

Research Article

First molecular confirmation of the *Dactylogyrus anchoratus* and *D. vastator* (Monogenea, Dactylogyridae) from *Carassius auratus* in western India

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Abstract

Dactylogyrus anchoratus and *Dactylogyrus vastator* (Monogenea, Dactylogyridae) are distributed worldwide as the most frequent ectoparasites of goldfish (*Carassius auratus*). This is the first report of *D. anchoratus* and *D. vastator* from India. The monogeneans were identified using morphometric measurements of hard parts, the morphology of the haptor parts and the shape of the male copulatory organ. Molecular characterization by phylogenetic analyses of 18S and 28S ribosomal RNA gene sequences supported the morphological identifications.

Key words: Monogenea, *Dactylogyrus* spp., molecular identification, 18S, 28S, *Carassius auratus*, India

Introduction

The genus *Dactylogyrus* Diesing, 1850 (Dactylogyridae) includes more than 900 nominal species (Gibson et al. 1996). These monogeneans are the world's most common gill parasites of freshwater fishes (Woo 2006), mainly infecting cyprinid fishes (Šimková et al. 2007). *Dactylogyrus* parasites cause serious infections in the gill filaments that impair respiration, their pathogenicity results in high mortalities (Jiang et al. 2013; Tu et al. 2015) and significant economic losses (Woo et al. 2002; Reed et al. 2009) in aquaculture.

The Goldfish *Carassius auratus* (Linnaeus, 1758) is a freshwater fish of the family Cyprinidae (order Cypriniformes). *C. auratus* was introduced to India from Japan many years ago, but the year of introduction is unknown (<http://www.fao.org/fishery/introsp/230/en>). It has been widely distributed by the booming aquarium industry, which has made it the most popular ornamental fish in the world (Morgan and Beatty 2007; Gupta and Banerjee 2009). Many studies have suggested that *C. auratus* is one of the most common host parasitized by *Dactylogyrus* spp.

(Šimková et al. 2004; Jalali and Barzegar 2005; Shamsi et al. 2009; Molnár 2009, Rasouli et al. 2012; Borisov 2013; Tu et al. 2015). In India, most studies of *Dactylogyrus* spp. have been based only on morphology. However, accurate identification of such a large group of species requires the use of molecular tools such as the ribosomal 18S and 28S genes that are used to identify and distinguish monogenean species (Chisholm et al. 2001; Šimková et al. 2004; Wu et al. 2007; Chaudhary and Singh 2012, 2013).

In the present study, *Dactylogyrus anchoratus* (Dujardin, 1845) Wagener, 1857 and *Dactylogyrus vastator* Nybelin, 1924 were identified from gill filaments of *C. auratus* in Meerut, Uttar Pradesh (U.P.), India using a morphological and, for the first time, a molecular approach.

Material and methods

Parasite collection

Thirty six Gold fish obtained from the aquarium market in Meerut (29°01'N; 77°45'E), U.P., India

were killed by a sharp blow on the top of the head, and their gill filaments were dissected out. For the collection of *Dactylogyrus* species, gill filaments were examined using a Motic SMZ-168 series stereomicroscope and parasites were removed. Parasites collected from the same individual fish were used for both morphological and sclerotized structure studies. For morphological examination permanent slides of whole individual parasites were prepared by staining with acetocarmine, dehydrating with ascending grades of alcohol and mounting in Canada balsam. For the study of sclerotized structures, whole parasites were cleared gradually in water and mounted in glycerin. Specimens were examined using light microscopy. Identification was via the morphology of haptor hard parts and the copulatory complex. Line drawings were made with a camera lucida connected to the Motic digital microscope (DMB series). All measurements were made directly from the drawings and also confirmed with the inbuilt measuring software (Motic Image Plus 2.0 for Windows). Measurements are given in micrometers unless otherwise stated.

