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Morphologic and Molecular Detection of *Dactylogyrus* Species in *Cyprinus Carpio* and *Ctenopharyngodon Idella* in Southwestern Iran

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ABSTRACT

Monogeneans gill parasites of the diversified *Dactylogyrus* genus are host-specific to freshwater fish of the Cyprinidae family. Accurate detection of various *Dactylogyrus* species is very time-consuming and requires highly experienced personnel; to overcome this problem, the genomic DNA technology has been recently used to detect *Dactylogyrus* spp. In this study, 23 *Cyprinus carpio* and 19 *Ctenopharyngodon idella* isolates were collected from different farms and local markets of Ahvaz, southwest of Iran. The gill arches of each fish were carefully examined under a light microscope. *Dactylogyrus* species was identified morphologically. Identical species were kept in ethanol for molecular study. Molecular analysis was performed using amplification of the ITS-1 region of the ribosomal RNA gene of the parasite. Then, the amplified PCR products were sequenced. The aligned nucleotide sequences were analyzed to construct the phylogenetic tree of the identified species. The results revealed two lineages including *D. extensus* isolated from common carp and *D. lamellatus* taken from grass carp fish. Phylogenetic analysis showed that the detected *D. extensus* and *D. lamellatus* isolates from Khuzestan region were clustered with some Iranian (Guilan), Chinese and Czech isolates. Importing fingerlings from Guilan Province could lead to introducing monogenean infections to native cyprinid fish.

Keywords: *Dactylogyrus*, Common Carp, Grass Carp, Fish, PCR.

İran'ın Güneybatısında Yer Alan *Cyprinus Carpio* ve *Ctenopharyngodon Idella*'daki *Dactylogyrus* Türlerinin Morfolojik ve Moleküler Tespiti

ÖZ

Çeşitlendirilmiş *Dactylogyrus* cinsinin solungaç monojenleri, Cyprinidae ailesinin tatlı su balıklarına ev sahipliğine özgüdür. Çeşitli *Dactylogyrus* türlerinin doğru tespiti çok zaman alıcı olduğundan ve çok deneyimli personel gerektirdiğinden, genomik DNA teknolojisi son zamanlarda *Dactylogyrus* spp. Bu çalışmada, Güneybatı İran'ın Ahvaz'ın farklı çiftliklerinden ve yerel pazarlarından 23 *Cyprinus carpio* ve 19 *Ctenopharyngodon idella* toplandı. Her balığın solungaç kemerleri, ışık mikroskobu altında dikkatle incelenir. *Dactylogyrus* türlerinin morfolojik olarak tanımlanması morfolojik olarak belirlenmiştir. benzer türler moleküler çalışma için etanol içinde tutuldu. Moleküler analiz, parazitin ribozomal RNA geninin ITS-1 bölgesinin amplifikasyonu kullanılarak yapıldı. Daha sonra amplifiye PCR ürünleri dizildi. Hizalanmış nükleotit sekansları, tanımlanan türlerin filogenetik ağacını yürütmek için analiz edilir. Sonuçlar, ortak sazandan izole edilen *D. extensus* ve çim sazan balıklarından alınan *D. lamellatus* da dahil olmak üzere iki soyun ortaya çıktığını göstermiştir. Filogenetik analiz, Khuzestan bölgesinden tespit edilen *D. extensus* ve *D. lamellatus* izolatlarının bazı İran (Guilan), Çin ve Çek Cumhuriyeti izolatlarıyla kümelandiğini gösterdi. Guilan Eyaletinden balıkların ithal edilmesi, yerli Cyprinid balıklarına monojenik enfeksiyonların ortaya çıkmasına neden olabilir.

Anahtar Kelimeler: Sazan, Çim Sazan, Balık, PCR.

