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# Morphological and Molecular Differentiation of *Clinostomum complanatum* (Digenea: Clinostomidae) Metacercariae Infecting *Cyprinus carpio* in Aras River

**Key words**

*Clinostomum complanatum*;
*Cyprinus carpio*;
Aras River;
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**Abstract:** Clinostomiasis is one of the parasitic diseases infecting freshwater fish caused by digenean trematode of the Clinostomidae family. In this study a total of 100 fish belonging to the nine species were collected from Aras River in 2021-2022. The collected fish then analyzed for infection with *Clinostomum complanatum* (*C. complanatum*) metacercaria. The partial mitochondrial COX-I gene were amplified for sequencing and phylogenetic analyses. Only one specimen belonging to *Cyprinus carpio* were found to be infected with metacercariae with the prevalence of 5%. The sequence analysis confirmed morphological identification as all metacercariae were belonging to *C. complanatum* with homology of 100% with other sequences of *C. complanatum* available in the NCBI database. The present study reports the first case of *C. complanatum* in *Cyprinus carpio* in Iran by morphological and molecular methods and emphasizes the possible risk of transmission for consumers.

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## Introduction

Fishes can be affected by a wide variety of parasites, such as protozoa, trematodes, cestodes, nematodes, and acanthocephala. Currently, studies have recognized over 40 distinct zoonotic parasite species that can be found in fishes (1). *Clinostomum complanatum* is zoonotic digenetic trematodes that infected oral cavity and esophagus of piscivorous birds as a definite host and fish or amphibians as intermediate host (2). The well-known condition "yellow grub disease" is caused by the presence of large, typically yellow, encysted metacercariae of *C. complanatum* within various areas of a fish's body (3). The attachment of the this parasite to the pharynx of human can cause Halzoun syndrome that may be led to fever, bloody phlegm and pain in swallowing (4, 5). Several human cases of yellow grub disease have been reported in different parts of the world, especially in Asia (6, 7). The possible reason behind the absence of reported cases of *Clinostoma* disease in Iran could

be due to limit awareness and understanding of this parasite among healthcare professionals in the local medical community (8). As a result of the significant variability in morphology among *Clinostomum* species and the close resemblance between them, various taxonomic revisions have been carried out in order to classify this parasite accurately. The limited understanding of the morphological characters of several existing species can lead to the incorrect identification of *Clinostomum* spp. (9). So, in the current study, the molecular analysis were used beside the morphological studies. The Aras River is located in the south of the Caucasus mountains and flows throughout the borders between Iran, Nakhchivan, Armenia and Azerbaijan. This river originates in Anatolia and flow towards the east, serving as a vital source of life for both human and aquatic communities (10). Until now, the yellow grub has been identified morphologically from various fish species found in Iranian freshwater environments, such as *Capoeta gracilis, Chalcalburnuc chalcoides, Aphanius aphanius, A. dispar, and Pseudorasbora parva* (11-13). But, there has not performed any survey related to molecular evaluation of yellow grub in Iran. This study was performed to morphological and molecular differentiation of yellow grub infecting edible fishes in Aras River from Iran.

## Materials and methods

### *Study area*

Ethical approval. This experimental study was approved by the Ethical Committee of Ardabil University of Medical Sciences, Ardabil, Iran (IR.ARUMS.REC.1398.287). The different fish species were collected from Aras River as a most important river from the northwestern of Iran during 2021-2022. The three highly populated provinces located on its southern coast, the populations of which have grown steadily during the last decades.

### *Fish and parasitological examination*

The fish samples including, *Silurus glanis* [12], *Hypophthalmichthys nobilis* [5], *Sander lucioperca* [3], *Cyprinus carpio* [20], *Luciobarbus capito* [20], *Hypophthalmichthys molitrix*[10], *Aspius aspius* [5], *Ratilus rutilus*[15], and *Abramis brama* [8] were collected and then analyzed for infection with *C. complanatum* metacercaria. The collected fish were transported on thick ice bags to the laboratory of parasitology of Ardabil University of Medical Sciences, where they were examined immediately. The collected samples were studied for helminthic parasite as described previously (4, 14, 15). The important criteria such as locality, total length, weight of collected fish and the infected organs, number of cysts per fish were recorded. The isolated metacercariae from recovered cyst were fixed with 70% ethanol and clarified with Amman's lactophenol and staining by Azocarmine and subsequently mounted. The measurement of collected metacercariae including body size, and oral sucker and acetabulum length and width were performed by an eyepiece micrometer.