Molecular analysis

Prior to DNA analysis, worms were identified on the basis of morphology and then preserved in 95% ethanol at -20°C in separate vials. Genomic DNA was extracted from a pool of ten parasites for both the *Dactylogyrus* species collected using the Qiagen DNeasy™ tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. The partial fragments of 18S and 28S ribosomal RNA genes were amplified with primers 18S (forward, 5'-CGGTTGCAATTTTTATGTGG-3' and reverse, 5'-GAGTGATCCACCACTTGCAG-3') (Chiary et al. 2014) and 28S (forward, 5'-TCTAGTAACGGCGA GTGAACG-3' and reverse, 5'-GGTGAAGGTCT ACCTCAGC-3') (Chiary et al. 2014). Amplification reactions were performed as reported by Chiary et al. (2014). PCR cycles were carried out for 35 cycles as follows: 3 min at 94°C (initial denaturation) followed by 30 sec at 94°C for further denaturation, annealing for 45 sec at 55°C (18S primer pair) or 59°C (28S primer pair), 1 min at 72°C followed by a final extension for 10 min at 72°C . PCR products were examined on 1% agarose-TAE gels, stained with ethidium bromide and visualized under UV transillumination. PCR products were purified by Purelink™ Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen, Löhne, Germany), according to the manufacturer's instructions, and sequenced using a Big Dye Terminator version 3.1 cycle sequencing kit in an ABI 3130 Genetic Analyzer

(Applied Biosystems, Foster City, California, USA) using both sets of primers.

The 18S and 28S rDNA sequence data were subjected to BLASTn searches for related species of *Dactylogyrus* in GenBank (<http://www.ncbi.nlm.nih.gov/genbank>) for phylogenetic analysis. Genetic divergences were calculated using a p-distance model for each gene region using MEGA v. 6 (Tamura et al. 2013). For the phylogeny of *Dactylogyrus* spp., related sequences were retrieved and aligned using the Clustal W (Thompson et al. 1994) multiple alignment option with default parameters. Maximum likelihood (ML) and Bayesian inference (BI) analyses were calculated under the GTR+I+G model, selected by Model Test, using MEGA v. 6 (Tamura et al. 2013) and TOPALI 2.5 (Milne et al. 2009) respectively. The data were tested for the nucleotide substitution model of best fit using Akaike's Information Criterion (AIC) (Akaike 1973). Bootstrap values were generated based on 1,000 resampled datasets. *Cichlidogyrus ergensi* (GenBank accession number HE792788) and *C. falcifer* (HQ010024) were used as respective outgroups in the 18S and 28S phylogenetic trees generated.

Results

Two species of monogeneans, *Dactylogyrus anchoratus* (Dujardin, 1845) Wagener, 1857 (Figure 1A–D) and *Dactylogyrus vastator* Nybelin, 1924 (Figure 1E–H), were identified from the gill filaments of goldfish with an overall prevalence of 78% (29 infected out of a potential 37 fish hosts). Over 100 *D. anchoratus* were found in 19 fish while *D. vastator* dominated with >250 collected from 24 infected hosts. Infected fish each carried 10–12 individual *D. anchoratus* parasites (mean: 10.4; SD: 0.76) while for *D. vastator* the frequency was 10–16 parasites per infected fish (mean: 11.3; SD: 2.06).

Voucher slides were deposited in the collection of the Museum of the Department of Zoology, Chaudhary Charan Singh University, Meerut, (U.P.), India under the voucher number HS/monogenea/2015/03 (*D. anchoratus*) and HS/monogenea/2015/04 (*D. vastator*); and in the Natural History Museum, Geneva, Switzerland, under the voucher number MHNG-INVE-91851 (*D. anchoratus*) and MHNG-INVE-91851 (*D. vastator*). 18S and 28S sequences were submitted to GenBank with accession numbers KT279400 and KT279401 (*D. anchoratus*) and KT279398 and KT279399 (*D. vastator*) respectively.