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INTRODUCTION

Various species of cyprinid fish have been imported from the Far East (China), Russia and Eastern European (Romania and Hungary) countries into the fish culture industry of Iran over the past 60 years. The growth rate of this sort of freshwater fish in aquaculture has been on the rise due to the increasing interest of Iranian consumers and the economic and nutritional values of this type of fish (Zolfinejad et al. 2017, Shamsi et al. 2009). When transferring fish to farm ponds and natural habitats, different pathogenic parasites are introduced to native species.

The cyprinid fish naturally harbor various species of monogenean gill parasites. *Dactylogyrus* Diesing 1850 (Monogenea), is the most common gill parasitic worm of freshwater fish (Woo 2006). This ectoparasite is known to be highly cyprinid fish-specific (Simkova et al. 2007). The host specificity of this species is very high such that each *Dactylogyrus* species attaches to a distinct niche of the gill arch. Sometimes, this specificity of the host and parasite species is such that they approach phylogenetically close to each other (Simkova et al. 2007, Kadlec et al. 2003). According to the studies by Shamsi et al. (2009) and Daghigh Roohi et al. (2019), each grass carp can carry at least two species of *Dactylogyrus* on its body surface. Severe parasitism of the gill filaments leads to epithelial hyperplasia followed by serious respiratory failure and negative effects on fish growth. It can also cause a high rate of mortality in small fish (Lu et al. 2012, Tu et al. 2015, Thoney and Hargis 1991). The infected fish are more susceptible to secondary infestation, which increases the intensity of pathogenicity of the *Dactylogyrus* species, and eventually, the economic loss (Woo et al. 2002, Tu et al. 2015). *Dactylogyrus* direct life cycle accelerates the rate of parasitic infection distribution, and it can cause disease transmission to reach its epizootic level (Thoney and Hargis 1991). Therefore, early detection of the causative microorganism plays a significant role in controlling and preventing fish infections and economic losses. Also, the taxonomic study of parasites in a specific geographical region helps to find common lineal species, and it informs fisheries organizations of the provinces or countries of where this parasitic infection originates from.

Morphological identification of *Dactylogyrus* species directly depends on the accurate observation of copulatory components with different shapes and sizes in various species. Since *Dactylogyrus* is a very small (usually less than 1 mm) parasite, it is difficult to differentiate various species, especially when flatted marginal hooks occur in viscous gill media during the mounting process (Sharma et al. 2011). Also, the exact identification of closely resembling species of *Dactylogyrus* based on morphological characteristics is extremely time-consuming and requires highly

experienced personnel. The genomic DNA technology has recently been used for the accurate detection of various microorganism species such as *Dactylogyrus*.

Previous molecular studies have shown that the ribosomal DNA (rDNA) of Platyhelminthes is a highly conserved region and can be used as a suitable marker for the identification and evolution study of different species (Olson et al. 2003). Most molecular studies have been carried out on the 28S rDNA region in cyprinid fish in Iran (Ahmadi et al. 2017, Mozhdeganlou et al. 2011, Daghigh Roohi et al. 2019), while there are limited studies on the internal transcribed spacer 1 (ITS-1) region of rDNA. In the present study, we evaluated the genomic sequence of the internal transcribed spacer 1 (ITS-1) region to detect closely-related *Dactylogyrus* species in Khuzestan Province, Iran.

MATERIAL and METHODS

Parasite collection

A total of 42 freshwater cultured common and grass carp, including 23 *C. carpio* and 19 *C. idella*, were acquired as dead but fresh from different aquacultures and local markets of Ahvaz, southwest of Iran. After transferring the fish to the parasitology laboratory of the Faculty of Veterinary Medicine of Shahid Chamran University of Ahvaz, the gill arches of each fish were placed on a plate and examined under stereomicroscope. Then, the identified *Dactylogyrus* species were separated and collected by the routine methods. Fish sampling continued until 10 *Dactylogyrus*-infected fish from each species were collected (of the 42 fish, 20 infected fish were found and investigated).

