Identification of *Gyrodactylus gurleyi* in *Carassius auratus* using morphometric and molecular characterization

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Key words:
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Abstract:

BACKGROUNDS: Gyrodactylus is a small monogenean ectoparasite that lives on the skin and fins of most of the world's fish species. Gyrodactylus appears to be one of the most prevalent parasites found in ornamental fish, especially in Cyprinids. Goldfish (*Carassius auratus*) are a popular ornamental fish that are highly contaminated by *Gyrodcatylus*. **OBJECTIVES:** The present study is aimed to identify morphological and molecular characteristics of the Gyrodactylus parasite on gold fish. **METHODS:** The morphological identification of Gyrodactylus specimens was performed using the measurements and drawings of opisthaptoral hard parts of the parasites. The molecular species description was based on a polymerase chain reaction (PCR) of partial sequence of the 5.8S region of ribosomal RNA, and a partial sequence of the internal transcribed spacer 2 (ITS2) of ribosomal RNA. The nucleotide sequences of the PCR products were compared with corresponding sequencing registered in GenBank. **RESULTS:** Based on the morphometric analysis and sequencing, the Gyrodactylus specimens were described as *Gyrodactylus gurleyi*. **CONCLUSIONS:** A combination of molecular techniques with morphological analysis seems to be the best approach for the identification of Gyrodactylus speices.

Introduction

Carassius auratus is a freshwater fish, in the family of Cyprinidae, known under the common name of goldfish. It is one of the most commonly kept aquarium fish. There are many different varieties of domesticated goldfish. A large number of different fish species, particularly goldfish, are annually imported to Iran from the Far-East (China), Russia and Eastern Europe (Shamsi et al., 2009). Also, goldfish are cultured and reproduced in several fish farms in Iran. Considering the economic importance and interest of people for this ornamental fish, the study dealing whit the identification of pathogenic parasites for aquarium fish seem to be both important and necessary.

The Gyrodactylus (Gyrodactylidae: Monogenea: Platyhelminthes) is a small monogenean ectoparasite (<1 mm) which lives mainly on the skin and fins of freshwater and marine fish (Collins et al., 2002). Ectoparasites of the genus Gyrodactylus can infect the most of the world's fish species. Gyrodactylus is one of the parasite organisms that can cause diseases with high mortality rate, (Bakke et al., 2002; Meinila et al., 2004). Gyrodactylus parasites damage the host's epidermis during attachment to the fish. Lesions in the epidermis are caused by the 16 marginal hooks and two anchors of the attachment organ. Ulcers generated by enzymatic digestion cause loss and inability to osmoregulate which seems to be the main

reason of the host mortality. The epidermal damage caused by bacteria or fungi give rise to the secondary infections that can play a considerable role in the pathogenicity of the Gyrodactylus (Collins et al., 2002). It is estimated that there are more than 20,000 species of Gyrodactylus (Bakke et al., 2007). A little more than four hundred valid Gyrodactylus species are described, from nearly 400 hosts in fish families. So, identification of 20-30 new Gyrodactylus species per decade is a very low increased number to be identified in every ten year period (Harris et al., 2004; Bakke et al., 2002).

Malmberg 1970 identified the morphological species of the Gyrodactylus based upon its opisthaptoral hard parts. In particular, the shape of the tiny marginal hook sickles is species specific, but, unfortunately, the morphology of the attachment organ is variable. Factors like host, geographical location, and temperature impress the haptors intraspecific phenotypic variation (Rokicka et al., 2007).

To resolve the problems of the identification of the Gyrodactylus specimens, molecular methods were introduced by Cunningham, et al., 1995. Molecular methods result in giving high quality systematic information, that is mostly based on nuclear ITS in Gyrodactylus (Ziêtara et al., 2008; Matìjusová et al., 2003). The nuclear ITS is used more commonly among Gyrodactylus species (Kuusela et al., 2008). It is thought that the combination of morphological analysis with a molecular method can result in the beter method to identify the Gyrodactylus species. This paper presents the identification of Gyrodactylus species on *Carassius auratus* using both morphological and molecular characteristics.