Body measurements (n=5 per species) mean and ranges were:

D. anchoratus: body 340.5 (335.0–345.0) long and 77.8 (75.0–80.0) wide. A single pair of anchors, dorsal anchor total length 114.8 (112.0–116.0) long,

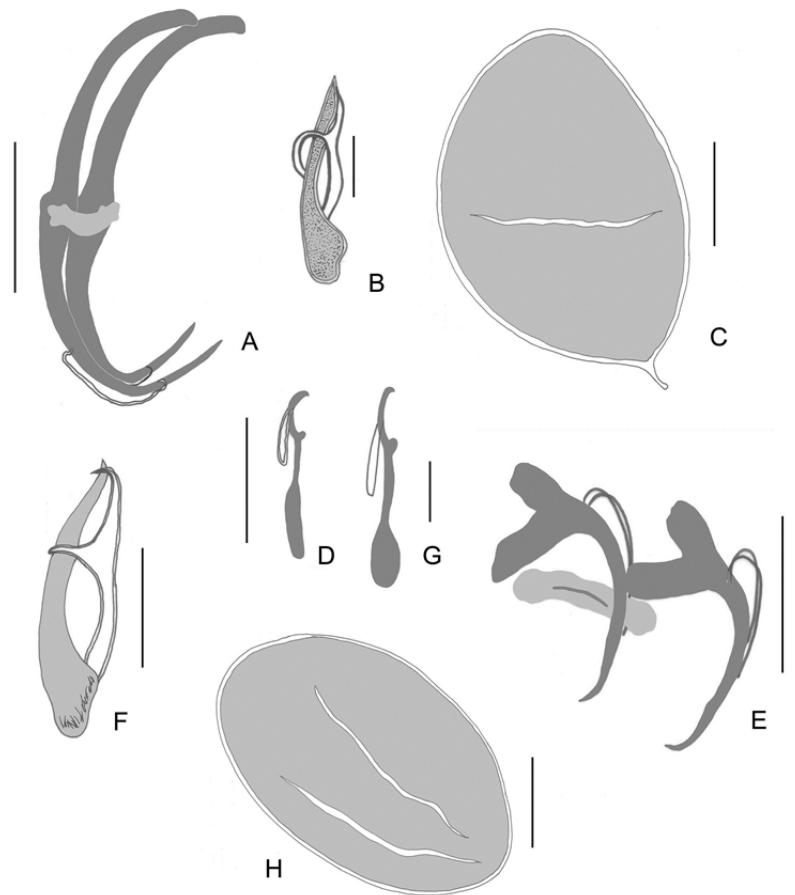


Figure 1. Line drawings of *D. anchoratus* (A–D) and *D. vastator* (E–H) infecting goldfish, *C. auratus*, from Meerut, India: dorsal anchor with dorsal bar (A and E); male copulatory organ (B and F); egg (C and H); and marginal hook (D and G). Scale bars = 35 μ m (A), 15 μ m (B, F), 25 μ m (C,G,H), 30 μ m (D), 40 μ m (E).

base length 44.0 (41.5–46.5) and recurved point length 25.6 (23.0–27.0); dorsal anchor elongate inner root 50.8 (48.7–52.0) long, outer root not evident. Dorsal bar total length 17.9 (15.0–21.0), width 5.06 (3.8–6.0). Marginal hook total length 24.3 (22.5–27.0); copulatory organ total length 27.8 (23.2–31.5). *D. vastator*: body 206.6 (195.0–215.0) long and 37.4 (35.0–40.0) wide. A single pair of dorsal anchors, total length 35.2 (35.0–36.0), base length 17.0 (16.0–18.0) and recurved point length 10.3 (08.0–12.5); dorsal anchor inner root elongate, 17.5 (17.0–18.0) long and outer root short, 8.5 (8.0–9.0) long. Dorsal bar total length 24.6 (24.5–25.0), width 3.5 (03.0–04.0). Marginal hook total length 22.4 (20.0–25.5); copulatory organ total length 50.6 (41.5–58.3).

