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Morphometric and molecular characterization of *Dactylogyrus lamellatus* isolated from farmed Grass carp, *Ctenopharyngodon idella* (Valenciennes, 1844), in Guilan Province, Iran

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Abstract

Grass carp is an herbivorous species, which is actually popular in Iran. Monogenean parasites, particularly members of the family Dactylogyridae, have been one of the main causes of mortalities in Iranian fish farms and therefore they have been subject of many studies in Iran. The main aim of the present study was to describe morphological and molecular characteristics of monogenean parasites recovered from the farmed grass carp (*Ctenopharyngodon idella*) in the Guilan Province during 2015 to 2016. A total of 80 grass carps have been examined for infestation with monogenean parasites. 10923 *Dactylogyrus* species were recovered from 95% of the farmed grass carp. The parasite was first classified based on their morphological characteristics and identified as *Dactylogyrus lamellatus*. The parasite was then subjected to PCR and sequencing of the 28S rDNA gene. The phylogenetic tree showed that *D. lamellatus* genetically was similar to those previously reported from other countries and Mashhad farms in Iran.

Keywords: Grass carp, *Dactylogyrus lamellatus*, 28S rDNA, Guilan, Iran.

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Introduction

Grass carp is a large member of the carp and minnow family (Cyprinidae) and is native to south eastern Russia and north western China. This herbivorous species has been deliberately introduced into many countries for vegetation control purposes (Cudmore and Mandrak, 2004) and also for aquaculture due to rapid growth rate (Zolfinejad *et al*., 2017). In Iran, this species was intentionally released for vegetation control in Anzali lagoon and introduced to the Guilan province for polyculture fish farming about 40 years ago. Today, this fish is artificially propagated in fish hatcheries of Guilan province and was sent to the whole of the country for cultivation.

Class Monogenea is a group of parasitic worms commonly found in fishes. These parasites feed of mucus and epithelial cells of the skin and gills and sometimes of the blood (Chaudhary *et al*., 2013). Infestation with monogenean parasites may lead to serious hyperplasia of the gill filaments epithelium, impairs respiratory function, negatively affects growth, and even associates with high mortalities, especially in small carps (Lu *et al*., 2012; Jiang *et al*., 2013; Tu *et al*., 2015). Dactylogyrus represents the most dominant genus among the Monogenea. These are common ectoparasites usually attached to the gills of freshwater fish of the family Cyprinidae (Gibson *et al*., 1996; Woo 2006). The fish heavily infected with *Dactylogyrus* spp. is susceptible to secondary infestations that can play a significant role in the pathogenicity of these parasites, which result in considerable economic losses (Dove and Ernst, 1998; Woo *et al*., 2002; Reed *et al*., 2009; Tu *et al*., 2015).

Thus far, grass carp is known to be parasitized by at least two species of *Dactylogyrus* (Shamsi *et al*., 2009; Daghigh Roohi *et al*., 2016) in both pond and natural lake habitats in Iran. Identification of the Monogenea in Iran has often been based on a morphological basis. Monogenean identification solely based on morphological features can be problematic and ambiguous for closely related species. For effective diagnosis, treatment and control of parasitic diseases, the accurate identification of parasites is necessary (Mcmanus and Bowles, 1996). Recently, molecular markers have provided a useful alternative to the identification and validation of monogenean species (Cunningham *et al*., 1995; Tu *et al*., 2015). Our knowledge on genetic character of Iranian monogenea is poor and little is known about molecular aspects of these parasites (Mozhdeganlou *et al*., 2011; Omidzahir *et al*., 2012; Mousavi *et al*., 2013; Omidzahir *et al*., 2015). In the present study, Dactylogyrids that recovered from cultured grass carp in the Caspian Sea region are described morphologically, then their genetic characterization was done based on sequences of 28SrDNA gene.

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Materials and methods

Fish collection

This work was carried out from April 2015 to November 2016 in Guilan Province, North of Iran (Fig. 1). A total of 80 grass carp (*Ctenopharyngodon idella*) were collected from 10 fish farms. Fish were one summer old with the average total length of 11.38±2.84 cm and an average weight of 15.89±12.69 g. The fish were placed in plastic tanks with water obtained from the collection sites and transferred to the Parasitology Laboratory of National Inland Water Aquaculture Research Center in Anzali where they were randomly distributed into several 100-L aquariums containing water from the fish farm and investigated within 72 hours. Fish were examined for the presence of Dactylogyrids parasites under a dissection microscope (4 to 40 magnifications). Live parasites were picked up from the gills scraping by using small needles. Then mounted in glycerine-jelly for morphological investigations (Gussev, 1983).



Figure 1: Distribution of studied ponds in Guilan Province, Iran.

