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Occurrence and description of *Clinostomum complanatum* (Rudolphi, 1819) metacercariae in freshwater fishes from Gheshlagh basin, West of Iran

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Clinostomum spp. have a long uncertain taxonomic history which also have attracted great attentions. This could be due to their zoonotic potential and the presence of yellow grubs in the fish as a second intermediate host. In the current study, a total of 3oo freshwater fish belonging to the nine species were collected from two stations in the Gheshlagh basin, Kurdistan Province. Four species including *Alburnus mossulensis*, *Capoeta damascina*, *Garra rufa* and *Squalius cephalus* were found to be infected with the metacercariae. The highest prevalence (4.1%) and mean abundance (0.31±0.37) were observed in *C. damascina*. The metacercariae were identified using molecular (Internal Transcribed Spacer (ITS)), SEM and morphological analysis as *Clinostomum complanatum*. The phylogenetic analysis of four sequences of ITS gene were conducted. The specimens were placed within a lineage of *C. complanatum* and formed a clade with other *Clinostomum* species in the Palearctic region. The current study revealed the *C. damascina*, *G. rufa* and *A. mossulensis* as new hosts for *C. complanatum* and first report of this metacercariae in the region. Furthermore, the present study demonstrate the first molecular and morphological data on *C. complanatum* of the Iranian freshwater fish.

Key words: *Clinostomum complanatum*, *freshwater fish*, *Gheshlagh basin*, *ITS gene*, *morphology*.

INTRODUCTION

Helminth parasites of the freshwater fish are the most known parasitic groups among Vertebrate. The fishes are host for three groups of worms including Platyhelminthes, Nematoda and Acanthocephala. At least, 30-thousand helminth species have been known from both freshwater and marine fishes worldwide (Kurochin, 1985). Digenetic trematodes are a large group of flatworms consisting of more than 2,500 nominal genera, and generally have 3 hosts, two intermediate and one definitive in their life cycle (Cho *et al*., 2014).

The Clinostomidae Lühe, 1901 as adult parasitize the buccal cavity or esophagus of birds, reptiles and mammals including humans. The first larval stage is in Gastopoda and second larval stage is in the muscles, abdominal cavity, fins, and gill cavity of freshwater fish, and amphibians (Kanev *et al*., 2002). *Clinostomum* Leidy, 1856 is a cosmopolitan genus with a complex life cycle. The species of *Clinostomum* can be infective as metacercariae and adult for their second intermediate and final hosts, respectively (Shamsi *et al*., 2013; Aghlmandi *et al*., 2018). The metacercariae are known as 'yellow grub' that can infect different parts of fish bodies (Gustinelli *et al*., 2010). Humans can also

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be infected after eating raw or uncooked fishes causing 'halazoune syndrome' (Park *et al*., 2009). Such parasites have great attention due to their zoonotic potential and capabilities to produce pathogenicity in their hosts.

The genus *Clinostomum* has had a relatively ambiguous taxonomic conditions, since the species show the low interspecific variations and high intraspecific variations and their taxonomy do not affect by host association character (Locke *et al*., 2015). The species diversity of *Clinostomum* has undergone significant changes by authors; for example, 24 species by Vianna *et al*. (2003), one by Feizullaev and Mirzoeva (1983), 13 by Ukoli (1966) and Mathews and Cribb (1998) and eight species by Locke *et al*. (2015) have considered to be valid. The morphological descriptions and species identification in helminth parasites were performed via the adult samples in the final hosts, but often the access to the final hosts is limited when they have usually protected by law. Likewise, the most reports on the given species are on the metacercariae in fish known as the second intermediate hosts. Since this larval stage possesses few diagnostic characters at species level, the molecular methods can help to link the larvae to adult stages. Therefore, species delamination in Trematoda are usually carried out by molecular methods (Mathew & Cribb, 1998). Despite the valuable studies in the last decades on clarification of the species diversity of *Clinostomum* more efforts still should be made in which use combined morphological and molecular information in combination with sampling at different geographic regions.

