Rapid Communication

Monogenoidea on exotic Indian freshwater fish. 4. *Dactylogyrus minutus* from Platinum Ogon, an ornamental variety of the common carp *Cyprinus carpio* (Cypriniformes, Cyprinidae)

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Abstract

Platinum Ogon, an ornamental variant of the common carp *Cyprinus carpio*, is one of the most expensive and sought-after pet fish. Multiple parasite species from *Dactylogyrus* and *Gyrodactylus* were recovered from Platinum Ogon specimens during an ongoing nationwide study of the monogenoidean parasites that infect ornamental fish imported into India. The morphological examination, combined with a DNA-sequencing analysis of the 18S–ITS 1 and 28S rRNA genes, identified *Dactylogyrus minutus* as one of these parasites, establishing this species' presence in India for the first time. This finding is significant for two reasons. First, *D. minutus* has been associated with high death rates in carp fry. Second, *Cyprinus carpio*, its host, has established itself in Indian riverine waters. The nucleotide sequence of the 28S rRNA gene of *D. minutus* was phylogenetically compared to that of 19 other *Dactylogyrus* species from three fish families: Cyprinidae (10 species), Leuciscidae (six species), and Xenocyprididae (three species). The phylogenetic tree analysis revealed the monophyletic lineage of *Dactylogyrus* species from goldfish and common carp, which is likely due to the genetic relatedness of their hosts.

Key words: Platyhelminth parasites, exotic fish, ornamental fish trade, 18S–ITS 1 and 28S ribosomal RNA genes, phylogenetic analysis, India

Introduction

Platinum Ogon, also known as Purachina Ogon in Japan, is an ornamental variant of the common carp *Cyprinus carpio* (Linnaeus, 1758) (Cypriniformes, Cyprinidae). This species is known for its characteristic metallic silver-white colour reflectance (Gur et al. 2014). The common carp is one of the most valuable cultured fish species in many parts of the world and has significant economic, recreational and cultural value (Cai et al. 2019). Meanwhile, its colour variants, such as koi carp and Platinum Ogon, are among the most expensive and sought-after fish in the pet fish industry. *Dactylogyrus minutus* Kulwiec (1927) is a well-known pathogen of *C. carpio*. This species was first described in Poland and has since been reported in 25 countries (Figure 1). Although the original description was incomplete and the illustrations were diagrammatic, subsequent researchers—particularly

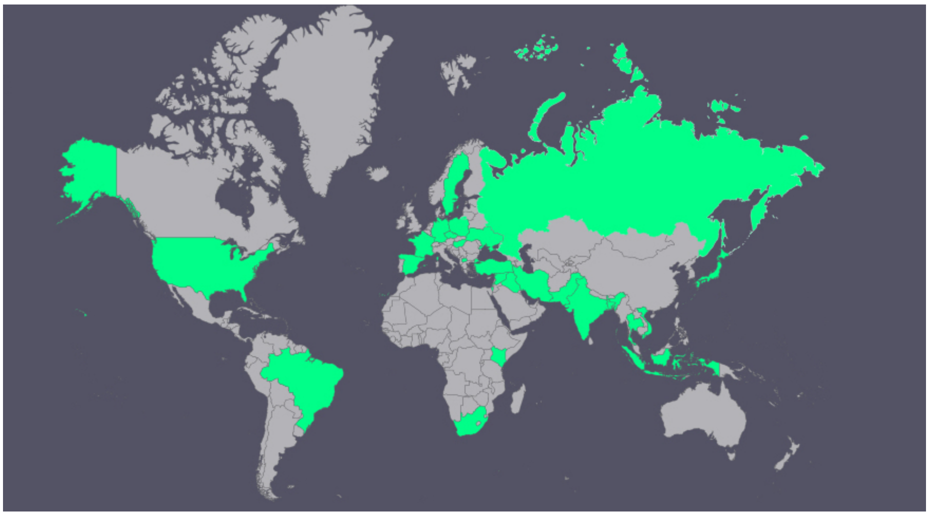

Figure 1. Worldwide distribution map of *Dactylogyrus minutus* Kulwiec, 1927 (shown in green colour) (after many contributions in Nybelin (1937); Gusev (1955); Paperna (1959); Markevich (1951); Allison and Rogers (1970); Lambert (1977); Ogawa and Egusa 1977; Molnár 1987; Samman (1989); Lux 1990; Buchmann et al. 1993; Lacasa-Millan and Gutierrez-Galindo 1995; Kritsky and Heckmann 2002; Barai et al. 2005; Tekin-Ozan and Kir 2005; Hironaka et al. 2006; Stojanovski et al. 2008; Tekin-Ozan et al. 2008; Theerawoot 2008; Dzika et al. 2009; Hoa et al. 2010; Koyun 2011; Mama and Abdullah 2012; Crafford et al. 2014; Otachi et al. 2014; Tancredo et al. 2019; Roohi et al. 2019; present study).