Parasites collection and morphological identification

Monogenean parasites were recovered from gill filaments of *Ctenopharyngodon idella*. Parasite identification was performed based on the character of copulatory organ, the haptoral sclerites like anchors, bars and hooks. Photographs of mounted parasites were captured at magnifications of 20X and 40X using a Nikon Eclipse 50i compound microscope with Nikon Digital Sight DS-L1 image analysis software and Nikon Digital Sight DS-SM camera. For morphometric analysis, a total of 7 point to point measurements were made on *Dactylogyrus* specimen photographs, using Image J software. Drawings were made with the aid of a drawing tube Nikon Y-IDT Japan. Finally, drawing of specimens and their measurement data were compared by parasites identification keys (Bykhovskaya-Pavlovskaya *et al*., 1962; Gussev, 1985). After species identification, the same parasite specimen was stored in %75 ethanol tube for molecular analysis.

Molecular Studies

DNA extraction and PCR

The genomic DNA was extracted from one specimen using the YTA Genomic DNA Extraction Mini Kit (Yekta Tajhiz Azma, Iran) according to the manufacturer's manual. The 28S rDNA region of specimens was amplified using primers (forward, 5'TCTAGTAACGGCGAGTGAACG-3') (Chiary *et al*., 2014) and the modified reverse primer (5'-

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GTGGGAAGGTCTACCTCAGC-3').
Each amplification reaction was performed in a final volume of 25µl containing 12.5 µl 2× Taq Mastermix, 1 µl of each primer (Macrogen, South Korea), and 2 µl of genomic template DNA. The amplification was carried out in a thermocycler (BIO-RAD, USA) under the following conditions: after an initial denaturation at 94 °C for 3 min, 35 cycles at 94 °C for 30 sec (denaturation), 59 °C for 30 sec (annealing), 72 °C for 1 min (extension) with a final extention at 72 °C for 10 min. The PCR products were analysed on 1.5% agarose gel and visualized under a UV illuminator, and the results were recorded.

DNA Sequencing

Purified fragments of PCR products were sequenced from both forward and reverse sites of each PCR product. Sequencing was carried out using the same primers as used for PCR amplification (Macrogen, South Korea).

Phylogenetic analysis

28SrDNA sequences of the *Dactylogyrus lamellatus* specimens in the present study were aligned separately using the Clustal W software and then manually adjusted to perform the phylogenetic analysis. Gaps and ambiguously aligned regions were removed. The phylogenetic tree was built using Mega 6.0 by the UPGMA method.

Statistical calculation and analysis

In this study, all calculations were performed using the following formulas (Bush *et al*., 1997):

Prevalence=Number of infected hosts /Total of hosts×100

Mean intensity=Total number of parasites / Number of infected hosts

Mean abundance=Total number of a particular parasite/Total number of infected and uninfected hosts

Dominance=Total number of a particular parasite/Total number of parasites × 100

The mean intensity of infestation and abundances of Monogenean among different seasons was tested by the Kruskal-Wallis test (KW, multiple comparisons) and Mann-Whitney test. The results were considered significant at the 95% level (p<0.05). Computations were performed using the SPSS.15 programme.

Results

Totally, 10923 *Dactylogyrus* parasites were collected from 80 pieces of grass carp. Morphological examination of taxonomically important features such as the shape of the anchors, connective bar and the shape of a copulatory organ (Fig. 2) as well as detailed morphometric (Table 1) revealed that the only one species is identified as *D. lamellatus* in the present study.

The selected rDNA region was successfully amplified and sequenced. The sequence was deposited in GenBank with accession number MG657261. The 28SrDNA sequence size for *D. lamellatus* was 624 base pairs (bp) (Fig. 3). To build a

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phylogenetic tree we used of different 28SrDNA sequences, which are brought in Table 2.

Table 1: Morphometrics (µm) of *Dactylogyrus lamellatus* recovered from *Ctenopharyngodon idella*.

Characters \ Species	*Dactylogyrus lamellatus* (n=10)	
	Present Study	**Bykhovskaya Pavlovskaya (1962)**
Body length	320.55±80.92 (221.48-444.63)	Up to 480
Body width	71.57±21.23 (44.58-94.76)	Up to 110
Length of marginal hooks	27.12±3.16 (20.62-32.47)	21-31
Length of median hooks	35.59±2.02 (32.08-37.64)	35-41
Connecting bar Length	26.95±1.91 (24.04-29.11)	28-30
Connecting bar Width	3.07±0.42 (2.57-3.82)	Up to 4
The total length of the copulatory organ	42.68±3.77 (35.78-46.86)	About 50

n = number of specimens measured and range in size given in brackets.