There is an acceptance on the separation of *Clinostomum* spp. into two clades, one from Palearctic area i.e. *C. complanatum* (Rudolphi, 1819), *C. cutaneum* Paperna, 1964, *C. phalacrocoracis* Dubois, 1930, *C. philippinense* Velasquez, 1959, *C. tilapiae* Ukoli, 1966 and those from Nearctic/Neotropical regions i.e. *C. marginatum* Rudolphi, 1819, *C. tataxumui* Sereno-Uribe *et al.* 2013, *C. detruncatum* Braun, 1899 and *C. attenuatum* Cort, 1913 (Caffara *et al*., 2011; Locke *et al*., 2015; Acosta *et al*., 2016; Caffara *et al*., 2017). *Clinostomum complanatum* as type species of *Clinostomum* has been reported from freshwater fish more than any *Clinostomum* species in the world. This species recognized as an important parasitic zoonosis for public health.

The Gheshlagh River is 95 km in length as a tributary of Tigris basin, originating from the North of Sanandaj city. This river together with Gavehroud River form the great Sirvan River which enters the Darbandikhan Lake in Iraq. Gheshlagh dam is constructed on this river at 12 km of the North of Sanandaj aiming to provide the urban water as well as fisheries activities (Jafari *et al*., 2009). The ichthyofauna of this region has been largely known and so far, 19 species have been recorded from both endemic and introduced species (Asarab, 2006; Bahrami Kamangar *et al*., 2012).

The aim of this study was to verify the taxonomic status of *Clinostomum* metacercariae found in the Gheshlagh River in Kurdistan Province. The morphological and molecular tools are employed in this study for the first time on the species of *Clinostomum* in the freshwater fish of Iran.

MATERIAL AND METHODS

Sampling

The study was carried out in the Gheshlagh River, Swarian station (35º 11□ N, 46º 88□ E) and reservoir of Gheshlagh dam (35º 44□ N, 46º 89□ E). A total of 300 freshwater fish belonging to nine species were collected from the Gheshlagh basin, in the vicinity of Sanandaj city, Kurdistan Province (Fig. 1) from September 2016 to August 2017. The fish species were identified as *Alburnus mossulensis* (Heckel, 1843) (61), *Barbus lacerta* (Heckel, 1843) (2), *Capoeta damascina* (Valenciennes, 1842) (98), *Capoeta trutta* (Heckel, 1843) (30), *Carassius auratus* (Linnaeus, 1758) (4), *Cyprinion tenuiradius* Heckel, 1847 (3), *Hypophthalmichthys molitrix* (Valenciennes, 1844) (18), *Garra rufa* (Heckel, 1843) (13), and *Squalius cephalus* (Linnaeus, 1758) (66). Fish were caught by the gill and cast net and transported alive

