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RESEARCH PAPER

The Study of Morphometric and Molecular *Paradiplozoon chazarikum* Parasite in Caspian Sea the *Rutilus frisii kutum*

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Abstract

Different species of monognegens cause different damage to the gills of different fish, one of which is *Paradiplozoon chazarikum* parasite in the *Rutilus frisii kutum*. The parasites were identified by isolating the gills below the stereo microscope. After providing wet slurry, clarifying and monteting them, under the optical microscope, morphological and morphometric indices were evaluated. To investigate the molecular Paradiplozoon chazaricum parasite from two upstream Ribosomal primers, the upstream F '5-GTCGTCGATTGGTTTGTT GTC-3 and downstream R' 5-GAGTTGCAGTATAAGCTAGGCTG-3, for DNA amplication with PCR. As a result, the morphometric study of the parasite using a valid parasitological key as well as the sequence obtained from *Paradiplozoon chazaricum* parasite was compared with the sequence recorded in the gene bank. Finally, the parasite was identified."

Introduction

Paradilozoon chazaricum is a member of Phylum Platyhelminthes, Class monogenea, Subclass Polyopisthocotylea, Family Diplozoidae, Subfamily Diplozoinae, Gense Diplozoon, and Species *Paradiplozoon chazarikum*. The most important characteristic of the Paradiplozoon genus is the narrow medial margin of the posterior end of the parasite. Five species of this genus have been reported in freshwater fish of Iran (Gussev, 1987; Gao *et al*., 2007). The *Paradiplozoon chazaricum* is composed of four pairs of hooks as the main adhesive structure in their opisthohaptor. In the larval stage, two infants stick together and create a single parasite. These parasites are united at maturity and always exist as X. The anterior end of the parasite has two vowels opening to the anterior portion of the gastrointestinal tract. The sticky plates are four-sided and have sticky clips. This parasite has no mating, and its larval forms are called diporpa (Matejusova, Koubkova, Gelnar, & Cunningham, 2002). Investigations on mangoed parasites in Iranian juvenile fishes began in 1949, and the results were also reported on monogens of Iranian freshwater fish (Baychowsky, 1949). A large number of studies have been carried out on various types of monogens in freshwater fish and Caspian Sea in Iran (Jalali and Molnar, 1990; Molnar and Jalali, 1992; Gussev, Jalali., & Molnar 1993; Jalali and Molnar, 1994; Shamsi and Jalali, 1997; Rahanandeh, *et al.,* 2010 and Rahanandeh, *et al.,* 2011). In another study, the *Paradiplozoon baychowsky* in the gill of the *Chalcalburnus chalcoides* and *Aspius aspius*, *Paradiplozoon homoion* in the *Abramis bramaorientalis*, *Rutilus rutilus caspius*, *Barbus brachycephalus*, *Capoeta capoeta gracilis* and carp in the Caspian Sea, *Paradiplozoon schizothorazi* in the Schizothorax fish,

and *Paradiplozoon Chazarikmum* in the *Rutilus frisii kutum*, *Eudiplpzoon nipponicum* reported in the *Capoeta capoeta gracilis* gills of the Mahabad River and the *Diplozoon Paradoxum* from gills of various species of fish. Although in the past, by using morphological studies, naming different species of Diplozoons which differed according to the species of fish in the geographical environment, but today, the best way to diagnose and categorically isolate parasites from each other, using their molecular detection method (Collins, Buchmann, & Cunningham, 2002; Bakke, Cable, & Harris, 2007; Ibrahimzadeh Mousavi, Omid Zahir, Shayan, Ebrahimzadeh Abkouh, & Mahmoudzadeh, 2015). Today, the application of molecular methods as a novel method for the detection and detection of various species of monogens has been introduced (Cunningham, McGillivray, Mackenzie, & Melvin, 1995). Since the application of the molecular method for species detection, many of the monogenic parasites that are morphologically similar are separated using this technique. The most important molecular methods used in monogens are the use of the ribosomal RNA gene (Harris, Shinn, Cable, & Bakke, 2004; Huyse, Malmberg, & Volckaert, 2004). The parasites of *Gyrodactylus derjavinoides* in *Salmo trutta trutta* and *Gyrodactylus derjavini* in *Salmo trutta caspius*, which were similar in morphology, were studied by using PCR and found that the two parasites are dmolecularly different (Malmberg, Collins, Cunningham, & Jalali, 2007). The 31 species of *Gyrodactilus monogenes* were isolated in five families of freshwater fish molecularly (Matejusova *et al*., 2002). Nowadays using molecular combination and morphology, many parasitic species can be studied and differentiated from different classes. In Iran, the use of the molecular method for the detection of parasites is used very little. By using morphological and molecular methods, it is possible to distinguish between many morphological monogens of differentiated morphology from each other (Malmberg *et al*., 2007; Matejusova *et al*., 2002).