Based on the morphological study of hard parts, *D. anchoratus* showed morphological similarities with *D. arcuatus* Yamaguti, 1942 and *D. formosus* Kulwiec, 1927, which also parasitize *C. auratus*. *D. anchoratus* can easily be distinguished from *D. arcuatus* by the morphology of the male copulatory

organ, which is slightly sinusoidal in *D. anchoratus*, but strongly curved in *D. arcuatus*. Additionally, *D. anchoratus* shows some similarities with *D. formosus* in the shape of the haptoral parts; however, all parts of the haptor of *D. anchoratus* are significantly larger than *D. formosus*. Based on the morphological study of the hard parts of haptor and male copulatory organ, *D. vastator* shows some morphological similarities with *D. intermedius*, another *C. auratus* parasite. However, *D. vastator* can be readily distinguished from *D. intermedius* on the basis of comparatively larger roots and hook length.

For *D. anchoratus*, the BLASTn search of the 510bp 18S fragment showed an overall 99% identity to the other two sequences of the same species isolated from *Cyprinus carpio* (AJ490161) and *Carassius auratus* (AJ564111) from the Czech Republic (Šimková et al. 2004). P-distance analysis revealed that the 18S rRNA gene sequence isolated from Indian *D. anchoratus* diverged from the *C. auratus* isolate by only 0.024% and from the *C. carpio* isolate by 0.026%; the former thus being