Paperna (1959) and Ogawa and Egusa (1977)—have added sufficient information.

In 2019, the Indian government tasked the senior author of this manuscript with conducting a nationwide survey of monogenoidean parasites found on exotic ornamental fish imported to India as part of a research project (SERB–EMR/2017/003232). During this survey, we collected multiple parasite species from Platinum Ogon. Among these parasites were at least four species of *Dactylogyrus* Diesing, 1850, namely *D. anchoratus* (Dujardin, 1845) Wagener, 1857; *D. extensus* Mueller and Van Cleave, 1932; *D. minutus* Kulwiec, 1927; and *D. vastator* Nybelin, 1924]. Two species of *Gyrodactylus* von Nordmann, 1832 were also detected, but the specific species have not been identified yet. Due to methodological limitations, this multi-species infection made it difficult to isolate genomic sequencing data from specific parasite species. Therefore, we decided to publish information about the first species we positively identified morpho-molecularly while continuing to work on identifying the other Platinum Ogon monogenoids. We did this to draw the attention of farmers, extension/conservation workers, ichthyologists, zoologists, and management agencies to the parasitological problems associated with common carp.

The present study is the fourth in a series aimed at increasing our knowledge of exotic and/or invasive parasitic monogenoids introduced into India via the ornamental fish trade. This is the first study to demonstrate the presence of *Dactylogyrus minutus* Kulwiec, 1927 in post-quarantine populations of Platinum Ogon in India. We supplemented our findings

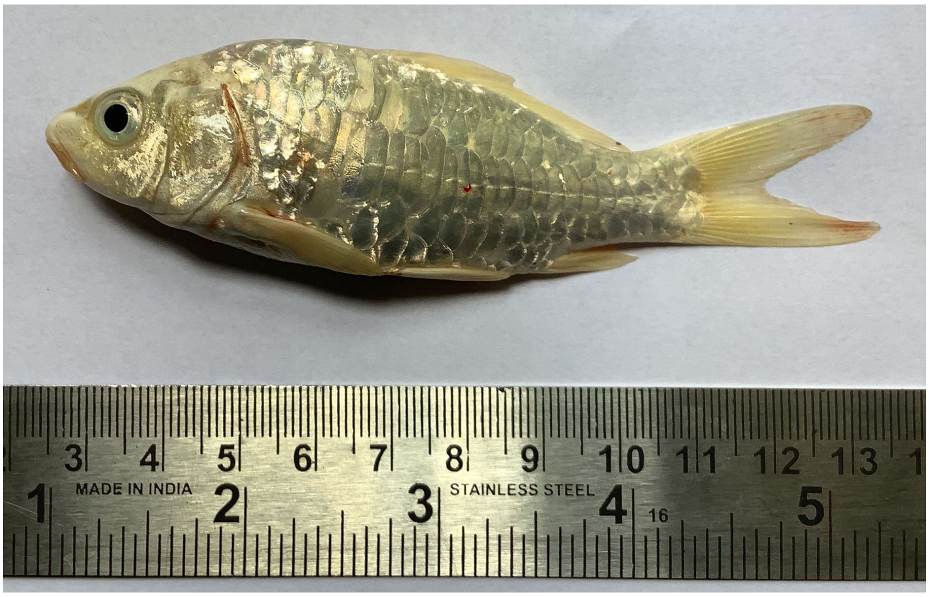

Figure 2. Freshly euthanised specimen (10.7 cm TL) of Platinum Ogon (*Cyprinus carpio* Linnaeus, 1758). Photograph by Chawan Matey.

using high-resolution images of haptoral sclerites and reproductive organs, as well as molecular data on the genes encoding the 18S rRNA gene and internal transcribed spacer 1 region (18S–ITS 1) and 28S rRNA.

Materials and methods

Sample collection and examination: Platinum Ogon is a genetically modified variety of koi carp, bred by crossing Wagoi (completely scaled) Purachina koi with Doitsu (partly scaled) Purachina koi (Boris Gomelsky, *pers. comm.*). Although it is unknown how Platinum Ogon was introduced into India, today, this fish is routinely imported by fish farmers and reared in their ponds or tanks before being sold to retailers who deal directly with consumers. Between January 2020 and March 2021, eight freshly dead and seven moribund Platinum Ogon (total weight: 6.5–9 g; length: 7–12 cm) (Figure 2) were collected from aquaria shops in Lucknow, New Delhi, and Kolkata, India. Individual monogenoids were removed from their gill lamellae under a stereomicroscope (Leica EZ4HD) and immediately fixed and stored separately in 5% lukewarm formalin and 95% ethanol (at −20 °C).