### DNA extraction

The total DNA of isolate metacercariae was extract using a Qiagen extraction kit (Valencia, CA) according to the manufacturer's instructions and preserved in 70% ethanol.

### *Molecular analysis*

The partial mitochondrial COX-I gene with 620 bp size was amplified using specific primers including Trem_Co1F 5and Trem_Co1R as described previously (3). The PCR program was performed as fallow: :95ºC for 10 min for denaturation, followed by 40 cycles of 94ºC for 20 seconds, 55ºC for 20 seconds, and 72ºC for 40 seconds, and the final extension of 70ºC for 10 minutes. The PCR products were sent for sequencing to Pishgam Company, Tehran, Iran.

### Data analysis and phylogenetic tree construction

The raw nucleotide sequences were analyzed using NCBI BLAST program and aligned using Bioedit software (version 7.0.9, Los Angeles, CA). Phylogenetic tree of the *C. complanatum* COI gene was accomplished using Maximum Likelihood method and Tamura-Nei model by the MEGAX (16). *Alaria mustelae* (JF904529) was set as outgroup.

## Results and Discussion

Totally, the metacercariae of *C. complanatum* were detected in 1 out of 100 (1%) isolated fishes from Aras River. The metacercaria were identified only from the specimens belonging to *Cyprinus carpio* with the prevalence of 5%. The metacercaria was collected from gill (14) and body cavity (10). The morphologic features of isolated *C. complanatum* metacercariae were as follows: narrowed around the ventral sucker, the small oral sucker, well developed oral collar, the extended intestinal ceca until ventral sucker, triangular testes, the well-developed cirrus sac that opens into the genital pore and irregular ovary. The isolated metacercariae (Figure 1) had similar morphology and their measurements were record at table 1.