Materials and Methods

Sampling and microscopic examination: In the present study, a total of fifty goldfish (*Carassius auratus*) were referred alive in their original water, to the Aquatic Animal Health Department laboratory (Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran). The body and fins of each fish were immediately examined by wet mounts, microscopic slides were prepared from parasite samples and the existence of Gyrodactylus was analyzed using a light microscope. Each parasite was separately photographed by digital camera (Sony, SSC-DC80P NO.401182)under the light microscope. Photographic images of parasites were used for morphological analysis. After taking photos, each *Gyodactylus* sample was drawn and measured. Morphological identification of Gyrodactylus species was performed using features of opisthaptoral characters (anchors, marginal hooks, ventral and dorsal bars) according to exiting Gyrodactylus identification keys. The characters and measurement criteria described in this study have been taken from Malmberg (1970) (Fig 1). Then, each Gyrodactylus specimen was removed from the fish under the light microscope, and was individually preserved in the sterile tube containing 70% (v/v) ethanol.

DNA extraction and PCR amplification: DNA was extracted by digesting a single parasite specimen using a DNA isolation kit (MBST, Iran) according to the manufacturer's instructions.

Polymerase chain reaction (PCR) amplification was performed using sense primer (5`-CGA TCA TCG GTC TCT CGA AC-3`) at position 687 to 706 of the 5.8S rRNA gene, and the antisense primer (5`-TTA AGG AAG AAC CAC TAG AG-3`) at position 1042 to 1061 of the ITS2 rRNA gene.

The amplification reaction contained 15 µl DNA sample, 10 µl of 10 × PCR buffer (Cina gene, Iran), 1.5 mM $MgCl_2$, 20 pg of each primer and 2.5 U of Taq polymerase (Fermentas) in a total volume of 100 µl. The amplification was carried out in a thermocycler (MWG, Germany) using step one at 95°C for 5 minutes; step two with 38 cycles, at 94°C for 45 seconds; 50°C annealing temperature for 45 seconds, 72°C for 45 seconds; and, the final step taken for 10 minutes at 72 °C. The PCR products were analyzed on 1.5% agarose gel and visualized under the UV illuminator and then the results were recorded.

PCR product purification and sequencing: The PCR products were purified using a PCR purification kit (MBST, Iran) following the manufacturer's instructions. Purified fragments were sequenced from both sites of each PCR product using a method based on Sanger (1977). Sequencing was carried out using the same primers as used for PCR amplification, by kowsar company.

Results

Gyrodactylus parasites were identified in 15 out

Table 1. Morphological measurements of opisthaptoral hard parts of *Gyrodactylus spp*. collected from *Carassius auratus* in present study and *Gyrodactylus gurleyi* reported by Ergens &Yukhimenko (1987) and Cones &d Wiles (1983).

Measurement (μm)	Present study	Ergens andYukhimenko(1987)	Cones and Wiles(1983)
Total length of anchor	**47.55-52.96**	**45-55**	**47-56**
Length of anchor root	**10.07-17.34**	**13-19**	**10-18**
Length of anchor shaft	**35.72-40.75**	**33-42**	**33-43**
Length of anchor point	**21.65-26.15**	**23-28**	**21-27**
Marginal hook sickle length	**4.98-5.11**	**5**	**5**
Marginal hook handle length	**21.77-22.3**	**-**	**22**
Total length of marginal hook	**24.22-27.5**	**23-28**	**24-28**
Length of ventral bar	**19.81-21.74**	**18-22**	**18-25**
Median width of ventral bar	**3.75-5.03**	**5-6**	**3-5**
length of ventral bar membrane	**10.26-12.52**	**10-13**	**9-14**
Total length of dorsal bar	**19.42-23.14**	**12-18**	**19-22**
Median width of dorsal bar	**1.99-2.15**	**1-2**	**-**