Morphological identification: Formalin-preserved flatworms were mounted in either glycerine (temporary mounts) or dibutylphthalate polystyrene xylene (DPX) (permanent mounts) as recommended by Kritsky et al. (1986). The mounted worms were examined, photographed and measured (in micrometres) at a magnification of 100x using a Leica DM4B upright microscope with LAS X integrated imaging system (Leica Microsystems, Wetzlar, Germany). The *D. minutus* specimens were identified using standard taxonomic criteria related to the hard parts of their haptor and reproductive organs, as described by Gusev (1976).

DNA extraction and PCR amplification: Genomic DNA (gDNA) was extracted from three monogenoids individually fixed in separate vials containing 95% ethanol, using Extracta DNA Prep for PCR-Tissue (Quantabio, Beverly, US) following the manufacturer's instructions. Partial fragments of the 18S rRNA gene and internal transcribed spacer 1 region were amplified by employing the universal primers – namely, forward s1: 5′-ATTCCGATAACGAACGAGACT-3′ (Sinnappah et al. 2001), reverse ir8: 5′-GCTAGCTGCGTTCTTCATCGA-3′) (Šimková et al. 2003). Additionally, partial fragments of 28S ribosomal RNA genes were amplified employing the specific primers (forward: F1: 5′-GCGAGTGAACGGAGATTAGC-3′; reverse R1: 5′-CCATTATTGACCGTGATGTATG-3′) (Ahmadi et al. 2017).

An automated thermal cycler (Himedia Laboratories, India) was used to perform a conventional PCR with reaction mixtures (final volume 20 μL) containing 4.0 μL of distilled water, 10.0 μL of 2x PCR TaqMixture (Himedia Laboratories, India), 10 pmol of each primer, and 4.0 μL of the sample DNA. The amplification profile consisted of three steps: initial denaturation at 95 °C for three minutes, followed by 35 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds, and extension at 72 °C for one minute, and a final extension at 72 °C for seven minutes.

Gel electrophoresis and PCR product amplification: The PCR products were electrophoresed in 1.2% agarose gel containing Sybr Safe Gel Stain (Invitrogen, USA) and visualised on a BioRad Gel Doc XR+ Imager (Bio-Rad, Hercules, USA). A QIAquick PCR Purification Kit (Qiagen, USA) was used to purify the PCR product for Sanger sequencing.

Sanger sequencing: A commercial facility (Eurofins Genomics India Pvt. Ltd.) sequenced the purified PCR product, both in the forward and reverse directions, using the same primers that generated the PCR products using an ABI 3730xl automated sequencer (Applied Biosystems, USA).

Phylogenetic analysis: The forward and reverse chromatograms were quality trimmed with SnapGene version v5.3 (http://www.snapgene.com) and assembled using the CAP3 Sequence Assembly Program (Huang and Madan 1999) to generate the consensus sequences. To confirm the molecular identity of our worms, we used NCBI's Basic Local Alignment Search Tool (BLASTN, https://blast.ncbi.nlm.nih.gov/Blast.cgi) to compare consensus sequences with related sequences available in the GenBank database. For the phylogenetic analysis, we aligned the newly generated consensus sequence of the 28S rRNA gene with the sequences of other *Dactylogyrus* species retrieved from the GenBank, NCBI (Supplementary material Table S1) using ClustalW in MEGAX, with all default parameters applied (Stecher et al. 2020). We used the maximum likelihood method, the optimal evolutionary model (GTR+G), and 1000 bootstrap iterations were used to determine phylogenetic relationships (Stecher et al. 2020). *Tetraonchus monenteron* (Wagener, 1857) Diesing, 1858 (GenBank: AJ969953.1) served as the outgroup to root the tree following Roohi et al. (2019).

Results

Taxonomic summary

Platyhelminthes: Monogenoidea: Dactylogyridae

***Dactylogyrus minutus* Kulwiec, 1927**

Host, localities, and collection date: *Cyprinus carpio* Linnaeus, 1758 (Cypriniformes, Cyprinidae); Aquarium shops in Kolkata (22.5726°N; 88.3639°E), Lucknow (26.8467°N; 80.9462°E), and New Delhi (28.6139°N; 77.2090°E), India; January 2020–March 2021.

Site of infection: gills.

Type host and locality: *Cyprinus carpio* Linnaeus, 1758 (Cypriniformes, Cyprinidae); Poland (Kulwiec 1927).