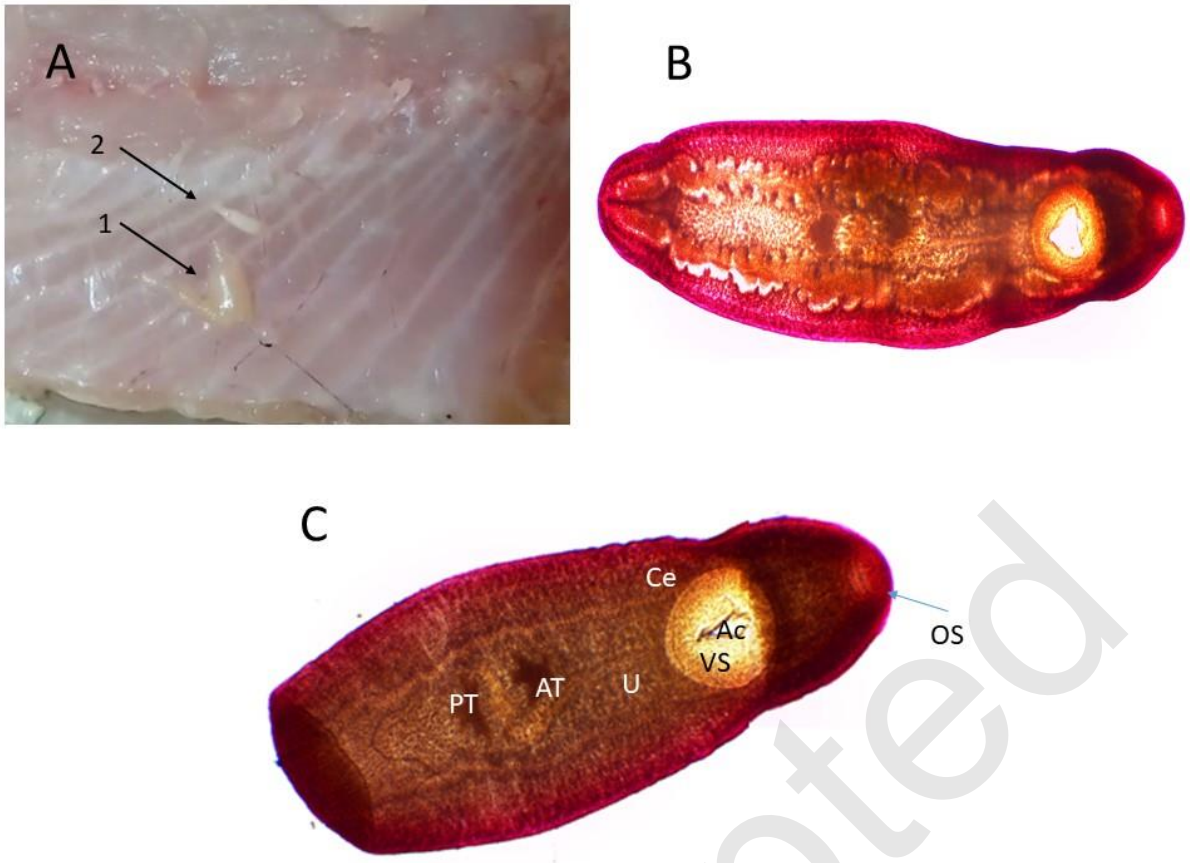


**Figure 1:** (A) 1. Encysted metacercariae, 2. Excysted metacercariae. (B-C) Stained C. complanatum metacercariae. OS, Oral sucker; VS, Ventral sucker; Ce, Ceca; A, Acetabulum; U, Uterus; AT, Anterior testis; PT, Posterior testis

**Table 1:** Morphological data from *C. complanatum* metacercariae range (mean ± SD) in µm

| | ***Clinostomum complanatum*** **(n = 10) min–max (mean ± SD) µm** |
|---|---|
| **Body length** | 5,675–7,521 (6,521 ±1,534) |
| **Body width** | 1,543–2,738 (2,041 ±125) |
| **Body length/width** | 2,148–3,978 (2.505 ± 463) |
| **Oral sucker (OS) length** | 212–396 (125± 50) |
| **OS width** | 294–495 (371 ± 51) |
| **Ventral sucker (VS) length** | 595–890 (726 ± 44) |
| **VS width** | 61–890 (751 ± 41) |
| **Anterior testis (AT) length** | 305–912 (570 ± 95) |
| **AT width** | 301–512 (452 ± 98) |
| **Posterior testis (PT) length** | 292–453 (395 ± 81) |
| **PT width** | 441–680 (571 ± 21) |
| **Ovary length** | 135–164 (149 ± 11) |
| **Ovary width** | 97–178 (129 ± 24) |

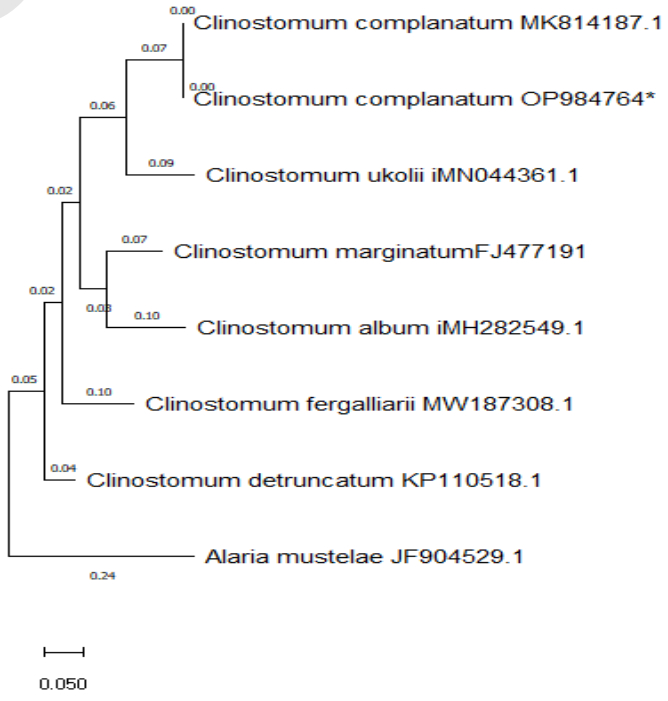


**Figure 2:** Phylogenetic tree inferred using maximum likelihood and Tamura-Nei model derived from cox1 data set

The partial mitochondrial COX-I gene were successfully amplified from isolated metacercariae. The sequencing of amplified fragments confirmed morphological study as all metacercariae were belonging to *C. complanatum* with homology of 100% with other sequences of *C. complanatum* available in the NCBI database. The amplified sequences were submitted to GenBank (accession no: OP984764). The phylogenic tree of the region corresponding to the COI of our sequence with other isolates from GenBank was showed in Figure 2.