Identification of each parasite was performed based on a survey of the structures of sclerotic attachment and reproductive organs according to Gusev (Gusev 1985). The parasites were observed on clear slides in glycerin using a light microscope equipped with digital image analysis (Carl Zeiss Axiovision LE 4.5). Some similar species were placed in 70% ethanol before DNA extraction.

Molecular studies

DNA extraction and PCR amplification

Each ethanolic specimen was washed in distilled water to remove alcohol from its tissues. The genomic DNA was extracted using the CinnaPureTM DNA kit (CinnaGen Co., Iran) according to the manufacturer's instructions. The ITS-1 region of ribosomal RNA genes was amplified with forward (5'-CTGCGGAAGGATCATTTATC-3') and reverse (5'-GATCCACCACTTGCAGTTGT-3') primers (Mozhdeganlou et al. 2011). Polymerase chain reaction (PCR) was carried out in a thermocycler (A&E, England) for 38 cycles as follows: 5 min at

94°C (initial denaturation) followed by 45 s at 94°C for further denaturation, annealing for 45 s at 56°C, 45 s at 72°C and a final extension of 72°C for 10 min. The PCR products were investigated on 1.5% agarose-Tris-acetate-EDTA (TAE) gel, visualized with safe stain under a UV illuminator. Distilled water was used instead of genomic DNA of PCR as negative control.

DNA sequencing

For morphologic confirmation, the amplified PCR products were purified and sequenced at Bioneer-Korea Co. using both sets of primers. After reading the sequences, the data were analyzed using BioEdit V. 7.0.5.3 software and the Basic Local Alignment Search Tool (BLAST) programs and databases of the NCBI (National Center for Biotechnology Information, BLAST: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The alignment sequences were analyzed by the Molecular Evolutionary Genetics Analysis (MEGA) software, version 10, using default parameters to construct the phylogenetic tree of the isolated species.

Phylogenetic analysis

The evolutionary history was inferred by using the maximum likelihood estimation method and Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-2522.07) has been evaluated. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The initial tree(s) for the heuristic search was obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then the topology with superior log likelihood value was selected. A discrete gamma distribution was used to model evolutionary rate differences among sites (5 categories [+G, parameter = 1.6401]). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 0.00% sites). The tree is drawn to scale, with branch lengths

measured in the number of substitutions per site. This analysis involved 23 nucleotide sequences. There were 227 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al. 2018).

RESULTS

Twenty *Dactylogyrus* parasites were isolated from 42 collected fish (19 grass carp and 23 common carp). Microscopic identification of the species revealed that two species of *Dactylogyrus* including *D. extensus* (Figure 1) and *D. lamellatus* (Figure 2) were isolated from the gills of common and grass carp, respectively. The PCR products with the bond lengths of 515 and 618 base pairs were visible on the gel-electrophoresis in *C. carpio* and *C. idella* samples, respectively (Figure 3).

The nucleotide sequence of the PCR products was aligned and compared with other registered *Dactylogyrus* species in the GenBank. According to the phylogenetic analysis, the detected isolates belonged to two distinct clusters. The Khuzestan isolate of *D. extensus* was 95% homologous with the Chinese (KM277459.1) and Czech (AJ564129) isolates of this species. The Khuzestan isolate of *D. lamellatus* was also 99% homologous with the Chinese (KX369221 and KX369218), Czech (AJ564141.1) and Guilan, Iran (MG907490.1) isolates of this species in the GenBank. The results of phylogenetic analysis on the ITS-1 region of rDNA showed that *D. extensus* (Khuzestan isolate) was grouped together with the same species isolates collected from China and Czech Republic, along with the closely related sister species, *D. minutus* isolate from Guilan, north of Iran. Also, *D. lamellatus* isolated from Khuzestan Province was clustered with the Iranian isolate (Guilan Province, north of Iran) and the same species isolates collected from China and Czech Republic with high bootstrap (99%) (Figure 4).