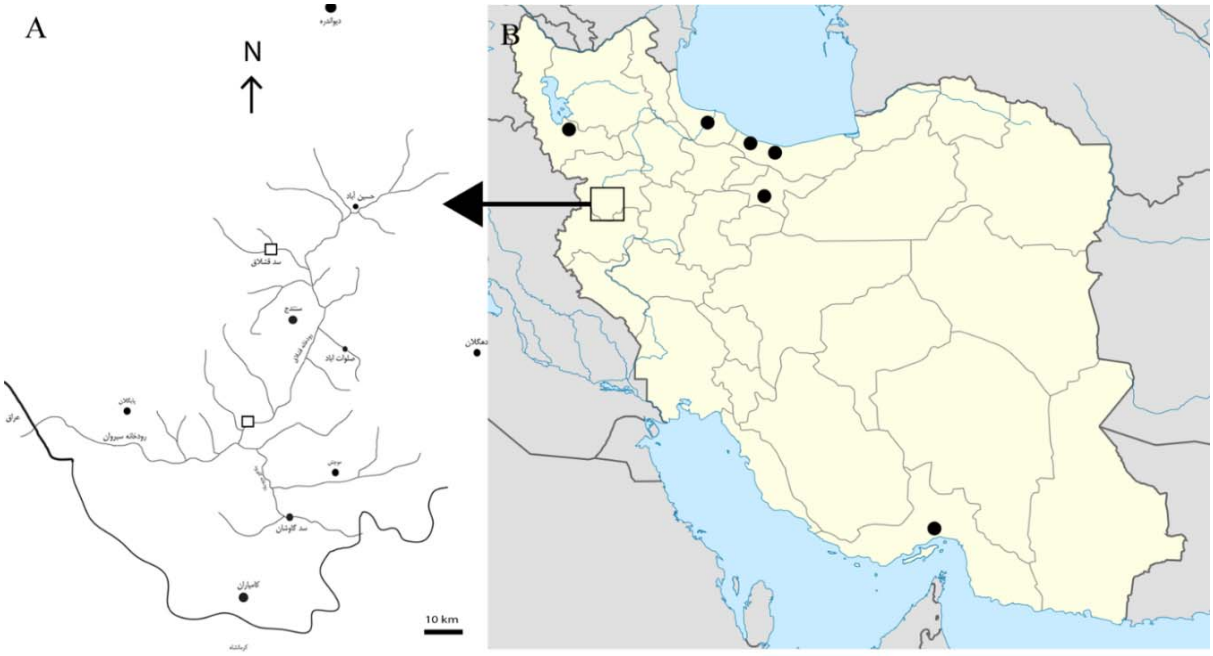

FIGURE 1. A. The map of Gheshlagh basin in the vecinity of Sanandaj city, the blank squres show the sampling stations. **B.** The location of sampling in the western Iran. The filled circles show the places from where *Clinostomum complanatum* has been reported.

to the laboratory where the length, weight, and sex of fish were recorded. All organs of the body were investigated freshly for helminth parasites under dissection microscope. Of nine collected fish species, four species including *C. damascina*, *A. mossulensis*, *S. cephalus* and *G. rufa* were infected with the metacercariae of *Clinostomum complanatum*. The specimens were excysted and preserved in ethanol 70% for morphological studies and a few samples were preserved in 99% ethanol for molecular studies.

Morphological and Molecular studies

Whole mount of 15 metacercariae were prepared as follows: the worms were cleared with Amman's lactophenol, stained by boracic carmine, dehydrated by graded ethanol series, cleared in methyl salicylate, mounted on glass slides in Canada balsam. The line drawing were made with the aid of a drawing tube connected to the Olympus BX51 microscope, measurements are given in micrometer. Measurements are mentioned as ranges followed by the mean, standard deviation and number of specimens examined, in parenthesis. Total DNA was extracted using a DNP^{TM} DNA extraction Kit (SINACLON, Iran) according to the instructions of the manufacturer. Internal transcribed spacer (ITS1, 5.8S, ITS2) □ 1000 bp was amplified using the primers reported by Gustinelli *et al.* (2010) and Cribb *et al.* (1998). Each Polymerase Chain Reaction (PCR) was performed in a total volume of 20 µl (10 µl master mix (PCR Master Mix 2x, SinaClon), 0.5 µl forward and 0.5 µl (5 pmol/ml) reverse primer, 1 µl DNA template and 8 µl ddH2O). The PCR was performed under the following thermocycling profile: 2 min initial denaturation at 94 °C; then 34 cycles of 30 s at 94 °C, 40 s at 57 °C, 90 s at 72 °C; and 10 min final extension at 72 °C. The PCR products were resolved using 1% agarose gel (EP5081, SINACLON, Iran) in 1X TBE. The products were sequenced with an Applied Biosystems 3730/3730xl DNA Analyzers Sequencing (Bioneer, Korea). The sequences were edited using BioEdit 7.0.5.3. The alignment of the sequences was performed using Clustal X ver. 1.85 with sequences of *Clinostomum* species available in GeneBank. Phylogenetic trees were reconstructed for

the gene data set using Maximum Likelihood (ML) and Bayesian Analyses (BA). Evolutionary model was calculated using the MODELTEST v 3.4 (Posada & Crandall, 1998) by the Akaike information criterion (AIC), and the resultant GTR+ G model were utilized. Robustness of the inferred trees was evaluated using bootstrap analysis on 1,000 pseudoreplications using RAxML 7.0.4 (Stamatakis *et al*., 2008). BA analyses were conducted using Mr. Bayes v 3.1.2 (Huelsenbeck & Ronquist, 2001). For BA, 5,000,000 cycles were implemented for four simultaneous Monte Carlo Markov chains, sampling the Markov chain at intervals of 100 generations. Log-likelihood stability was attained after 100,000 generations; the first 5,000 trees were discarded as "burn-in" in each analysis. Support for BI tree nodes was determined based on values of Bayesian posterior probabilities. Final trees were visualized in the FigTree v.1.4.2 program (Rambaut, 2009). *Euclinostomum heterostomum* was regarded as outgroup. The nucleotide sequences of ITS gene of *C. complanatum* were deposited in GeneBank databases with accession numbers MH845233-MH8452336.