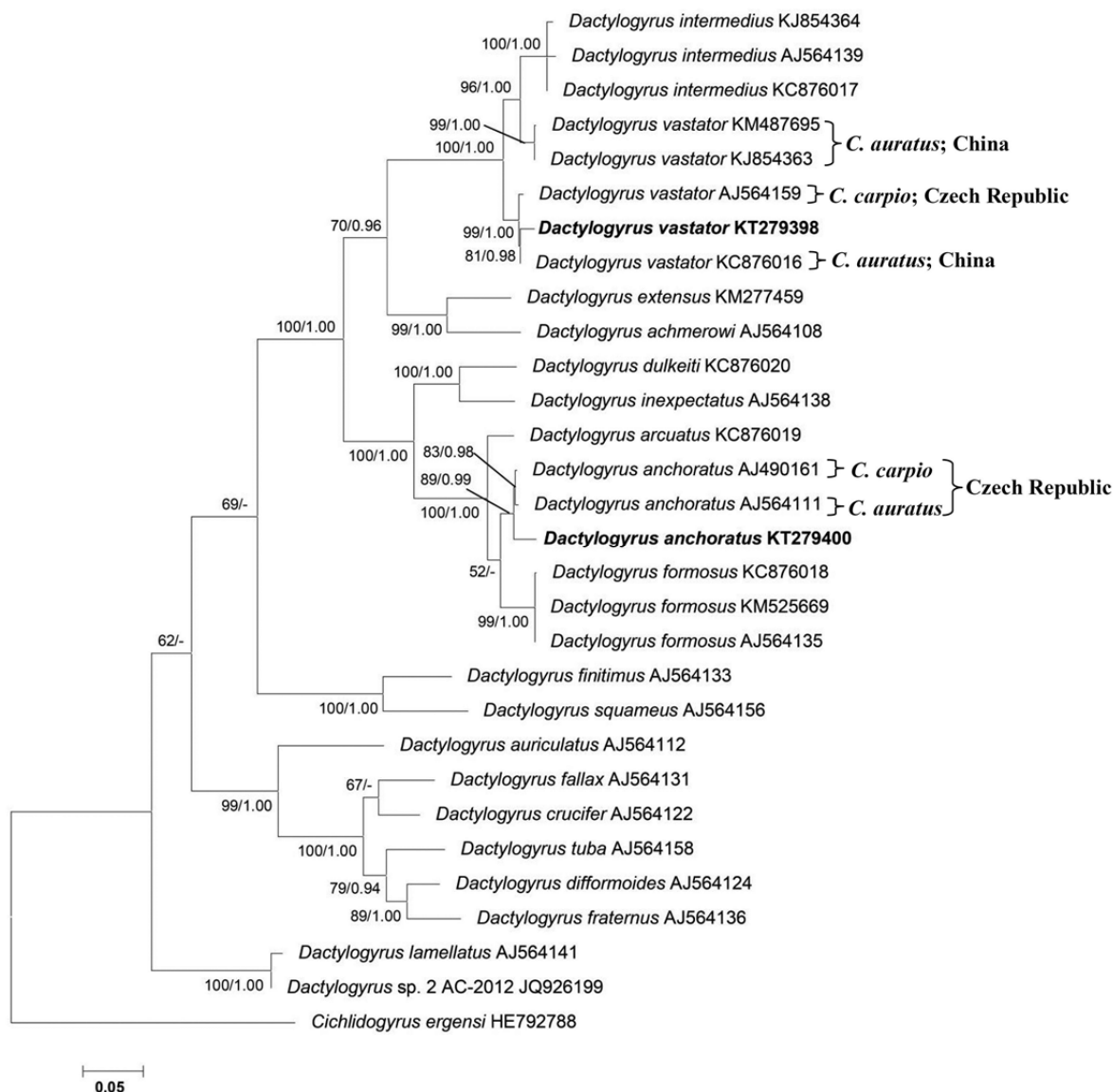


Figure 2. Phylogenetic tree generated by maximum likelihood analysis based on 18S rDNA sequences for selected species of *Dactylogyrus* with *D. anchoratus* (KT279400) and *D. vastator* (KT279398) from India. Accession numbers for all species is shown in tree next to the species name. Supported values at the branch are shown as ML/BI. Nodes unsupported by BI are marked with a hyphen. Species newly sequenced in this study are in bold. *Cichlidogyrus ergensi* was used as outgroup.

genetically more similar to the present specimens. Besides this, the 18S sequence of *D. anchoratus* generated in this study also displayed 98 to 99% identity to *D. formosus* and *D. arcuatus*, and these share some morphological resemblance. In the 18S phylogenetic analysis, *D. anchoratus* (Indian isolate) grouped together with the same species isolates collected from the Czech Republic, along with the closely related sister species, *D. formosus* and *D. arcuatus*, using both ML and BI analyses (Figure 2).

The 790 bp 28S rRNA gene sequence of the Indian *D. anchoratus* lineage also shows 99% identity to two available sequences of the same species from Iran (JX524546 from *Cyprinus carpio* and JX524547 from *Ctenopharyngodon idella*). Further analysis of the 28S rRNA gene sequence showed that the Indian isolate differed only by 0.01%. Moreover, both ML and BI methods clustered *D. anchoratus* with the two Iranian isolates with high bootstrap support (99/1.00), with this clade grouped