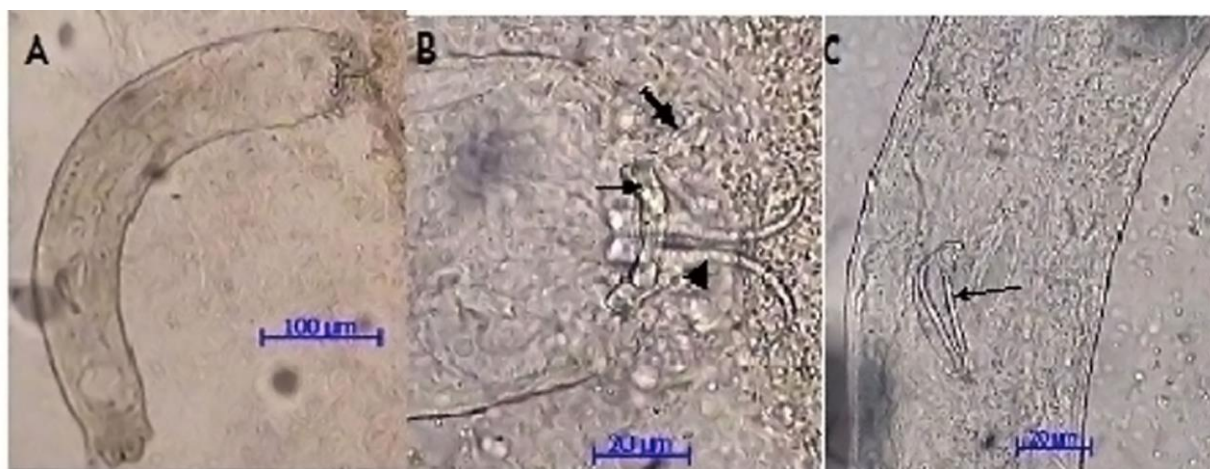


Figure 1. Photomicrograph of *Dactylogyrus extensus*: A: Adult whole mount worm, B: Haptor of *D. extensus*; Anchors (arrow head), Dorsal bar (narrow arrow), marginal hook (thick arrow), C: male copulatory organ (arrow).



Figure 2. Photomicrograph of *Dactylogyrus lamellatus*: A: Adult whole mount worm, B: Haptor of *D. lamellatus*; Anchors (arrow head), Dorsal bar (narrow arrow), marginal hook (thick arrow), C: male copulatory organ (arrow).

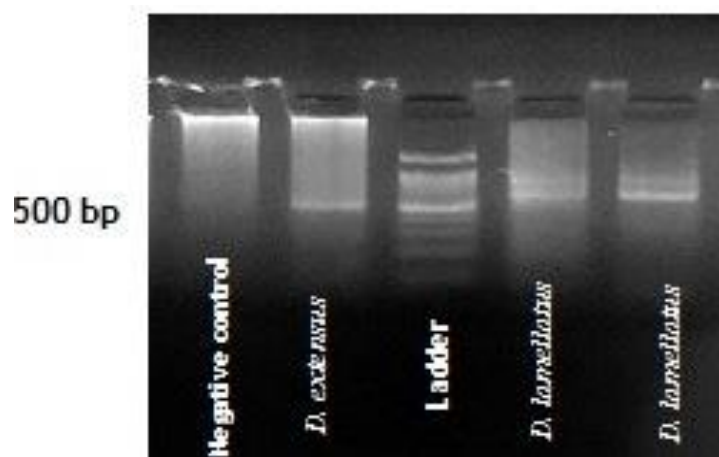


Figure 3. Agarose gel electrophoresis of PCR amplification products from *D. extensus* and *D. lamellatus* under UV light.