In the present study, Statistical analysis was used for *C. complanatum* metacercariae infection in the infected fish. The prevalence, mean intensity and mean abundance of each host species were evaluated (Bush *et al*., 1997). First, the normality of data was tested using Shapiro-Wilk test, then the significant differences were calculated by Kruskal-Wallis test. The *p*-value was considered significant when < 0.05.

RESULTS

Of the nine examined fish species which belonged to Cyprinidae, four were infected with the metacercariae (yellow grubs) including *Alburnus mossulensis*, *Capoeta damascina*, *Garra rufa*, and *Squalius cephalus*. The metacercariae were observed as encysts and excysted worms in the muscles, buccal cavity, sub operculum, and pectoral fins bases. The highest prevalence of the metacercariae was in *C. damascina* with 4.1%, and the highest mean abundance and mean intensity were in *C. damascina* with 0.31±0.37 and 9±7.3, respectively (Table 1). There was not any significant difference for prevalence, mean intensity and abundance of *C. complanatum* among the examined fish species. In total, of 76 examined fish specimens in the reservoir, no infected fish were observed and of 224 examined fish specimens in the river, 5% were infected with *C. complanatum*. The relationship between prevalence and intensity of infection with fish length and sex were measured. There was a positive relationship between the length of *C. damascina* and prevalence of *C. complanatum* (Kruskal-Wallis=16, p=0.001), but there was not any relationship between the host sex and prevalence of infection.

TABLE 1. The prevalence and mean intensity and abundance of *Clinostomum complanatum* in freshwater fish from Gheshlagh River

Fish species	Fish No.	Number of infected (Prevalence)	χ^2 (P)	Parasite intensity	Mean abundance	Kruskal-Wallis test (P)	Mean intensity	Kruskal-Wallis test (P)
C. damascina	98	4 (4.1%)		0-31	0.31±0.37		9±7.3	
S. cephalus	66	2 (3%)	1	0-1	0.03±0.02	7.6	1±0	0.6
A. mossulensis	61	3 (5%)	(0.8)	0-2	0.07±0.04	(0.4)	1.3±0.3	(1.85)
G. rufa	13	2 (15%)		0-2	0.23±0.16		1.5±0.5	

MORPHOLOGY

(Figs. 2, 3, 4)

Taxonomy: Subclass Digenea Carus, 1863; Order Strigeida Poche, 1926; Family Clinostomidae Lühe, 1901; subfamily Clinostominae Lühe, 1901; Genus *Clinostomum* Leidy, 1856.

Second intermediate host: Levantine scraper *Capoeta damascina* (Valenciennes, 1842), common chub *Squalius cephalus* (Linnaeus, 1758), Mossul bleak *Aburnus mossulensis* Heckel, 1843, Doctor fish *Garra rufa* (Heckel, 1843).

Site of infection: Muscles, buccal cavity, sub operculum, gill cavity and pharynx.

Locality: Swarian station, Gheshlagh River

The morphological characters of the 15 metacercariae are: body stout, oval, 1917–2700 (2331±205, 15) long, 646–1058 (851±119, 15) wide; covered by thin cuticular spine. Oral sucker 143–231 (178±26, 15) long by 147–243 (193±24, 15) wide, smaller than ventral sucker, ventral sucker 336–449 (395±34, 15) long by 322–422 (379±29, 15) wide. Intestinal cecae lateral to ventral sucker and genital complex, with small diverticula more evident at level of genital complex. Testes arranged in tandem at posterior side of middle third of body. Anterior testes 110–209 (155±28, 15) long by 89–261 (182±50, 15) wide, triangular in shape, posterior testes 101–192 (141±28, 15) long by 107–326 (212±53, 15) wide, triangular shaped with anterior margin concave and with two main lateral lobes and one posterior lobe. Cirrus sac 47–139 (101±26, 15) long by 50–123 (71±22, 15) wide, at right margin of anterior testes, genital pore at right anterior side of ootype. Ovary irregular, round, 45–108 (75±23, 15) long by 23–70 (45±22, 15) wide, located in intertesticular space. Uterus extending from right of anterior testes to near ventral sucker, with no lateral digitation.

The morphometric characters here are almost smaller than those previously described metacercariae of *C. complanatum* (Table 2), but the descriptive characters are consistent with *C. complanatum*. However, the identity should also be confirmed by molecular data.