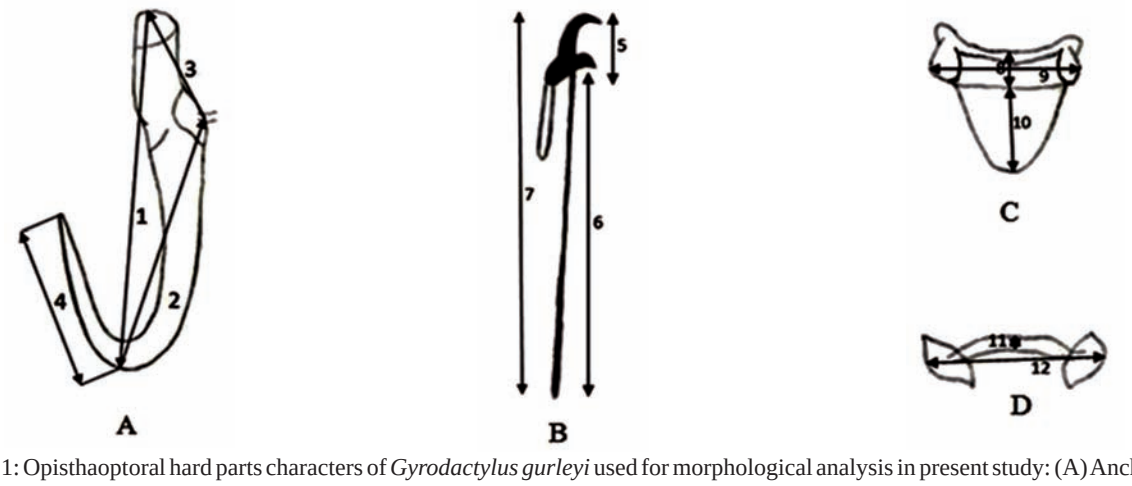

Figure 1: Opisthaoptoral hard parts characters of *Gyrodactylus gurleyi* used for morphological analysis in present study: (A) Anchor: (1) total length of anchor; (2) length of anchor shaft; (3) length of anchor root; (4) length of anchor point. (B) Marginal hook: (5) length of sickle; (6) length of handle; (7) total length of marginal hook; (C) Ventral bar: (8) median width of ventral bar; (9) length of ventral bar; (10) length of ventral bar membrane; (D) Dorsal bar: (11) median width of dorsal bar; (12) total length of dorsal bar.

of the 50 fish samples. Gyrodactylus specimens collected from goldfish samples were identified as *Gyrodactylus gurleyi* by both morphometric and molecular analysis.

Morphological description: The morphological characteristics of Gyrodactylus samples were compared with available Gyrodactylus morphology. An analysis was made of the micrographs and drawings of the morphological characteristics of the Gyrodactylus specimens. The opisthaptoral hard parts of specimens was measured with the following data collected and reported as: Total length of anchor 47.55-52.96; Length of anchor root 10.07-17.34; Length of anchor shaft 35.72-40.75; Length of anchor point 21.65-26.15; Total length of marginal hook 24.22-27.5; Marginal hook sickle length 4.98-5.11; Marginal hook handle length 21.77-22.3, Length of ventral bar 19.81-21.74; Median width of ventral bar 3.75-5.03; Total length of dorsal bar 19.42-23.14; and, Median width of dorsal bar 1.99-2.15. Light micrographs and drawings of opisthaptoral hard parts are shown in Figures 2 and 3. Morphological characteristics and morphometric measurement of haptoral hard parts of *Gyrodactlus*