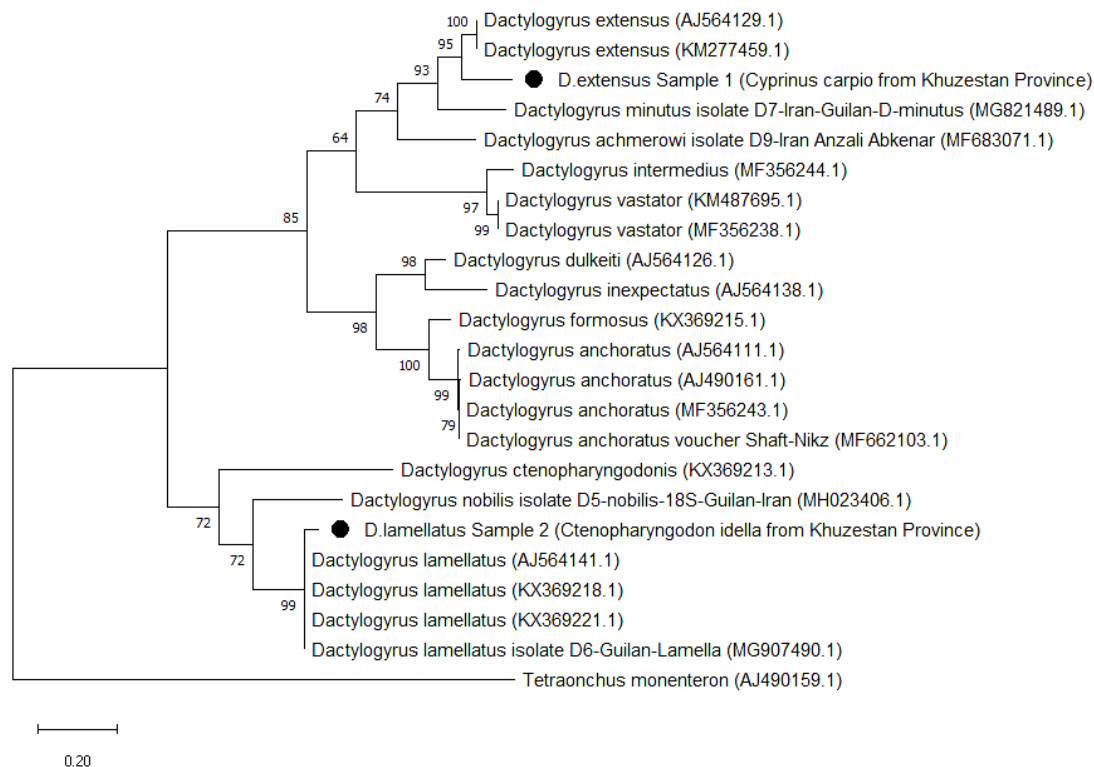


Figure 4. Phylogenetic tree based on the partial sequence of 18S, ITS-1 region and 5.8S rDNA, constructed according to the maximum likelihood method. Black dots represent the Iranian *Dactylogyrus* isolates (Khuzestan province). Numerals above the branches indicate bootstrap values (%) from 1000 replicates. The scale bar indicates the proportion of sites changing along each branch.

DISCUSSION

The routine identification of *Dactylogyrus* species was performed based on morphological characteristics during the process of appropriate preservation, fixation and staining of genital organs of the parasite (Strona et al. 2009). For this purpose, highly experienced personnel and powerful microscopic instruments are often required due to the minute size of parasites. Thus, the identification of *Dactylogyrus* species is difficult and time-consuming. Today, molecular techniques using genomic DNA of parasites are used as a more accurate and reliable method, which has significantly aided with the detection of these parasites. In the freshwater fish of Iran, more than 70 species of *Dactylogyrus* have been identified (Mozhdeganlou et al. 2011), which are mainly specific to cyprinid fish. These parasites have been introduced to the Iranian aquaculture and farming systems during the import of the cyprinid fish from the Far East (China), Russia and Eastern Europe (Romania and Hungary) over the past 40 years (Shamsi et al. 2009).