Despite examining of different fishes species from Aras River, only one fish species related to *C. carpio* were infected to clinostomiasis suggesting that this river can be suitable place for the completion of the parasite's life cycle. In a previous study that was performed in Iran, the high prevalence rate of adult and metacercaria of *C. complanatum* were reported from piscivorous birds and fishes, respectively (8). In other part of the world, the metacercaria of *Clinostomum* have been isolated from fishes, toads, frogs, salamanders and tritons (17). In the past, morphological methods were used to identify *Clinostomum spp.*, which were often confusing; Therefore, molecular techniques were developed in order to more accurately identification of clinostomastidae (18). So, we described *Clinostomum* infections in *C. carpio* from northwestern part of Iran using the combination of morphological and molecular techniques. Based on previous study that was performed in Iran, many species have been introduced as an intermediate host for *C. complanatum* (8). In the present study, *C. complanatum* metacercaria were isolated from the gill and abdominal cavity. The previous studies reported such cysts distributed in the base and anterior of anal, between pectoral and pelvic fins, pectoral fins, gill, operculum and muscle and head region (19). The larva of *C. complanatum* can cause pharyngitis or laryngitis in human if they were consumed. Human laryngitis caused by *Clinostomum* is a rare disease. However, in different part of the world specially in Asian countries where it is common to eat raw fish such as sushi, people may become infected with this parasite (5). The most effective method to kill or inactivate the fish parasites is freezing and heating (20). It is suggested to carry out more studies to determine the parasitic infections in other fish species. Also, it seems necessary to educate medical practitioners and those who work in fish transportation and especially fish cookers about the risk *C. complanatum*.

## Conclusion

The present study reports the first case of *C. complanatum* in *Cyprinus carpio* in Iran by morphological and molecular methods and emphasizes the possible risk of transmission for consumers. Furthermore, the present study demonstrates the first molecular and morphological data on *C. complanatum* of the Aras river freshwater fish.



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## References

1. Raissy M, Masoumeh B, Ansari M, Momeni H. Fish-borne parasites: a review on the reports from Iran. J Food Microbiol 2020; 6(4): 88–102.

2. Menconi V, Manfrin C, Pastorino P et al. First report of *Clinostomum complanatum* (Trematoda: *Digenea*) in European perch (*Perca fluviatilis*) from an Italian subalpine lake: a risk for public health? Int J Environ Res Public Health 2020; 17(4): 1389. doi:

3. Bonett RM, Steffen MA, Trujano-Alvarez AL, Martin SD, Bursey CR, McAllister CT. Distribution, abundance, and genetic diversity of *Clinostomum spp. metacercariae (*Trematoda*: Digenea*) in a modified Ozark stream system. J Parasitol 2011; 97(2): 177–84. doi: 10.1645/GE-2572.1

4. Caffara M, Locke SA, Echi PC. A new species of *Clinostomum Leidy, 1856* based on molecular and morphological analysis of metacercariae from African siluriform fishes. Parasitol Res 2020; 119(3): 885-92. doi: 10.1007/s00436-019-06586-2

5. Hara H, Miyauchi Y, Tahara S, Yamashita H. Human laryngitis caused by *Clinostomum complanatum.* Nagoya J Med Sci 2014; 76(1/2): 181–5.

6. Park CW, Kim JS, Joo HS, Kim J. A human case of Clinostomum complanatum infection in Korea. Korean J Parasitol 2009; 47(4): 401--4. doi: 10.3347/kjp.2009.47.4.401

7. Lee GS, Park SW, Kim J, Seo KS, You KW, Chung JH, MoonHC, Hong GY (2017) A case of endoscopically treated laryngopharyngitis resulting from *Clinostomum complanatum* infection. Korean J Gastroenterol 2017; 69(3): 177–80. doi:10.4166/kjg.2017.69.3.177

8. Aghlmandi F, Habibi F, Afraii MA, Abdoli A, Shamsi S (2018) Infection with metacercaria of *Clinostomum complanatum (*Trematoda*: Clinostomidae)* in freshwater fishes from Southern Caspian Sea Basin. Rev Med Vet 2018; 169(7/9): 147–51.