For molecular works, four sequences of ITS were obtained. A sequence for each host species (*C. damascina*, *G. rufa*, *S. cephalus* and *A. mossulensis*) was used. The ITS sequence was 1031 bp. The BLAST analysis was resulted in following identity with *C. complanatum* specimens: 100% with JF718624.1, 100% with JF718623.1, 100% with JX235337.1 and 99% with FJ609420.1. The intraspecific distance of ITS gene was 0 and the interspecific distance between our samples and *C. marginatum*, *C. tataxumui*, *C. phalacrocoracis* was calculated (0.51%, 0.67% and 0.71%, respectively). The phylogenetic tree resulted from Maximum Likelihood (ML) (Fig. 5) and BI showed our samples within *C. complanatum* clade, and this species formed a clade along with species like *C. phalacocoracis*, *C. tipaliae* and *C. cutaneum* from the Palearctic region.

DISSCUSSION

The present study is the first report of *C. complanatum* from fish in the Kurdistan Province and the first record of the metacercariae in *C. damascina*, *A. mossulensis* and *G. rufa*. *Clinostomum complanatum* has previously been reported from *S. cephalus* (another host in the present study) in Iran (Pazooki & Mansourian, 2012; Aghlmandi *et al*., 2018) and Turkey (Simsek *et al*., 2018). So far, the metacercariae of this parasite have been reported from 14 fish species in Iran (Table 3). Therefore, the metacercariae of *C. complanatum* exhibit the low host-specificity, reported from four families of Iranian fish so far (Cyprinidae, Cyprinodontinae, Gobiidae and Cobitidae). The recorded fish for this metacercariae globally exceed 100 species (Acosta *et al*., 2016; Perez-Ponce De Leon *et al*., 2016). In the last years, the most reports of clinostomid metacercariae in the fishes were introduced as *C. complanatum,* so it was recognized a cosmopolitan species (Nolan & Cribb, 2005). Today, the most studies have shown that this species is a 'European species' restricted to the Palearctic region (Caffara *et al*., 2011; Lock *et al*., 2015). Likewise, the *C. complanatum* metacercariae has been reported solely based on the morphology in more than 60 fish species in Mexico, may need further investigation (Perez-Ponce De Leon *et al*., 2016).

TABLE 2. Measurements of *Clinostomum complanatum* metacercariae in the present study in comparison with previous studies. Values indicated with µm

characters	*C. complanatum* (n=12) Simsek *et al.* (2018)	*C. complanatum* (n=27) Li *et al.* (2018)	*C. complanatum* (n=11) Caffara *et al.* (2011)	*C. complanatum* (n=16) This study
Body length	3998-6718 (5108±43.6)	2470-3287 (2924±224)	4495-7874 (5741±1223)	1917-2700 (2331±205)
Body width	1197-2131 (1697±0.16)	1071-1507 (1273±121)	1635-2434 (1934±239)	646-1058 (851±119)
Body length/width	2580-3526 (3045±0.21)	1.85-2.68 (2.3±0.2)	2.2-4.3 (2.9±0.6)	2.29-3.45 (2.77±.333)
Oral sucker (OS) length	243-319 (272±11)	156-285 (212±33)	259-337 (294±27)	143-231 (178±26)
OS width	261-483 (336±36)	235-390 (285±36)	284-507 (401±74)	147-243 (193±24)
Ventral sucker (VS) length	581-821 (682±36)	385-571 (457±44)	637-910 (795±77)	336-449 (395±34)
VS width	679-893 (763±32)	430-672 (546±59)	766-952 (839±60)	322-422 (379±29)
VS width/OS width	1.84-2.6 (2.3±0.16)	1.33-2.38 (1.93±0.2)	1.78-2.6 (2.1±0.3)	1.63-2.45 (2±0.24)
Distance between suckers	823-1012 (915±28)	513-732 (632±63)	860-1115 (1020±84)	356-583 (419±31)
Anterior testis (AT) length	290-711 (472±65)	212-310 (280±24)	316-957 (484±178)	110-209 (155±28)
AT width	203-498 (409±45)	178-347 (240±52)	273-559 (412±74)	89-261 (141±28)
AT width/length	0.7-1.07 (0.88±0.21)	0.61-1.21 (0.835±0.12)	0.4-1.2 (0.9±0.2)	0.55-2.19 (1.21±0.43)
Posterior testis (PT) length	219-398 (321±24)	178-347 (240±52)	245-441 (328±63)	101-192 (141±28)
PT width	384-564 (456±28)	225-325 (260±25)	408-602 (493±56)	107-326 (212±53)
PT width/length	1.3-1.75 (1.43±0.01)	0.7-1.58 (1.13±0.27)	1.08-1.8 (1.5±0.2)	0.83-2.74 (1.55±0.52)
Distance between testis	225-447 (324±35)	278-370 (329±25)	214-527 (353±91)	215-391 (360±47)
Ovary length	120-158 (139±5.7)	57-97 (76±12)	135-164 (149±11)	35-108 (60±24)
Ovary width	81-149 (110±9.9)	77-114 (89±8)	97-178 (129±24)	40-80 (53±22)
Ovary width/length	0.67-0.94 (0.78±0.01)	0.81-1.53 (1.2±0.2)	0.59-1.08 (0.8±0.13)	0.43-1.28 (0.86±0.28)
Cirrus sac (CS) length	213-391 (278±26)	132-250 (180±34)	209-405 (296±59)	77-140 (103±21)
CS width	119-180 (151±8.9)	117-205 (140±24)	124-197 (157±24)	61-103 (78±13)
CS length/body length	0.051-0.054 (0.054±0.01)	0.05-0.09 (0.061±0.012)	0.034-0.071 (0.053±0.013	0.02-0.06 (0.044±0.01)