Material and Method

In this study, 40 the *Rutilus frisii kutum* were transported live to the aquatic health and aquaculture ponds after being caught from the sea. After examining the gills, the gills were removed from the fish body using scissors and scalpel and placed in a 0.6% physiologic serum solution. The parasites were identified by a microscope and separated from the gills. They were placed on a slide with the help of a Sampler to provide wet spreading, clarification, and melting with a Malmberg solution (Ammonium solution of picrates and glycerin) to be stabilized and studied morphologically under a light microscope. In order to identify the morphology of the *Paradiplozoon chazaricum* parasite, the length of the anterior and the posterior, the opisthohaptor, the diameter of the sucker and the pharynx were taken using a camera mounted on a Nikon optical microscope with a magnification of 4x, 10x, 40x and 100x, and then with the Malumberg method, the morphometric indices of the isolated samples were measured. Finally, the diagnosis of *Paradiplozoon chazaricum* parasite species was performed using a valid parasitology key. Different areas of the opisthohaptor, total body length, anterior and posterior body, sucker diameter and pharynx diameter were measured using the software (Carl Zeiss Axio Vision LE Rel.4.5). To prepare the parasite for molecular detection, the specimen of the parasite was placed in a 70-percent sterile alcohol test tube. The collected samples were placed at a temperature of -20 ° C. DNA extraction from DNA extraction from *Paradiplozoon chazaricum* parasite was carried out using the Sina gena Extract Kit according to the company's instructions. In this study, according to gene sequence reported in genbank (DQ098892) and using the Gene Runner software, a pair of predominant sequences of the B. ribosomal RNA gene upstream of F '5-GTCGTCGATTGGTTTGTT GTC-3 and downstream R' 5-GAGTTGCAGTATAAGCTAGGCTG-3 was designed (Table 1). PCR test was performed using specific primers designed for the parasite on each extracted DNA specimen. After preparing the final volume of the solutions to 100 µL, the PCR test was performed on the Eppendorf Thermocycler machine. In order to check the PCR product, an agarose gel was prepared and placed on electrophoresis wells at 100 volts. To evaluate the genes obtained from the marker (100 bp plus DNA Ladder) made by Fermentas, it was used in a gel. Finally, isolated bands that were visible using UV rays were captured. After obtaining the results of sequencing, the sequences were analyzed using chromas software and after the blast in the collection of gen bank, the isolated species were identified. The primers were designed according to the sequence of the bRibosomal RNA gene (Table 1). The gene used by the World Bank Gene.

Results

In this study, *Paradiplozoon chazaricum* parasites were first identified with morphological characteristics after separation. These parasites have two lips and four

Table 1. Sequence of primers used for PCR from *Paradiplozoon chazaricum* parasite

Paradip - F	5 ´ - GTC GCG GAT TGG TTT GTT GTC - 3´
Paradip - R	5 ´ - GAGT TGC AGTATAAGCTAGGCTG - 3´

pairs of hooks in the posterior region of their opisthohaptor, and in the anterior part they also contain the sucker and pharynx (Figure 1). Total body length 6.8 to 7.9 µm, length of anterior 5.6 - 6.7 µm, the length of posterior 1.2 -2.1 µm, the diameter of the pharynx 0.08-0.14 µm. The diameter of sucker was between 0.15 at 14.1 µm and 14.0 at 12.2 µm, and the length and width of the hooks were 0.05 µm / 0.05 µm (Table 2). Following the morphology of the parasite, the sequence of the *Paradiplozoon chazaricu*m parasite nucleotides isolated using PCR, primers designed for DNA replication were evaluated, and thus, the molecular species of *Paradiplozoon chazaricum* parasite species was identified. The nucleotide sequencing of the PCR product of *Paradiplozoon chazaricum* parasite with a length of 513 bp was determined that 123 bp from the upstream F region of the ribosomal genes and 390 bp was related to the lower portion of the R region of the ribosomal genes (Figure 2). The nucleotide sequence obtained from the parasite with other nucleotide sequences, the Paradiplozoon parasite was compared to the universal gene mutation and was 100% similar to *Paradiplozoon chazaricum* parasite species.