Infection parameters: Prevalence: 100% (15 out of 15 *C. carpio* examined); Intensity: 20–42 (mean = 25.86 parasites /infected fish).

Voucher specimens deposited: (1661786-89) (four specimens stained with Horen's and Gomori's trichrome and mounted on glass slides in DPX) deposited at the Smithsonian Institution.

DNA sequences deposited: 18S rRNA–ITS 1 region, OK042091; 28S rRNA, OK037582.

Morphological data (based on 23 specimens)

Comparative morphometry of hard parts of our specimens' haptor and reproductive organs to those of the original description and subsequent redescriptions/records identified the specimen as *D. minutus* (Figures 3–4; Table S2). The morphology of the dorsal bar and copulatory complex was enhanced in images obtained using phase-contrast. The dorsal bar demonstrated a horizontal fenestration, which had not been observed in previous studies. The copulatory tube is a long, thin tube that tapers anteriorly. There are two accessory pieces. One of these pieces is about the same size as the copulatory tube itself and has a faucet-shaped side branch that extends from its centre; the other (shorter) piece originates from the opposite side of the tube and is closely applied to its base. The copulatory complex exhibited a wide range of morphometric variations of increasing complexity and size related to the worm's maturity. Specifically, the larger and stouter the copulatory complex (especially the side branch), the more mature the individual was (Figure 5). The vagina was a lightly sclerotised sac, dextral, surrounded by a sclerotised eye-shaped ring that was visible only in mature worms (Figure 4). However, the sclerotised ring described by Paperna (1959) and Allison and Rogers (1970) at the distal end of the uterus was not found.

Molecular phylogenetic data

The purification and sequencing of PCR products yielded 940-bp- and 547-bp-long sequences for the 18S rRNA–ITS region and 28S rRNA genes,

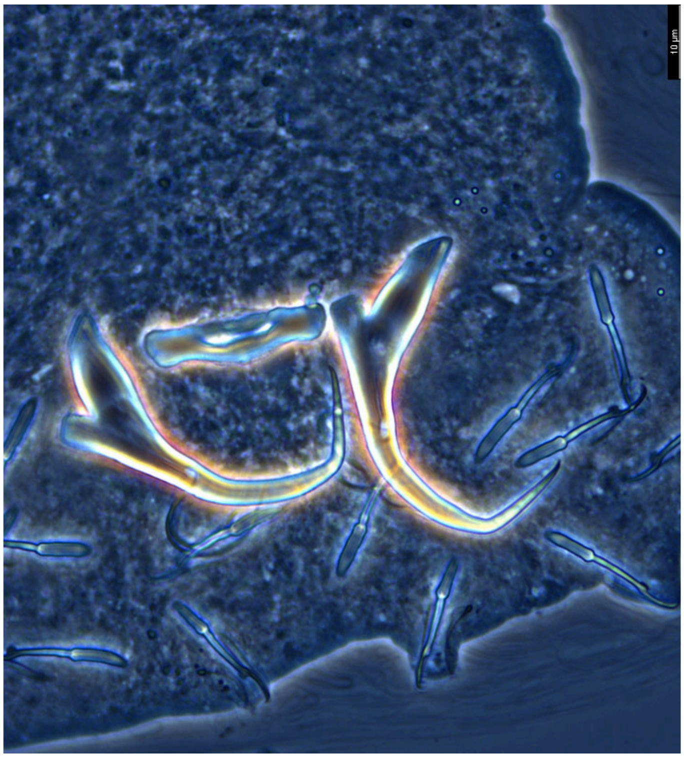

Figure 3. *Dactylogyrus minutus* Kulwiec, 1927. Phase contrast image of haptoral hard parts (anchor-bar complex and hooks). Photograph by Amit Tripathi.

