Ribosomal DNA and morphological analysis of *Dactylogyrus* species from freshwater fishes of India

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Abstract *Dactylogyrus* species (*Dactylogyridae*: Monogenea) are a group of monogenean gill parasites these parasites are highly host specific to freshwater fish of family Cyprinidae. A method is presented for the isolation and morphometric analysis of hamuli marginal hooks and bars form individual dactylogrids monogenoidea using phase contrast microcopy and camera lucida drawing, while simultaneously processing for rDNA analysis using the polymerase chain reaction. The identification and taxonomy of the worms in question was done by the morphometrical methods and rDNA analysis. ITS-1 regions of the rDNA gene of worms were amplified, sequenced and then the discrimination was recorded at the species level. The studied segments were compared with the published data on such segments of other *Dactylogyrus* species and were found to have significant relationship in the genes and spacer regions of the rDNA.

Keywords *Dactylogyrus anchoratus* · 18S rDNA · Monogenea · PCR · Morphometric analysis

Introduction

The taxonomy of dactylogrids monogeneans depends upon accurate descriptions of small differences in the size and shape of copulatory complex. However, marginal hooks are difficult to flatten in viscous mounting media, and the taxonomically informative variations in shape and size are close to the limits of microscopy. Malmberg (1957)

introduced saturated ammonium picrate in glycerine as a combined fixative/mountant for Gyrodactylids, improving contrast and resolution of the marginal hooks and is being widely used. Since the introduction of molecular tools into taxonomy, systematic, and phylogeny, many species descriptions are re-evaluated, new species are described while some are assigned new taxonomic position. In classical morphological analysis, srptic speciation may lead to an underestimation of the number of species while phenotypic plasticity may induce the reverse effect. In parasitic organisms, the morphological identification can be furthermore obliterated by convergent evolution (Price 1980). In this study, we assess the validity of molecular markers, comparative morphometric analysis and statistical classifiers in discriminating closely related *Dactylogyrus* species.

However, when using light microscopy based images, the total complement of sclerites is required. Cunningham et al. (1995) introduced molecular markers, namely the rDNA region with the V4 region and the internal transcribed spacers (ITS), as a new tool for species identification. By using RFLP and DNA probe hybridization a relatively rapid screening for potential pathogenic *G. salaris* specimens became possible. However, recently it has become clear that these molecular tools are not always as straight forward as generally accepted. DNA probe hybridization with the amplified V4 region misidentified *Gyrodactylus teuchis* samples as *G. salaris*. Direct sequencing remains the most reliable method for *Gyrodactylus* identification to date (Cunningham et al. 2001).

In the present study *Dactylogyrus* specimens living on carp of the genus *Cyprinus carpio* gills have been used as a model. These fishes are exotic freshwater fishes playing an important role in the food fishes in aquaculture. This is related to common gold fish with which it is capable of

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inter breeding. It gives its name to the carp family Cyprinidae. Originating in Asia, the fish has been introduced into environments worldwide.

First we collected the ITS rDNA sequences of several specimens isolated from all host species. Subsequently, we collected and compared morphological data from *Dactylogyrus anchoratus* like species of all hosts which were previously described by Gusev (1985). In order to quantify the morphological differentiation among the population, the morphometric analyses have been done. Since there was not sufficient material available for *Dactylogyrus anchoratus*, only the molecular analysis was carried out.

Cunningham and coworkers (Cunningham et al. 1995; Cunningham 1997) developed methods for DNA sequence analysis of gyrodactylids, which, although employed primarily for the diagnosis of the pathogen *Gyrodactylus salaris*, provide an opportunity for molecular analysis complementing the existing morphological taxonomy. However, polymerase chain reaction (PCR) amplification of the ribosomal DNA cassette is optimal from ethanol-fixed specimens, which mounted for critical analysis, an essential step, if sequence data are to be correlated with morphological taxonomy.

The objective of this study was to investigate *Dactylogyrus anchoratus* combined morphological observation and established by ribosomal DNA analysis and therefore present manuscript describes the comparison of morphological observation and rDNA analyses of *Dactylogyrus anchoratus*.

Materials and methods

Parasite collection and identification

Fifteen species of *D. anchoratus* belonging to the Cyprinidae were collected from *Cyprinus carpio*. Parasite determinations were performed based on the hard parts of the parasite haptor and reproductive organs, according to Gusev (1985). After species determination, the same

parasite specimen was stored in absolute ethanol SPETR-ANAL (Allied-Signal, Riedel-deHaen, Seelze, Germany) at 4°C for molecular analysis.

Molecular analyses

DNA extraction and PCR

Individual parasites were removed from ethanol, dried and DNA was extracted using the standard phenol–chloroform method (Sambrook et al. 1989). For general study of species, individuals from different hosts were investigated. Total DNA obtained from different parasites was resuspended in 20 µl distilled water. Partial 18S and entire ITS-1 region were amplified in first reaction with the help of using primer S1 (5' ATTCCGATAACGAACGAGACT 3') that binds with the terminal region of the 18S gene and H7 (5' GCTGCGTTCTTCATCGATACTCG-3') (Sinnappah et al., 2001) that anneals within the 5.8S rDNA. Each amplification reaction was performed in a final volume of 25 µl containing 1.5 U of Taq DNA polymerase, 1× Buffer, 1.5 mM MgCl₂, 200 µM of each dNTP, 0.8 µM of each primer and 3 µl of DNA. The standard PCR condition (4 min at 95°C, 1 min 95°C, 1 min 55°C and 1 min 30 s. at 72°C and 10 min of final elongation at 72°C) was used to amplify the product. 1.5% agarose gel was used for electrophoretic separation and DNA was stained with ethidium bromide for visualization. The DNA fragment amplified with these primers comprises the 3' terminal end of the 18S rDNA, the entire internal transcribed spacers1 (ITS-1) and a very small fragment of 5.8S rDNA. PCR products were cut from the gel and purified. DNA was precipitated and resuspended in nuclease free water and independently sequenced for each individual species.