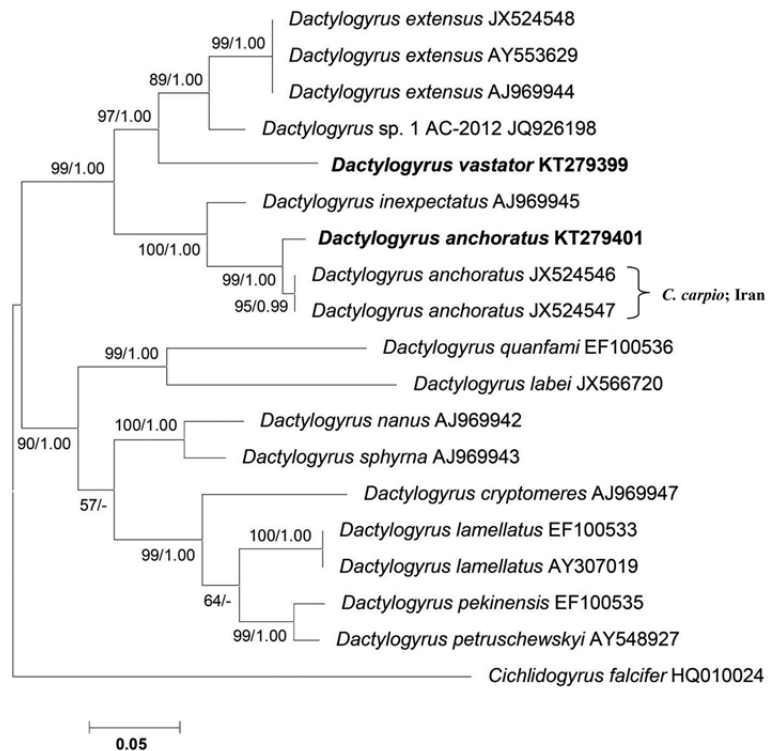


Figure 3. Maximum likelihood tree of selected *Dactylogyrus* species based on 28S sequences with Indian isolates (accession numbers, KT279401 (*D. anchoratus*) and KT279399 (*D. vastator*). Posterior probabilities for BI are given after the bootstrap values for ML. Nodes unsupported by BI are marked with a hyphen. Species newly sequenced in this study are in bold. Accession numbers are given next to the species name. *Cichlidogyrus falcifer* was used as outgroup.

along with another sister species, *D. inexpectatus* (supported by 100% bootstrap values) (Figure 3).

The BLASTn search of the 730bp 18S fragment for *D. vastator* showed 92–96% overall identity to other isolates of *D. vastator* from the Czech Republic (AJ564159) and China (KM487695, KJ854363 and KC876016), which were collected from *Cyprinus carpio* and *Carassius auratus*, respectively (Figure 2) (Simková et al. 2004; Tu et al. 2015; Ling et al. 2016). Pairwise distance analysis of the aligned 18S sequences revealed a low genetic difference, with an intraspecific variation of only 0.04–0.06% (Chinese isolates) and 0.05% (Czech Republic), in comparison with the Indian isolate, which shows maximum similarity with a Chinese isolate (0.04%; KC876016) collected from *C. auratus*. The Indian isolates of *D. vastator* cluster together with the four other isolates of the same species, along with another sister species, *D. intermedius*, in a clade with high bootstrap support (100/1.00) in agreement with Tu et al. (2015) (Figure 2).

In the analysis of 28S fragment (490bp), as no sequence of the same species is available on GenBank, *D. vastator* grouped with other isolates of *Dactylogyrus* with high bootstrap support (99/1.00) based on both ML and BI methods (Figure 3).

Discussion

Dactylogyrus is a group of monogenean parasites, having more than 900 described species (Gibson et al. 1996). Morphologically, monogeneans found in this study were identified as *D. anchoratus* and *D. vastator*. These parasites are native to Southeast Asia, i.e. China, but they have the potential to spread to other regions through introductions (Molnár 2009). Morphometric characters of *D. anchoratus*, such as total length and inner root, are larger than the most closely-related *Dactylogyrus* species, *D. formosus*. Differentiating *D. vastator* from its most closely related species, *D. intermedius*, is mostly via the dorsal anchor total length, and shaft and root, which are slightly larger in *D. vastator* than in *D. intermedius*. Both parasites were identified based on the shape of their haptor armature and male copulatory complex, which are visible under the microscope (Figure 1). Besides morphology, molecular characterization permits validation and comparison with other congeners of *Dactylogyrus* ensuring correct identification. *D. anchoratus* and *D. vastator* are established on goldfishes worldwide as highly pathogenic gill parasites, causing hyper-trophy of gill tissue and ultimately death (Dove and Ernst

1998; Řehulková and Rehulka 1999; Di Cave et al. 2000; Řehulková and Gelnar 2005; Molnár 2009; Shinn and Tun 2013, Fridman et al. 2014). Most species of *Dactylogyrus* are strictly host-specific or show specificity for phylogenetically related cyprinid species (Šimková et al. 2006). As Indian freshwaters contain a high cyprinid fish biodiversity (Johnson and Arunachalam 2009; Basu et al. 2012; Baliarsingh et al. 2013; Pawara et al. 2014). The introduction of *D. anchoratus* and *D. vastator* poses considerable risks to native cyprinids because these two species are categorized as generalist parasites, which have already been shown to be capable of parasitizing at least two unrelated host species (Jarkovský et al. 2004; Šimková et al. 2013).

Conclusions

Our study reports the occurrence of *D. anchoratus* and *D. vastator* from cultured goldfish in Meerut, U.P., India, using morphological and molecular methods. Further investigation is required to identify more species of *Dactylogyrus* molecularly for the diagnosis and prevention of the diseases caused by these parasites.

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