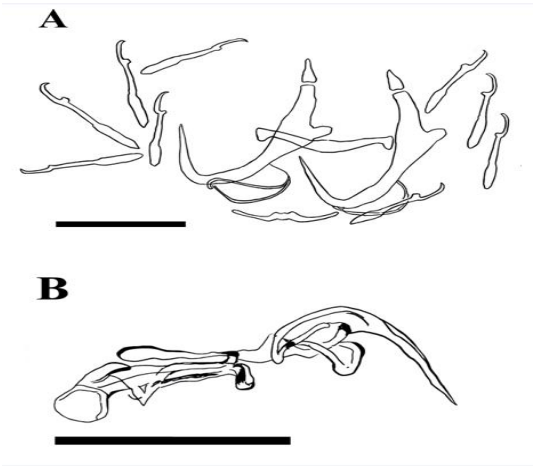


Figure 2: Drawing of *Dactylogyrus lamellatus*: the opisthohapthoral central hook complex (A); copulatory organ (B).

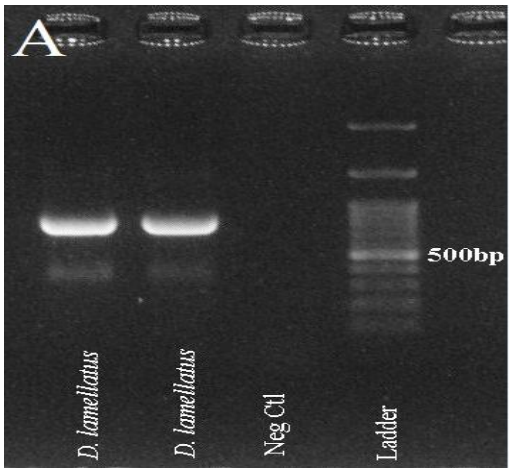


Figure 3: Agarose gel electrophoresis of PCR amplification products from *Dactylogyrus lamellatus* in the present study following exposure to UV light.

Table 2: List of *Dactylogyrus* species used in this study with their host species, Genbank accession numbers and locality to build a phylogenetic tree.

Taxa	Host name	GenBank Accession number	Locality of collection
Dactylogyrus extensus	*Cyprinus carpio*	AJ969944	Zcech Republic
Dactylogyrus nminutus	*Cyprinus carpio*	MF926269	Guilan, Iran
Dactylogyruns nachmerowi	*Cyprinus carpio*	MF979966	Guilan, Iran
Dactylogyrus vastator	*Cyprinus carpio*	MF928712	Guilan, Iran
Dactylogyrus aristichthys	*Aristichthys nobilis*	MH023397	Guilan, Iran
Dactylogyrus nobilis	*Aristichthys nobilis*	MH023399	Guilan, Iran
Dactylogyrus hypophthalmichthys	*Aristichthys molitrix*	EF100532	China
Dactylogyrus suchengtaii	*Aristichthys molitrix*	MG825765	Guilan, Iran

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Table 2 continued:			
Dactylogyrus anchoratus	*Cyprinus carpio*	JX524546	Mashhad, Iran
Dactylogyrus inexpectatus	*Carassius auratus*	AJ969945	Zcech Republic
Dactylogyrus quanfami	*Cirrhinus moliorella*	EF100536	China
Dactylogyrus labei	*Catla catla*	JX566720	India
Dactylogyrus sphyrna	*Rutilus rutilus*	AJ969943	Zcech Republic
Dactylogyrus cryptomeres	*Gobio gobio*	AJ969947	Zcech Republic
Dactylogyrus lamellatus	*Ctenopharyngodon idella*	AY307019	China
Dactylogyrus lamellatus	*Ctenopharyngodon idella*	MG657261	Guilan, Iran ●
Dactylogyrus lamellatus	*Ctenopharyngodon idella*	KY039154	Mashhad, Iran
Dactylogyrus lamellatus	*Ctenopharyngodon idella*	EF100533	China
Dactylogyrus lamellatus	*Ctenopharyngodon idella*	AG969948	Zcech Republic
Dactylogyrus pekinensis	*Megalobrama amblycephala*	EF100535	China
Dactylogyrus petruschewskyi	*Megalobrama amblycephala*	AY548927	China
Tetraonchus monenteron	*Esox lucius*	AJ969953	Zcech Republic

(● This study)

In the present study, 95% of the fish were found to be infected with *D. lamellatus*. The highest rate of infestation has been observed with an average of 505 parasites per fish in summer. In Table 3 the prevalence, mean intensity, range and total of *D. lamellatus* in grass carp in different seasons is compared (Mann-Whitney test, $p<0.05$). As shown in Table 3 this parasite was present during all seasons and cultivation period, however, the mean intensity was significantly higher in summer than other seasons ($p<0.05$). The low intensity of this parasite was observed in autumn.

Phylogenetic trees showed 100% similarity between our isolates and other *Dactylogyrus lamellatus* specimens registered in GenBank (Fig. 4).