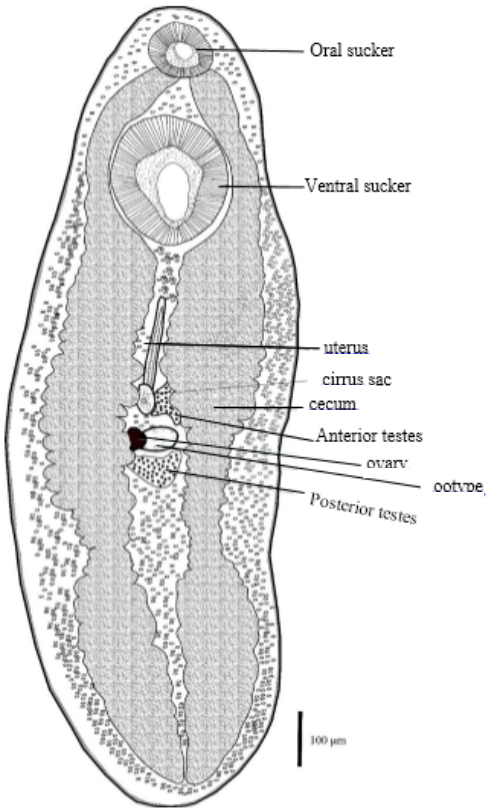

FIGURE 2. Drawing line *of Clinostomum complanatum* metacercariae from Gheshlagh River

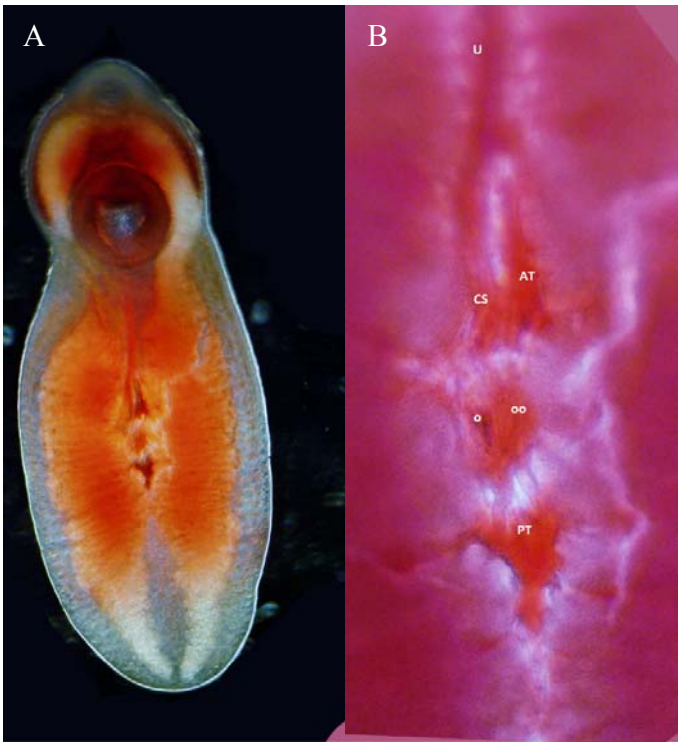

FIGURE 3. Image of *Clinostomum complanatum* metacercariae, **A.** whole worm, **B.** genital complex. AT: anterior testis, CS: cirrus sac, O: ovary, OO: ootype, PT: posterior testis.

