% prevalence in *C. catla* in an experimental challenge study with gill monogenean with a corresponding intensity in parasite load of 34.75, 38.25, 41.5 and 45 at 1st, 2nd, 3rd and 4th weeks post-challenge, respectively. However, Dash *et al.* (2014) noticed severe acute haemorrhagic inflammation in the gills with mortality in rohu co-habitationally infected with *D. catlaius*, although the infection was noticed only from day 4 onwards. Possibly strain variations within the species with different morphotypes along with other host factors might be governing the host-parasite interaction.

The present study provides a holistic information regarding *Dactylogyrus* prevalence in the state of Odisha. Further the study illustrates host-parasite relation of *D. catlaius* among Indian major carps which strengthens knowledge about our understanding regarding *Dactylogyrus* for developing any prophylactic measure in future.

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Add-on Information

Authors' contribution: A. Paul: Field observation, data collection, Experiments data analysis and compilation; **P.K. Sahoo:** Guide the experiment and manuscript, concept, data analysis and compilation.

Research content: The research content of manuscript is original and has not been published elsewhere.

Ethical approval: The research samoles were collected from farms following institute (ICAR-CIFA) ethical guidelines and clearance.

Conflict of interest: The authors declare that they have no conflict of interest.

Data from other sources: Not applicable

Consent to publish: Author agree to publish the paper in *Journal of Environmental Biology.*

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