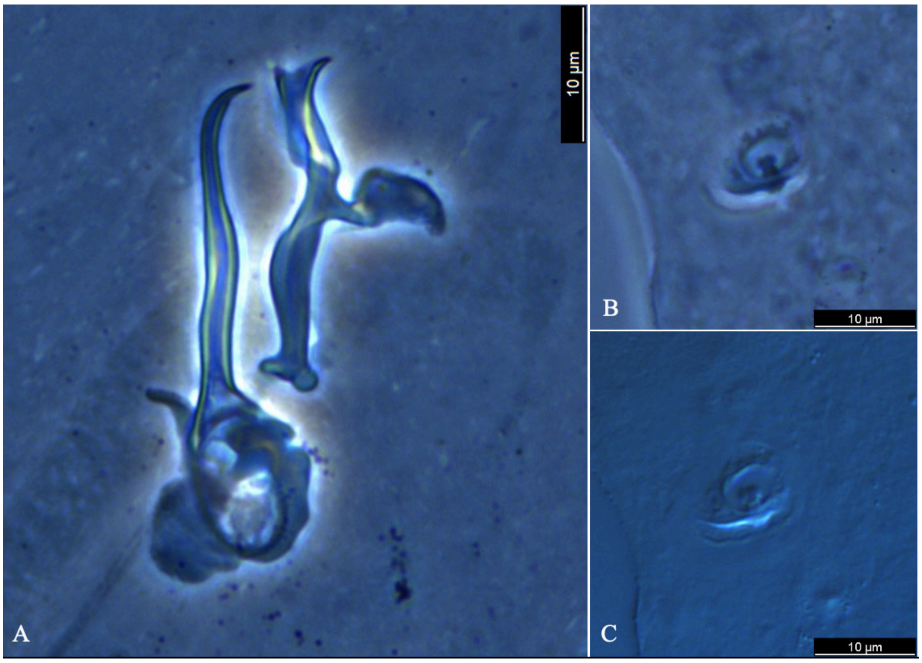

Figure 4. *Dactylogyrus minutus* Kulwiec, 1927. Photomontage of phase contrast and DIC images of hard parts of reproductive organs. A. Copulatory complex. B and C. Vagina. Photograph by Amit Tripathi.

respectively. For the 28S rRNA sequence, the BLASTN search returned a hit identical to *Dactylogyrus* sp. B13 MG-2018 from *Carassius auratus* in China (MK335464.1, query cover = 100 %, identity = 100%). The second-highest BLAST score was retrieved for *D. minutus* from *C. carpio* in Iran (MF926269.1, query cover = 100%, identity = 98.35 %). The BLASTN search

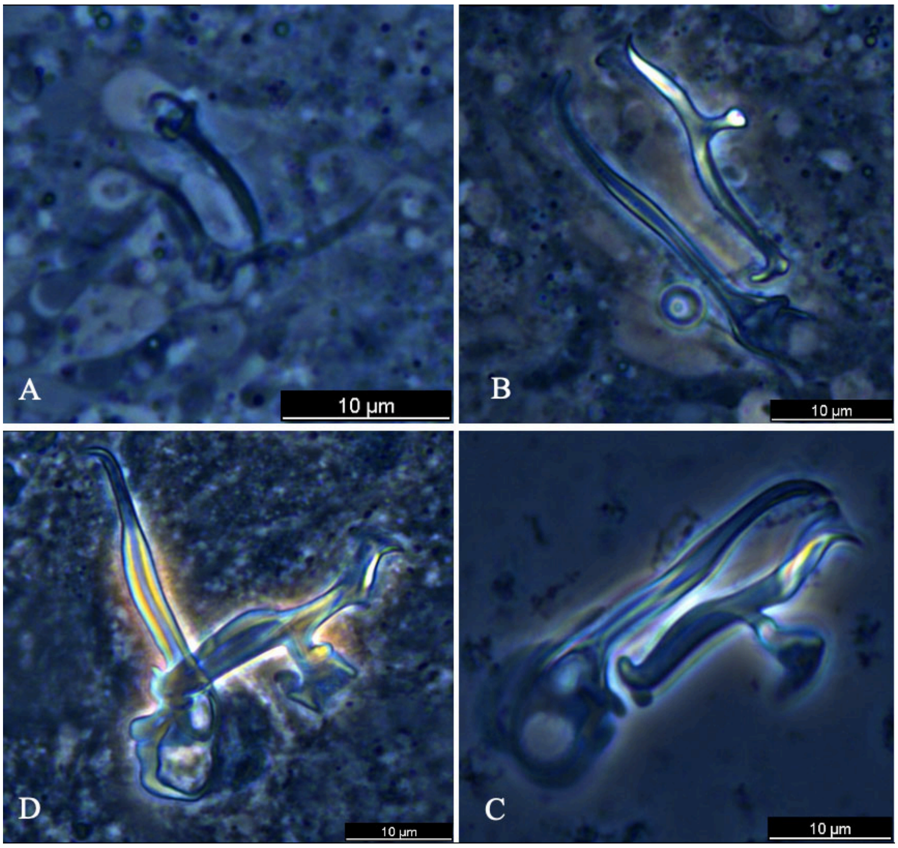

Figure 5. *Dactylogyrus minutus* Kulwiec, 1927. A–D. Morphometric variations in copulatory complex vis-vis worm's maturity. Photograph by Amit Tripathi.

returned no identical hits for the 18S rRNA and ITS region; the closest hits for this sequence were for *Dactylogyrus* sp. B13 MG-2019 from *C. auratus* in China (MK440299.1, query cover = 100%, identity = 99.60%) and *D. minutus* from *C. carpio* in Iran (MG821489.1, query cover = 100% coverage, identity = 96.69%), which represented the two highest BLAST scores. Thus, according to the NCBI BLAST search results, the two taxa closest to *D. minutus* from India were unidentified *Dactylogyrus* sp. B13 MG-2018 and 2019 from *C. auratus* in China and *D. minutus* from *C. carpio* in Iran.