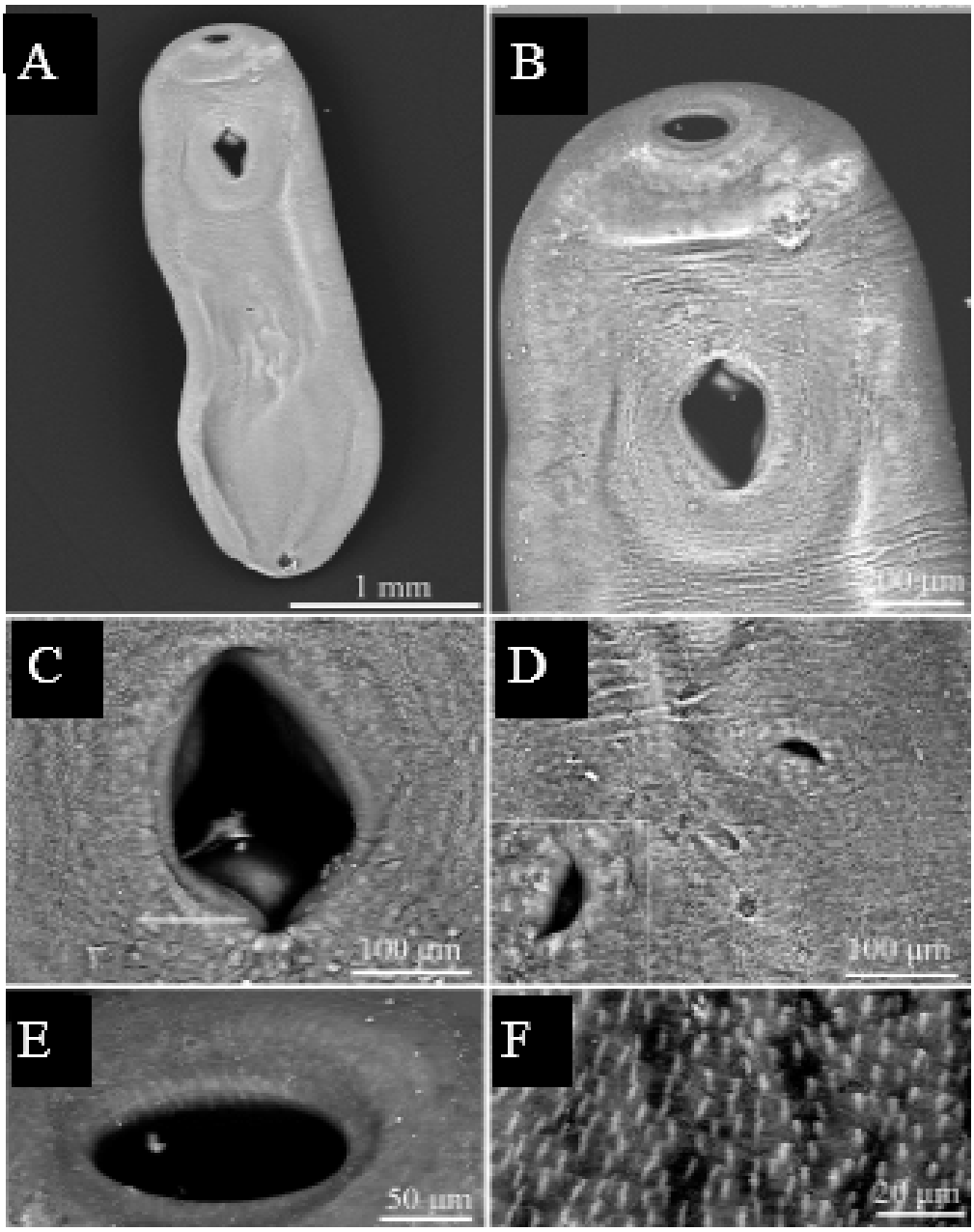

FIGURE 4. Scanning electron micrographs of the metacercariae of *Clinostomum complanatum*, **A.** Whole worm, **B.** enlarged view of ventral and oral suckers, **C.** enlarged view of ventral sucker, **D.** genital pore, **E.** oral sucker, **F.** cuticular spines on body surface.