Discussion

Different species of monogene parasites are scattered in freshwater and salty water fish of the world. Among the parasite species, the Ductylogyridae family, in contrast to other parasitic families in class monogenea, are specific to their host (Hodova, Matejusova, & Gelnar, 2010; Molnar, 1984) Monogenic parasites have different degrees of sensitivity to the physical and chemical parameters of water, which can

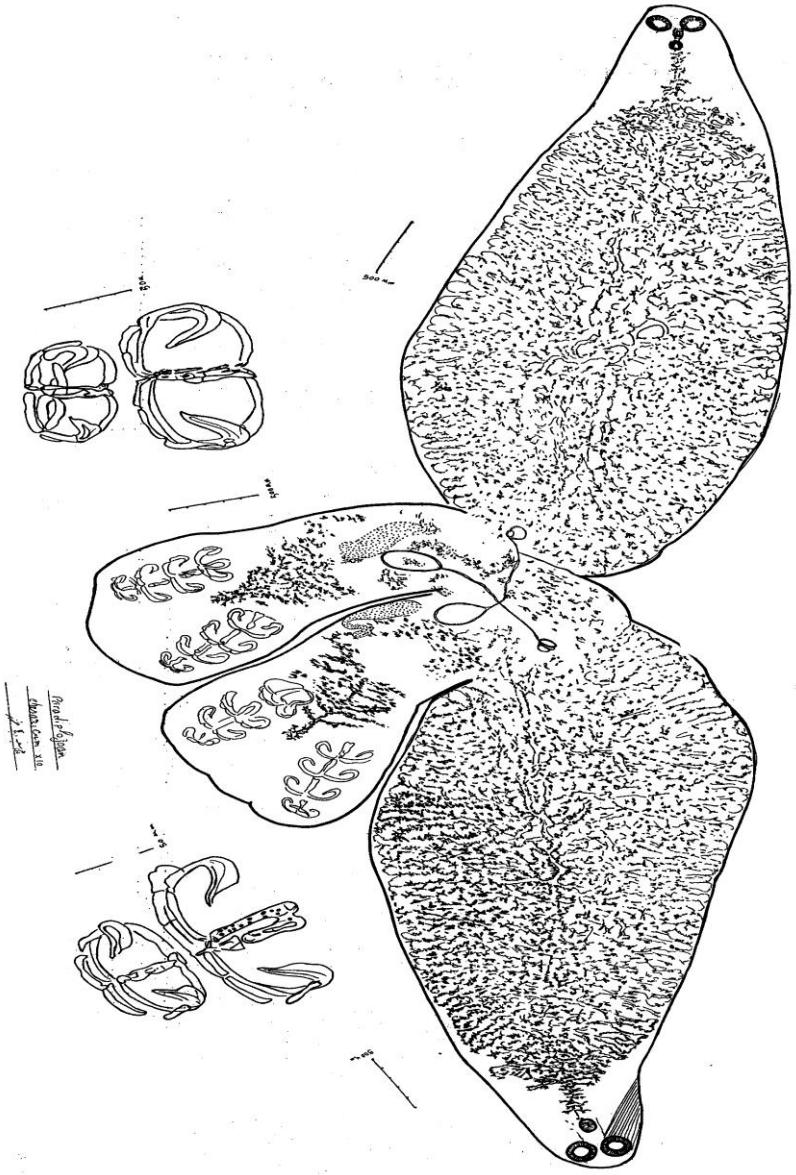

Figure 1. Microscopic drawing of the morphology of the *Paradiplozoon chazaricum* parasite.

Table 2. Morphometric specifications of *Paradiplozoon chazaricum*

Morphometric profile of parasite to micrometer	Unit of measurement (µm) In the present study	Unit of measurement (µm) Gussev(1985)
6.7 -7.7	6.8 - 7.9	Total body length
0.14- 0.15 at 0.12- 0.13	0.15 at 0.17 and 0.14 at 0.16	The diameter of sucker
0.09 – 0.12	0.08 - 0.14	Diameter of pharynx
	0.5 at 0.07	Length and width of hooks
	5.7-5.8	The length of anterior
	2.1 - 2 . 2	The length of posterior

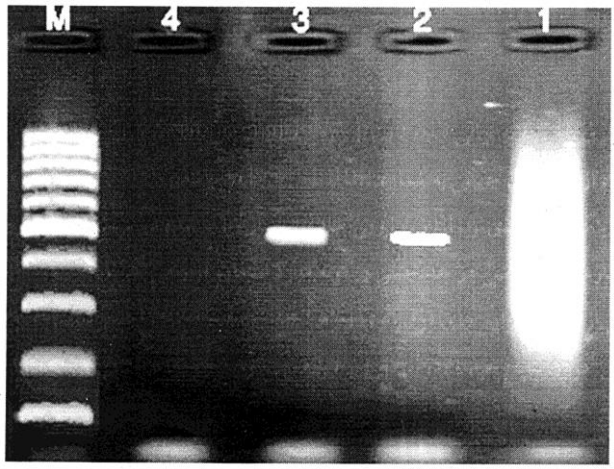

Figure 2. PCR product electrophoresis on DNA genome *paradiplozoon* parasite.