9. Karimova S. Some fish species of fishery importance in Nakhchivan part of aras reservoir. Bull Sci Prac 2022; 8(4): 72–80. doi: 10.33619/2414-2948/77/08

10. Gholami Z, Mobedi I, Esmaeili HR, Kia EB. Occurrence of *Clinostomum complanatum* in aphanius dispar (Actinoptrygii: *Cyprinodontidae*) collected from Mehran river, Hormuzgan province, South of Iran. Asian Pac J Trop Biomed 2011; 1(3): 189-92. doi: 10.1016/S2221-1691(11)60025-7

11. Hosseini S. The study of infection of fish with *Clinostomum sp.* in the lake of south of Tehran. Dissertation. Teheran: University of Tehran, 1987.

12. Malek M, Moubedi I. Occurrence of *Clinostomum Complanatum* (Rudolphi, 1819)(Digenea: *Clinostomatidae*) in *Capoeta capoeta gracilis* (Osteichthys: *Cyprinidae*) from Shiroud River Iran. Iran J Public Health 2001; 30(3/4): 95-8.

13. Fernando CH. Methods for the study of freshwater fish parasites. Biology series no. 12. Waterloo: University of Waterloo, 1972.

14. Matthews D, Cribb T. Digenetic trematodes of the genus *Clinostomum Leidy, 1856 (*Digenea*: Clinostomidae*) from birds of Queensland, Australia, including *C. wilsoni n. sp*. from *Egretta intermedia.* Syst Parasitol 1998; 39(3): 199-208. doi: 10.1023/A:1005982530560

15. Tamura K, Nei M. Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. Mol Biol Evol 1993; 10(3): 512-26. doi: 10.1093/oxfordjournals.molbev.a040023

16. Miller DL, Bursey CR, Gray MJ, Smith LM. Metacercariae *of Clinostomum attenuatum* in *Ambystoma tigrinum* mavortium, *Bufo cognatus* and *Spea*

*multiplicata* from west Texas. J Helminthol 2004; 78(4): 373-6. doi:10.1079/JOH2004248

17. Locke SA, Caffara M, Marcogliese DJ, Fioravanti ML . A large-scale molecular survey of *Clinostomum* (Digenea, *Clinostomidae*). Zool Scr 2015; 44(2): 203-17. doi:10.1111/zsc.12096

18. Simsek E, Yildirim A, Yilmaz E et al. Occurrence and molecular characterization of *Clinostomum complanatum (*Trematoda*: Clinostomidae)* in freshwater fishes caught from Turkey. Parasitol Res 2018; 117(7): 2117-24. doi: 10.1007/s00436-018-5898-3

19. Adams A, Murrell K, Cross J (1997) Parasites of fish and risks to public health. Rev Sci Tech 1997; 16(2): 652-60. doi: 10.20506/rst.16.2.1059

# Morfološka in molekularna diferenciacija metacerkarij *Clinostomum complanatum* (Digenea: *Clinostomidae*), ki invadirajo *Cyprinus carpio* v reki Aras

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**Izvleček:** Klinostomoza je ena od parazitskih bolezni sladkovodnih rib, ki jo povzročajo digeni trematodi iz družine *Clinostomidae*. V raziskavi je bilo v letih 2021–2022 v reki Aras ulovljenih 100 rib, ki pripadajo devetim vrstam. Ulovljene ribe so nato analizirali na prisotnost metacerkarij *Clinostomum complanatum* (*C. complanatum*). Delni mitohondrijski gen COX-I je bil pomnožen za sekvenciranje in filogenetske analize. Z metacerkarijami je bil invadiran le en primerek, ki je pripadal *Cyprinus carpio*, in sicer s 5-odstotno prevalenco. Analiza zaporedja je potrdila morfološko identifikacijo, saj so vse metacerkarije pripadale *C. complanatum* s 100-odstotno homologijo z drugimi zaporedji *C. complanatum*, ki so na voljo v podatkovni bazi NCBI. Pričujoča študija poroča o prvem primeru *C. complanatum* pri *Cyprinus carpio* v Iranu z morfološkimi in molekularnimi metodami ter poudarja morebitno tveganje prenosa na potrošnike.

**Ključne besede:** *Clinostomum complanatum*; *Cyprinus carpio*; reka Aras; Ardabil