To understand the phylogenetic relations of *D. minutus* from India with unidentified *Dactylogyrus* sp. B13 MG-2018 and 2019 from *C. auratus* in China and *D. minutus* from *C. carpio* in Iran, we constructed a phylogenetic tree in which we aligned the 28S rRNA gene sequences of *D. minutus from India* with those of 19 other *Dactylogyrus* species from three families—namely, Cyprinidae (10 species), Leuciscidae (six species), and Xenocyprididae (three species)—with *Tetraonchus monenteron* serving as an outgroup (Table S1). The final dataset included 21 nucleotide sequences and a total of 867 positions. These sequences clustered into two phylogenetically distinct and host-family specific clades (Figure 6). Clade 1 comprised 10 *Dactylogyrus* species, all of which colonised exclusively cyprinid hosts (*Cyprinus carpio* and *Carassius auratus*), whereas Clade 2 consisted of nine *Dactylogyrus* species that were parasitic either on xenocyprid (3 species) or leuciscid (6 species) hosts.

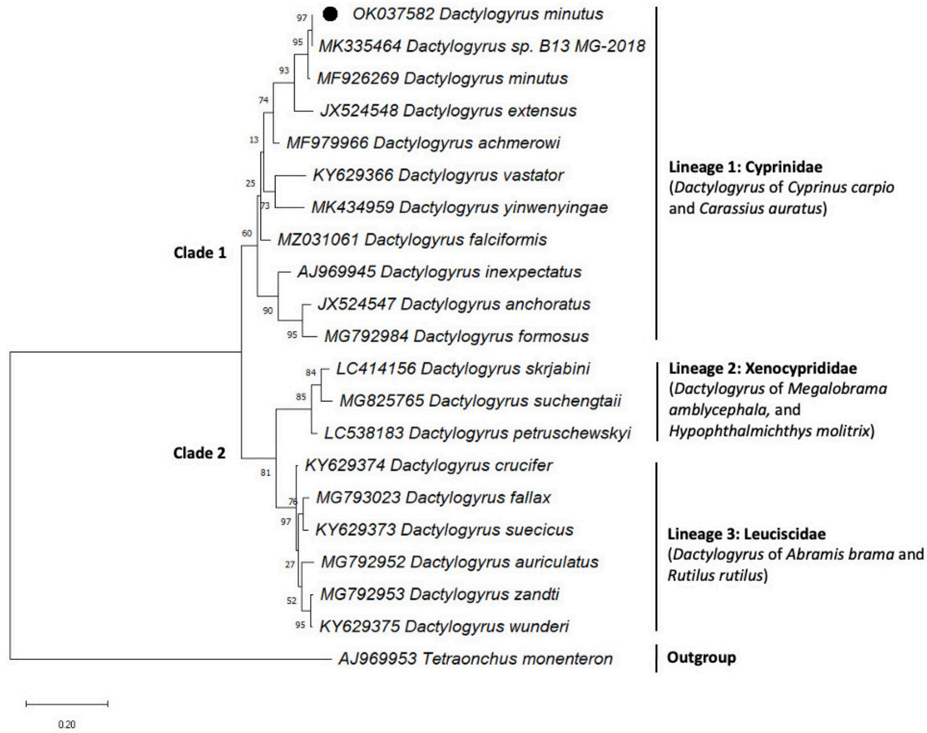

Figure 6. Maximum Likelihood tree based on GTR+G (General Time Reversible) model inferred from analysis of partial 28S rRNA gene sequences of 19 *Dactylogyrus* species from three carp families (Leuciscidae, Xenocyprididae, and Cyprinidae) with *Tetraonchus monenteron* taken as outgroup. Numbers along branches indicate bootstrap percentages resulting from the analyses. The taxon denoted by a black dot corresponds to the sequence generated in this study.