Shamsi et al. (2009) studied the geographical distribution of *Dactylogyrus* species in cyprinid fish of different fauna of Iran. They could isolate the species

of *D. anchoratus*, *D. extensus* and *D. vastator* from *Barbus sharpeyi*, *Carassius auratus gibelio* and *C. carpio*, respectively, in Khuzestan region (Mesopotamian fauna). Therefore, the present study is the first report of the presence of *D. extensus* in common carp and *D. lamellatus* in grass carp fish of Khuzestan Province, Southwestern Iran. Borji et al. (2012) detected the species of *D. extensus* and *D. anchoratus* in *C. carpio* (Borji et al. 2012) under a light microscope from the ponds around Mashhad, Northeastern Iran. Also, in another study in Northeastern Iran, the species of *D. anchoratus* from *C. carpio* and *C. idella*, *D. extensus* from *C. carpio* and *D. lamellatus* from *C. idella* were detected using molecular analysis (Ahmadi et al. 2017). The identified species in Northeastern Iran were similar to Khuzestan species, but since the molecular study was performed on the 28S rDNA region, it has not been considered for comparison with Khuzestan isolates in the GenBank. In the north of Iran (Guilan Province), the *D. lamellatus* species was reported in 95% of the farmed grass carp using molecular methods (Daghighi Roohi et al. 2019). The molecular study of *Dactylogyrus* species in Guilan Province was the only study from Iran on the ITS-1 region of rDNA. As the freshwater fish of farming systems and local markets in Khuzestan Province are provided by internal and external imports from Guilan Province and other

countries, monogenean infections are also introduced to native cyprinid fish of the southwest of Iran.

Molnar found that the evolution and maturation time of the *D. lamellatus* parasites increase with rising water temperature (Molnar 1971). Khuzestan Province is located in southwest of Iran at 29°57'-33°0'N and 47°38'-50°32'E and this area has a hot desert climate. In the present study, the maximum and minimum temperatures of the Province during the fish sampling period (December 2017 to March 2018) varied from 13 to 31°C on different days of months. Therefore, to provide an appropriate habitat for *D. lamellatus* in the intensive farming system, the proliferation of the parasite and the rate of fish involvement should increase. Among *Dactylogyrus* species, *D. extensus* has the lowest host specificity and broadest host range. Thus, the parasite can be easily transmitted from other species of fish to common carp (Gibson et al. 1996). Also, *D. extensus* has a high tolerance to a wide range of temperatures (from 1 to 30°C) and salinity (up to 1.3%) of water throughout the year (Jalali 1998, Mahdipour et al. 2004, Jalali and Barzegar 2005). Therefore, the incidence of *D. extensus* infection in these favorable environmental conditions for the parasite habitat is acceptable.

The phylogenetic analysis showed that the *D. extensus* and *D. lamellatus* isolates detected from Khuzestan region had a close affinity with some Iranian (Guilan), Chinese and Czech isolates. Since Khuzestan fish farmers usually provide the need of their cyprinid fish from East (China), Russia and Eastern Europe (i.e., Romania and Hungary) as fry or fingerlings, the *Dactylogyrus* species can be easily introduced to Iranian water resources and native fish of southwest of Iran. The biodiversity of the detected *Dactylogyrus* species might be caused by the host specific features and high diversity of these monogenean parasites in various geographical and environmental conditions.

CONCLUSIONS

This was the first study to demonstrate the occurrence of *D. extensus* and *D. lamellatus* in common and grass carp from Khuzestan Province, Southwestern Iran. Also, we found that PCR is a reliable and feasible diagnostic tool for the detection of *Dactylogyrus* species, and it can be applied for large-scale assessments in screening programs of customs and fisheries organization to ensure the health of imported aquatic species. Further molecular investigations are needed for the diagnosis and prevention of *Dactylogyrus* infections in other freshwater fish.

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

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