limit the geographical distribution of parasites. Of course, marine fish are also infected in world (Klinger, & Francisbyd, 2005; Jalali and Molnar, 1990). In the present study, the morphological and molecular work of *Paradiplozoon chazaricum* parasite isolated from the *Rutilus frisii kutum* in the southern part of the Caspian Sea was performed for the first time in Iran. Diplozoidae different species have been identified and reported in various fish of Iran and other countries (Milne & Avenant-Oldewage, 2006; Pecinkova, Matejusova, Koubkova, & Gelnar, 2007; Raymond, Champman, & Lanciant, 2006). *Paradiplozoon homoiom* were reported in fish such as in *Abramis brama*, *Chalcalburnus chalcoides*, *Barbus capito*, *Capoeta capoeta*, *Paradiplozoon chazaricum* in *Rutilus frisii kutum*, *Eudiplozoon nipponicum* in *Capoeta capoeta* and *Diplozoon paradoxum* in gill of various species of fish in the Sefidrud River in Iran (Molnar and Jalali, 1992 ; Jalali, 1995; Gussev, 1985; Rahanandeh *et al*., 2011). The family parasites of Diplozoidae, although they may be similar in terms of morphology, especially in terms of the location of the joints, the sucker and the shape of the hooks, but their classification was based on the general structure of their opisthohaptor (Vallgurova, Hodova, Sonnek, Koubkova, & Gelnar, 2010; Milne *et al*., 2006; Zurawaski, Mair, Maule, Gelnar, & Halton, 2003). Differentiation of species was identified according to the characteristics of the hooks in the diplozoidae. After a precise examination of morphology, especially their molecular structure, it was observed that the species of this type of parasite are fundamentally different. Observations on the molecular structure of parasites are one of the methods for accurately identifying different species in different parasitic families (Matejusova *et al*., 2001 and Matejusova et al., 2002). Using PCR, the molecular structure of Gyrodactylus was examined in four species of *Oncorhynchus mykiss*, *Salmo trutta*,

Thymallus thymallus and *Hucho hucho* in Poland (Rokicka, Lumme, & Ziêtara, 2007). The researchers stated that a new molecular method is necessary for the detection and differentiation of various species of Gyrodactylus and is a reliable method for identifying the different species Gyrodactylus genus. The use of morphological and molecular methods in *Gyrodactylus kobayashi* parasite was reported in golden fish (Yildirim, Zeren, Genc, Erol, & Konas, 2010; Ibrahimzadeh Mousavi *et al*., 2015). The *Gyrodactylus granoe* parasite, reported from *Cobitis granoei* fish in China, is morphologically very similar to the *Gyrodactylus micracanthus* species, but the molecular analysis of the regions ITS1, 8 / 5S and ITS2 introduced the new species of *Gyrodactylus granoei* in *Cobitis granoei* fish (You, Guo, King, & Cone, 2011). The length of the PCR product can be useful for the detection of Eudiplozoon and Paradiplozoon from other Diplozoidae (You *et al*., 2010; Matejusova *et al*., 2001). The study of PCR in three

species of *D. kashmirensis*, *D. aegyptensis* and *D. guptai* showed clearly that their nucleotide pair sequence was different and different from molecular structure (Bakke *et al*., 2007; Ibrahimzadeh Mousavi *et al*., 2015). The rDNA genes, in particular the 28S gene, have generally been useful in parasitic molecular and phylogeny classification (Zhu, Gasser, & Chilton, 1998). The changes of the nucleic acid between species in the internal transcriptional region of rDNA (ITS) were used to detect diploptic parasites (Gao *et al*., 2007; Matejusova *et al*., 2001). The parasites of the species *Gyrodactylus microdactylus*, *Gyrodactylus poeciliae* and *Gyrodactylus actzu* which were very similar in morphology, but whose molecular study showed different species (García-Vásquez, Razo-Mendivil, & Rubio-Godoy, 2015). Examples of the morphological and molecular analyzes of different species of parasites in the world show the importance of using morphological and molecular detection for the identification of different species of parasites. This study was first performed using a morphological and molecular method on the *Paradiplozoon chazaricum* parasite in the *Rutilus frisii kutum* of the southern part of the Caspian Sea in Iran. The present study showed that the application of morphological and molecular techniques is the best way to detect different species of parasites in a family. In this research, primers designed specifically for *Paradiplozoon chazaricum* parasite were used and it was determined that morphological characteristics correspond to the molecular structure of *Paradiplozoon chazaricum* parasite.

Conclusion

First, the findings of this study showed that the *Paradiplozoon chazaricum* species varies in terms of the genetic and morphological structure with other parts of the diplozoidae. Secondly, due to the above differences, this species is properly located in the diplozoidae. Third, the molecular diagnosis of this species is due to the importance of its epidemic in the *Rutilus frisii kutum* of the Caspian Sea and is reported for the first time in Iran.

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