Table 3: The prevalence, mean intensity, range and total number of *Dactylogyrus lamellatus* in *Ctenopharingodon idella*, in the present study and different seasons.

Parameters / Season	**Prevalence (%)**	**Mean±SE**	**Range**	**Total**
Spring N=47	100	98.17±66.04[a]	3-836	4614
Summer N=12	100	505±146.51[c]	61-1527	6060
Autumn N=21	80.95	14.64±5.92[b]	1-101	249

N: number of examined fish, SE: Standard Error, * Data with different letters in a column are statistically significant at the $p<0.05$ level

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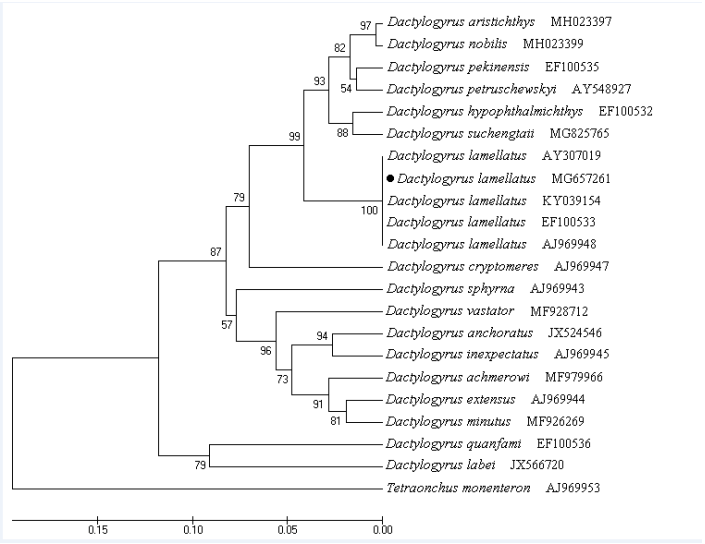


Figure 4: Phylogenetic tree constructed by UPGMA analysis based on 28S rDNA for selected species of *Dactylogyrus*, with *Tetraonchus monenteron* used as the outgroup.

Discussion

Result indicated that only one species of monogenean, *D. lamellatus*, was recovered from *Ctenopharyngodon idella* in the Guilan province fish farms. The parasite identification was performed by morphometric and molecular analysis.

Grass carp is readily infested by several monogeneans, among them *D. lamellatus* is the most harmful parasite, which is spread throughout the country and causes high mortality in fingerlings (Jalali and Molnar, 1990). This survey demonstrated, *D. ctenopharyngodonis* as another *Dactylogyrus* on grass carp which introduced from China in 1991 and has been reported from some of the water resources in the country (Shamsi *et al*., 2009; Daghigh Roohi *et al*., 2016) has not been spread out in fish ponds yet. The study of grass carp parasites in Zarrineh rud River showed that 11.87% of the fish were infested with *D. lamellatus*, whereas in this study, the rate of fish infestation was determined about 95% due to the high density of host fish in these ponds. As mentioned in table 3 the mean intensity of this parasite in Guilan fish ponds was also higher than natural waters like Anzali wetland in Guilan province or Khandaghloo Dam Lake in Zanjan province (Pazooki *et al*., 2006; Dghigh Roohi *et al*., 2016). Phylogenetic tree showed that *D. lamellatus* is genetically identical to those previously reported from other countries and also other parts of the country like Mashhad region which reported by Ahmadi *et al*. (2017). On the other hand, this phylogenetic tree shows that *D. lamellatus* is closely related to parasites of other Chinese carp like big head and silver carp. Because of grass carp is an alien fish, which introduced to Iran for cultivation so, we can conclude its private parasite, *D. lamellatus* probably has introduced to Iran due to lack of proper quarantine for their host. Therefore, as suggested by other authors a widely distributed species has

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the ability to adapt itself to a broad range of climates.

As shown in Table 3, this parasite infestation was significantly higher in summer than other seasons. It seems that the parasite intensity significantly depends on the water temperature. According to the previous studies, the time of *D. lamellatus* sexual maturation depends on the temperature of water. It is indicated that the egg production in *D. lamellatus* began 8, 6 and 4 days after infection at temperatures of 17-19 °C , 20-24 °C and 22-26 °C respectively (Molnar, 1971).

Therefore, due to the temperature conditions in Guilan province, the different seasonal infection intensity, which obtained in our study, is justifiable.

This study provides further insights into the biodiversity and taxonomic status of monogenean parasites in Iran. Iran is a country with various zoogeographical regions, resulting in a high diversity of monogenean parasites reported from the country. Therefore, there is a huge potential for further work on the genetic character of monogenean fauna in the region and to understand the taxonomic relationship between Iranian monogenea with those from the rest of the world.

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Conflict of interest statement

We declare that we have no conflict of interest.

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