Discussion

We confirmed the identity of our parasite as *D. minutus* based on comparative morphology and measurements of the hard parts of the haptor and reproductive organs. Paperna (1959) and Allison and Rogers (1970) described a sclerotised ring at the distal end of the uterus. However, most other studies—most notably, those of Kulwiec (1927) and Ogawa and Egusa (1977)—do not describe such a ring. The ring was not visible in our specimen, either. Without calling Paperna's (1959) and Allison and Rogers's (1970) observations into question, there could be two forms of *D. minutus*: one with a sclerotised ring and another without this ring. The presence or absence of a sclerotised ring might also be related to the maturity of worms, which also resulted in morphometric variations in the copulatory complex and the visibility of the vagina. This assumption is based on the well-established fact that the hard parts of monogenoids develop continuously throughout their lives, and thus, their length can be used as a reliable predictor of age/maturity (e.g., Ogawa 1984; Kearn 1990; Whittington and Ernst 2002). Regardless of size differences within the copulatory complex, the overall morphology is fairly consistent, making it a highly useful characteristic for reliably distinguishing *D. minutus* from the nominal *Dactylogyrus* species.

Dactylogyrus minutus from India shared a high nucleotide similarity with unidentified *Dactylogyrus* sp. B13 MG-2018 (for 28S rRNA genes) and B13 MG-2019 (for 18S rRNA genes + ITS region) from *C. auratus* in China. Since the goldfish is also a known host for *D. minutus* (Table S3), the *Dactylogyrus* sp. B13 MG-2018 and 2019 sequences likely represent *D. minutus.* We sent an email to the submitters of *Dactylogyrus* sp. B13 MG-2019 to confirm their parasite species identity but, unfortunately, received no response. Surprisingly, Indian specimens of *D. minutus* displayed nucleotide variances ranging from 1.65% to 3.31% from *D. minutus* from *C. carpio* in Iran. Although no direct evidence is available, it is possible that a cryptic *(*morphologically similar, but genetically distinct*)* lineage of *D. minutus* might exists. This notion is corroborated by the presence or absence of a sclerotised ring at the distal end of the uterus in some regions of the world. However, the validation of this possibility requires additional research combining genetic and morphological characterisation of *D. minutus* specimens obtained from different parts of the world, including Iran in particular.

According to Tan and Armbruster (2018), the suborder Cyprinoidei is divided into five primary groups: Cyprinidae, Leuciscidae, Danionidae, Gobionidae, and Xenocyprididae. The 28S rRNA gene sequences of *D. minutus* from India were aligned with published sequences of *Dactylogyrus* species from the Cyprinidae, Leuciscidae, and Xenocyprididae found in the NCBI nucleotide database to construct a phylogenetic tree. Two lineages of *Dactylogyrus* species were recognised in which parasitic distributions matched with strong support values with those of their fish host families, implying their discrete coevolutionary origins (Figure 6). Clade 1 was unique in that it included 10 *Dactylogyrus* species from the family Cyprinidae (common carp *Cyprinus carpio* and goldfish *Carassius auratus*). This result confirms the monophyly of *Dactylogyrus* spp. of cyprinid hosts as suggested by Šimková et al. (2004), both using the 18S rRNA gene alone and combining 18S rRNA and ITS1, and Šimková et al. (2006) using the 28S rRNA gene. This monophyly can be explained by two competing hypotheses. First, it could be that the 10 *Dactylogyrus* species evolved on the common carp and then radiated to phylogenetically related goldfish (or vice versa) by descent. Second, because of their nested environmental niches (Froese and Pauly 2021), common carp and goldfish may have clustered their *Dactylogyrus* species (convergent evolution). However, a convergent trend does not appear to support their monophyly, as all 10 *Dactylogyrus* species share ancestral morphological traits (synapomorphies) typical of the genus *Dactylogyrus*, with no morphologically derived characters that are unique (apomorphies). Apparently, the host phylogeny has a stronger influence than the host geographic range on the distribution of congeneric monogenoids on their hosts. Because closest evolutionary related hosts share their genetic makeup and, thus, their immune responses, it is natural for them to be susceptible to the same pathogens. A similar correlation between host species' relatedness and parasite similarities has been demonstrated in primates and humans (Davies and Pedersen 2008). Concerning *D. minutus* from India, the phylogenetic analysis confirmed the BLAST results by forming a well-supported sister taxon to *Dactylogyrus* sp. B13 MG-2018 from China, where *D. minutus* from Iran was recovered as a basal clade.

The common carp, native to Eastern Europe and Central Asia, is one of the most widely distributed freshwater fishes in the world. It has been introduced in over 135 countries (see Froese and Pauly 2021) for farming, aquaculture, sport fishing, and ornamental trade (Copp et al. 2005; Balon 2006). While the human-assisted movement of this species was primarily intended to increase fish production, it has frequently had negative impacts, such as deteriorated aquatic habitats, disrupted ecosystems, the gradual disappearance of native fishes, and the development of unfit water for swimming or livestock drinking (see Global Invasive Species Database 2021 and the references mentioned therein). Moreover, the species is host to at least 310 parasite species (Barus et al. 2002), which is probably more than any other fish. These parasites include viral, fungal, bacterial, protozoan, helminth, and arthropod infections (Hoffmann 1999; Froese and Pauly 2021). Unsurprisingly, the species has been added to the International Union for Conservation of Nature's (IUCN's) database of the world's 100 worst invasive alien species (Global Invasive Species Database 2021).