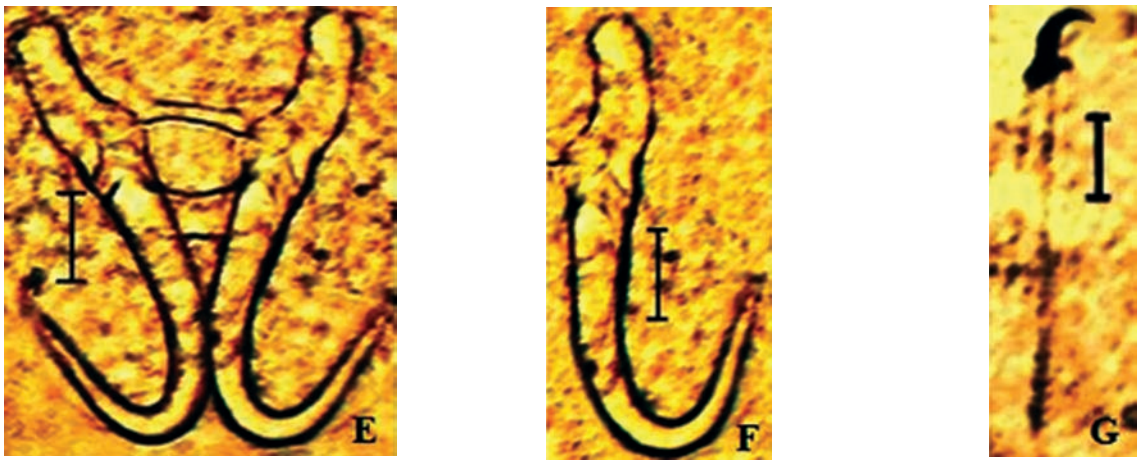

Figure 2: Light micrograph of opisthaptoral hart part of *Gyrodactylus gurleyi* in present study: E: Central hook complex; F: Anchor. G: Marginal hook. Scale bars: E, F= 10 µm, G= 5 µm.

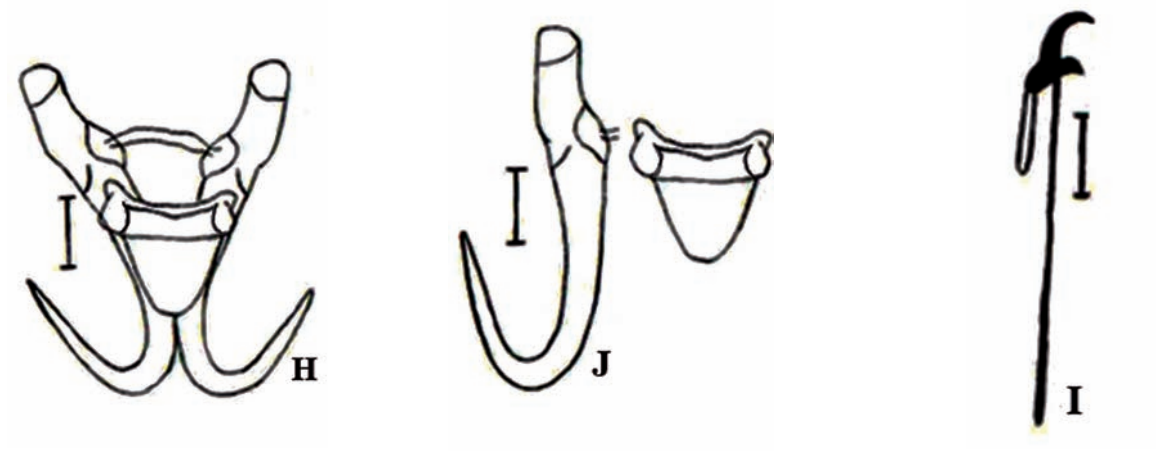

Figure 3: Drawings of the opisthaptoral hard parts of *Gyrodactylus gurleyi* in present study: H: Central hook complex; I: Anchor; J: Marginal hook; H, I= 10 µm, J =5 µm.

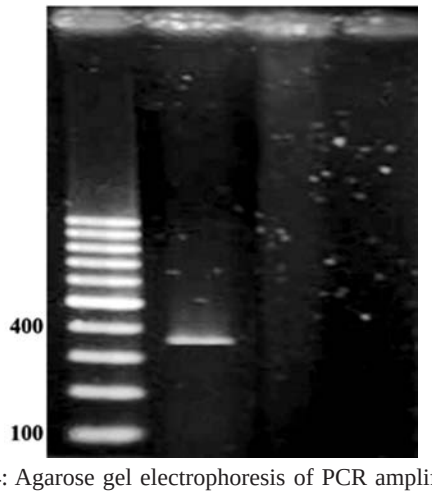

Figure 4: Agarose gel electrophoresis of PCR amplification product from *Gyrodactylus gurleyi* in present study following exposure to UV light.

samples were compared with those reported by Cone & Wiles (1983) and Ergens & Yukhimenko (1987) (Table 1).