Sequence alignment was first performed with Clustal W (Thompson et al. 1994) and improved by eye with Se Al (v1d1 and A. Rambaut, University of Oxford 1995). After alignment, ambiguous regions (i.e. containing gaps) were removed. Analysis was carried out on all substitutions.

Fig. 1 Diagrammatical representation of regions for PCR products and primer selection in *Dactylogyrus* intergenic spacers

Fig. 2 *Dactylogyrus anchoratus*, dorsal view (Dujardin 1854) Wagener 1857 whole mount (a) dorsal anchor (b) dorsal bar (c) hooks (d) copulatory complex (e)

Result

Species description

Family: Dactylogyrinae

Genus: *Dactylogyrus anchoratus* (Dujardin, 1854)
Wagener, 1857

Host: *Cyprinus carpio* (Common Carp)

Local on host: On gill arches

Locality: Aashivan Unnav

Water temperature: 20°C

Salinity: 12 ppm

Morphological examination: 21 specimens collected live

Dactylogyrus anchoratus was first described by Dujardin in 1854 from France as *Gyodactylus anchoratus*, but was later transferred under the genus *Dactylogyrus* by Wagener (1857). Later on this species was recorded on cyprinid hosts in many regions of the world. Further *Dactylogyrus anchoratus* has been divided into three subspecies viz., *Dactylogyrus anchoratus* by Kulwiec, 1927 on *Cyprinus carpio* in Poland, *Dactylogyrus anchoratus carassii* by Bychowsky, 1993 on *Carassius carassius* in Northern Russia and also recorded by Muller in 1936 on *Carassius auratus* in the USA, *Dactylogyrus anchoratus geii* Yin and

Fig. 3 PCR amplification of 18S ribosomal DNA *Dactylogyrus anchoratus*

Sproston, 1948 on *Carassius auratus* from Shangh region and also by region and also by Yamaguti in 1942 on *Carassius carassius* from Kyoto area, Japan who regarded it to be a distinct species as *Dactylogyrus arcuatus*. Paperna (1959) did not agree in splitting the species *Dactylogyrus anchoratus* in to three subspecies described *Dactylogyrus anchoratus* from Israel on *Cyprinus carpio* and on *Tyognathus steinizorum* from Sea of Galilee. The present specimens of *Dactylogyrus anchoratus* were collected on different host *Cyprinus carpio*. It is characterized by ‘Anchoratoid–Wegeneri’ type anchor with very long inner. Specimens of *Dactylogyrus anchoratus* resembles with the account as given by Paperna (1959) in most of the morphological details differencing in some minor features. Paperna (1959) in his description of *Dactylogyrus anchoratus* has not observed small protuberance on inner side of point of anchor. The appended work (Fig. 2) shows a comparison in the body measurements of *Dactylogyrus anchoratus* as given by Paperna (1959) and present specimens.

Molecular analyses

ITS-1, 18S and 5.8S gene sequences were pervious obtained from NCBI (accession number AJ564111). DNAs were amplified by PCR, using primers H7 and L1 from 28S to 18S regions, respectively (as shown in Fig. 1). These were 1.0–1.5 Kb in sizes based on the intensities in ethidium bromide staining. The fragments were sequenced and the ITS sequences of *D. anchoratus* has been submitted to NCBI nucleotide database under accession number EU342146. The alignment of ITS sequences has shown that gene 5.8S was identical for all specimens (Fig. 3).

Discussion

The method presented here greatly improve the reliability and usefulness of morphology of whole individual, Haptor, hooks, and copulatory complex. The DNA sequence analysis and morphology were very similar as reported with the other species (Cunningham et al. 1995; Cunningham 1997).

The use of single worm as source of DNA for 18S along with ITS-1 and 5.8S amplification and subsequent sequencing proved the evidence. Using the primers described here, it was possible to obtain up to 1.5 Kb of sequence from single worm spanning the region between primer sites H7–L1 i.e. the entire rDNA gene between the end of SSU gene and the beginning of LSU and encompassing ITS-1, ITS-2, and 5.8S regions.

The sequence obtained was directly comparable with that amplified from this region in other *Dactylogyrus* sps. Furthermore, the PCR products can be subjected to restriction fragment analysis allowing a decision to be taken about sequencing. Ribosomal DNA was amplified, and sequence data were obtained from 50%, a success rate that was considered acceptable given the small size of Dactylogyrids and the small quantity of DNA available for use as template.

This method makes possible the combined morphological and molecular analysis of field samples preserved in ethanol, and it will allow using the samples of museum specimens, fixed with ethanol and their respective hosts. This method could extend the range of monogenean material available for critical analysis. We suggested that field workers routinely fix Dactylogyrids in ethanol, and that the combined of hooks and analysis of the ITS-1, 5.8S, and ITS-2 regions of the new Dactylogyrids species.

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