According to Molnár (2009), 12 *Dactylogyrus* species have been described from common carp: seven in the Far East (i.e., China, Mongolia, and the Amur Basin of Russia) (*D. achmerowi, D. falciformis, D. lopuchinae, D. molnari, D. mrazeki, D. sahuensis,* and *D. yinwenyingae*); four in Europe (*D. anchoratus, D. crassus, D. minutus* and *D. vastator*), and one in North-America (*D. extensus*). *Dactylogyrus minutus* has been linked to high mortality rates in carp fry (Buchmann et al. 1993; Stojanovski et al. 2008). Indeed, all *Dactylogyrus* species are potentially pathogenic, especially to fingerlings, thus causing high mortality rates, and representing a liability for producers (Buchmann et al. 1993; Bretzinger et al. 1999; Kritsky and Heckmann 2002).

The common carp was introduced in India in 1939 from Sri Lanka and Thailand (Froese and Pauly 2021) for aquaculture purposes (Dey et al. 2005, as cited in Singh et al. 2010). Attempts were made to cultivate the carp as a compatible species in composite fish cultures with three prevalent Indian major carps (*Catla catla, Labeo rohita,* and *Cirrhinus mrigala*) and two Chinese carps (*Ctenopharyngodon idella* and *Hypophthalmichthys molitrix*) (Jena et al. 2002). However, the introduced fish escaped from confinement and established self-sustaining populations in several Indian river systems, including the Jhelum, Mahanadi, Yamuna (Singh and Lakra 2006; Singh et al. 2008), and the Ganges (Singh et al. 2010).

To the best of our knowledge, only one study from India has investigated the parasites of common carp. Swaminathan et al. (2016) investigated a disease outbreak in common carp at a fish farm in Kerala, finding a "minor infestation of *Dactylogyrus* sp., opportunistic bacteria, and carp oedema virus" from the gills of infected common carps. Because the negative ecological effects of its introduction have been relatively inconsequential so far, the common carp population in India is currently viewed as a viable and profitable species rather than an exotic nuisance. However, since no systematic studies have been conducted on the topic, it is too early to assess the complete ecological impact of common carp and associated parasite fauna in Indian rivers. Aside from being a well-known threatening pest, the common carp has introduced several parasitic diseases that could spread to other fishes. *Dactylogyrus minutus*, for example, has been reported on the bleak *Alburnus alburnus* (Linnaeus, 1758) in the Porsuk River in Turkey (Koyun 2011), indicating its potential to infect additional fish species.

The Indian government requires that exotic ornamental fish undergo several import procedures, including pre-quarantine, quarantine, and post-quarantine, and meet various requirements (National Fisheries Development Board 2021). The presence of *D. minutus* in post-quarantine Platinum Ogon populations despite these regulations, should be viewed as a warning of the parasitic threats that invasive carp pose to Indian fisheries. However, the presence of monogenoids, including *D. minutus*, in wild common carp in India has yet to be demonstrated. Thus, we are currently investigating this matter in our laboratory. As the invasive common carp expands its habitat range in India, the risk that it will negatively impacts on the native fishery industry will increase. We expect that quarantine officials and fishery managers will use the information contained in this report to develop preventive measures that will limit the spread and impact of parasitic fauna associated with common carp in India and elsewhere. We also expect that the molecular data generated here will aid assessments of the sequence variation within established populations of *D. minutus* worldwide.

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Authors' contribution

AT: research conceptualisation; experiment design; analysis of data; writing final draft. CM: collection of fish and parasites; generation of morpho-molecular data; preparation of figures and tables. NA: contributing reagents and/or materials, writing initial draft.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Details of monogenoid species and GenBank accession numbers of their (28S ribosomal RNA gene) sequences used in phylogenetic analyses.

Table S2. Comparative measurements (in µm) of reproductive organs and haptoral armaments of *Dactylogyrus minutus* Kulwiec, 1927 from India (present study) and other geographical locations.

Table S3. *Dactylogyrus* species shared by *Cyprinus carpio* and *Carassius* spp. (*C. auratus* and *C. carassius*). (Because this table is not intended to be exhaustive, only a few pertinent references have been included).

Appendix 1. References for supplementary tables.

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