Molecular characterization: In this study, the amplification of nucleotide sequence with 375 base pairs (bp) in lenght, including 5.8S rRNA gene 70 bp and ITS2 305 bp, were obtained from the individual Gyrodactylus parasite. After purification and sequencing of the PCR product, the nucleotide sequences were compared with other Gyrodactylus sequences available in GenBank. By a BLAST search (Basic Local Alignment and Search Tool) in GenBank, *Gyrodactylus gurleyi* (registered under accession number AJ001842) was identified. The sequence of Gyrodactylus parasites isolated from *Carassius auratus* in peresent study were deposited

in Gen Bank under accession number JQ922264.

Discussion

This study presents both morphological and molecular descriptions of Gyrodactylus species on *Carassius auratus*. Species of the monogenean genus Gyrodactylus are difficult to identify morphologically (Cable et al., 1999). Some species of Gyrodactylus are morphologically similar but molecularly clearly different. The large number of species, small size (<1 mm) and the tiny marginal hook sickles make the identification of Gyrodactylus species very difficult and uncertain. Until the introduction of molecular methods, the difficulties of Gyrodactylus species identification were resolved (Rokicka et al., 2007).

More than thirty three *Gyrodactylus spp* have been found on the gills and the body surface of both wild and farmed freshwater fish in Iran. Among the known species, *Gyrodactylus derjavini* (Mikhailov, 1975) has been found on the gills, caudal and anal fins of the Caspian Salmon (Salmo trutta caspius) and Rainbow Trout (*Oncorhynchus mykiss*). In warm water cultured fish, *Gyrodactylus sprostona*, with widest host range, infects Common Carp (*Cyprinus carpio*), Silver Carp (*Hypophthalmichthys molitrix*) and Big Head Carp (*Aristichthys nobilis*) in almost all of the Iranian fish farms (Jalali et al., 2005).

Ebrahimzade Mousavi, (2003) examined several ornamental fish for parasites, in Iran, and reported *Gyrodacylus kobayashii* on *Carassius auratus*. *Gyrodactylus chinensis* and Gyrodactylus sp. were reported from imported *Carassius auratus*, pear scale variety, by Ebrahimzade Mousavi et al. (2009).

Gyrodactylus specimens collected from goldfish samples in the present study were identified as *Gyrodactylus gurleyi*. In 1891, R. R. Gurley collected specimens of Gyrodactylus from the fins of "Japanese Fantail" (*Carassius auratus*) in Texas. He identified the material as *Gyrodactylus elegans*. Price (1937) examined Gurley´s wholemount slides of worms fixed in situ on host fins which ended up in the Helminthological Collection, United States Department of Agriculture, Beltsville, Maryland, and concluded the specimens were not *Gyrodactylus elegans* and described them as *Gyrodactylus gurleyi* (Cone and Wiles, 1983). The description was vague; therefore Cone and Wiles measured morphological characterization of *Gyrodactylus gurleyi*, again, and redescribed those specimens. Ergens and Yukhimenko (1987) reported *Gyrodactylus gurleyi* from *Carassius auratus* and *Cyprinus carpio* haematopterus. With the description of *Gyrodactylus gurleyi* reported by Cone & Wiles (1983) and Ergens & Yukhimenko (1987), the detailed morphometrical data of this species is now available.

For the first time, the nucleotide sequence of *Gyrodactylus gurleyi* for the internal transcribed spacers (ITS1 and ITS2) and 5.8S ribosomal DNA was published by Cable, et al., 1999. In this study the sequences of Gyrodactylus samples' was described as *Gyrodactylus gurleyi*.

The results obtained from this microscopic study were confirmed by sequence analysis. A combination of morphological description with a molecular technique seems to be the best practice for identifying Gyrodactylus species. This study present the first report of *Gyrodactylus gurleyi* using both morphological and molecular methods in Iran.

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مجله طب دامی ایران، ۱۳۹۱، دوره ۶، شماره ۱، ۴۶-۴۱

شناسایی انگل ژیرو داکتیلوس گرلیی در ماهی کاراسیوس اوراتوس با استفاده از بررسی مورفومتریک و ملکولی

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چکیده

زمینـه مـطالعه: انگل ژیرودا‌کتیلوس از دسته کرم‌های پهن مونوژن (تک میزبانه) و جزء انگل‌های خارجی مهم ماهی‌های پرورشی و ماهی‌های آزاد آبهای شیرین و آبهای شور و ماهی‌های زینتی محسوب می‌شود که می‌تواند سبب ایجاد بیماری و خسارات اقتصادی قابل توجهی گردد. این انگل یکی از شایع‌ترین انگل‌های ماهی به ویژه در خانواده کپور ماهیان می‌باشد. ماهی کاراسیوس اوراتوس (ماهی طلایی) یکی از ماهی‌های خانواده کپور ماهیان است که بسیار مستعد آلودگی و بیماری با انگل ژیرودا‌کتیلوس می‌باشد . **هدف:** هدف از این مطالعه شناسایی گونه انگل ژیرودا‌کتیلوس بر اساس ویژگی‌های مورفومتریک و مولکولی در ماهی زینتی کاراسیوس اوراتوس می‌باشد. **روش کار:** در این مطالعه انگل‌های ژیرودا‌کتیلوس از سطح بـدن و بـاله‌های نمونه‌های ماهی کاراسیوس اوراتوس جداسازی شدند. ویژگی‌های مورفولوژیک (ریخـت شنـاسـی) هـر یـک از انگل‌ها بر اساس کلیدهای تشخیصی با استفاده از عکسبرداری، ترسیم و اندازه گیری قسمت‌های مختلف اپیستهاپتور انگل انجام شد. بررسی مولکولی گونه انگل ژیرودا‌کتیلوس با استفاده از روش PCR قسمتی از توالی ۵/۸S ژن ریبوزوم RNA و قسمتی از توالی ITS۲ ژن ریبوزوم RNA مورد ارزیابی قرار گرفت. سپس توالی به دست‌آمده از محصول PCR با توالی‌های ثبت شده انگل ژیرودا‌کتیلوس در ژن بانک مورد مقایسه قرار گرفت. **نتایج:** بر اسـاس بررسی مورفومتریک و تعیین توالی، نمونه‌های انگل ژیرودا‌کتیلوس در این مطالعه ژیرودا‌کتیلوس گرلیی معرفی شدند. **نتیجه‌گیری نهایی:** با توجه به این مسئله که شناسایی انگل ژیرودا‌کتیلوس تنها بر اساس خصوصیات مورفولوژی به علت اندازه کوچک انگل (کمتر از یک میلیمتر)، تغییر مورفولوژی ناحیه اپیستهاپتور انگل تحت شرایط مختلف جغرافیایی، نوع میزبان و درجه حرارت آب، تعداد بسیار زیاد گونه های این انگل (بیش از بیست هزار گونه) و شناسایی بسیار اندک این گونه ها (حدود تنها چهارصد گونه تاکنون) کار مشکل و غیر مطمئنی است، بررسی ملکولی تشخیص مورفولوژی را مورد تایید قرار می‌دهد. بنابراین استفـاده از بـررسـی مـورفـومتـریـک به همراه استفاده از تکنیک مولکولی بهترین روش برای دستیابی به شناسایی صحیح گونه‌های انگل ژیرودا‌کتیلوس و معرفی گونه‌های جدید این انگل می‌باشد.

واژه‌های کلیدی: کاراسیوس اوراتوس (ماهی طلایی)، ژیرودا‌کتیلوس گرلئی، بررسی مورفومتریک، بررسی ملکولی، PCR.

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