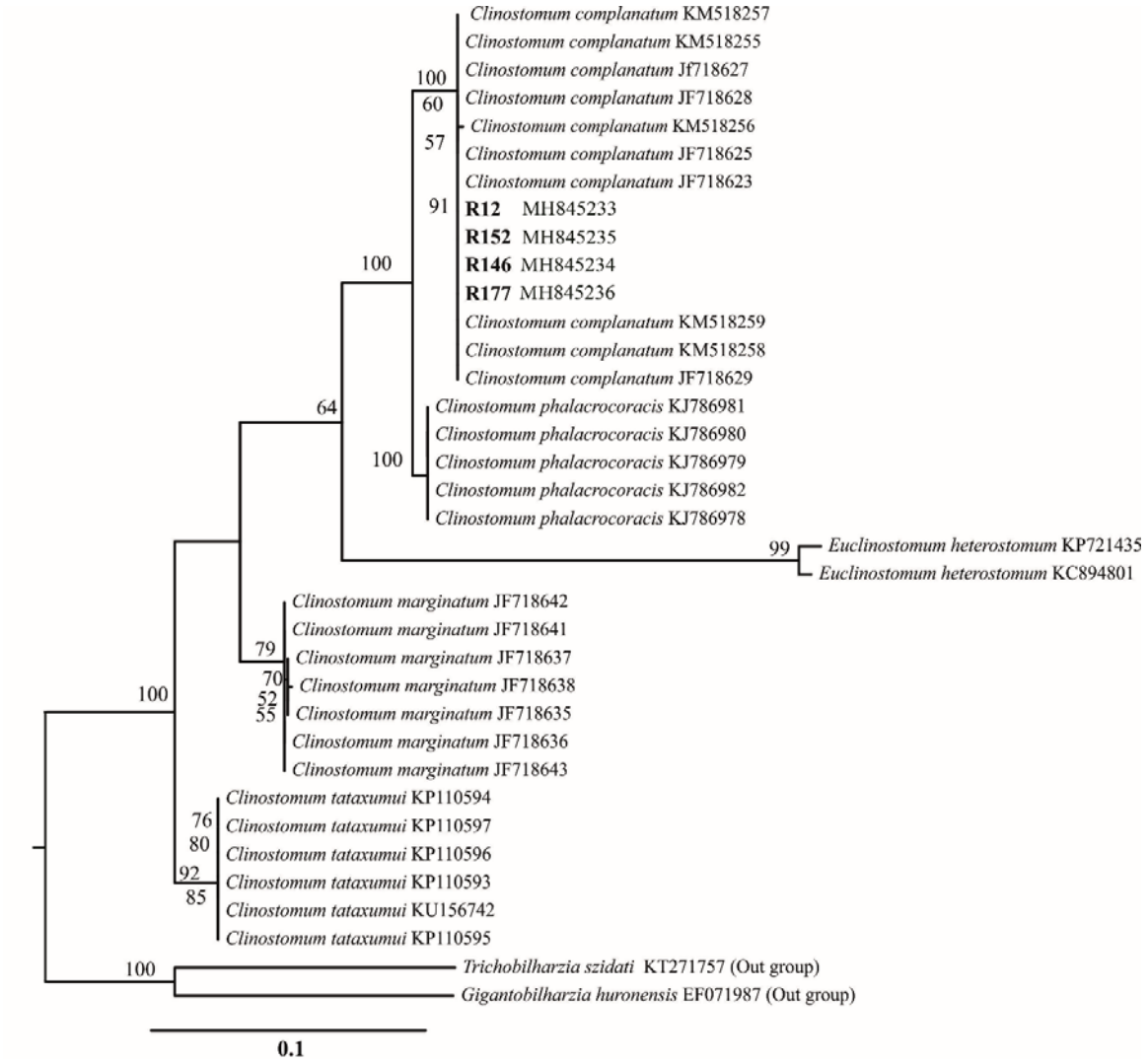

FIGURE 5. Phylogenetic tree inferred from Maximum Likelihood (GTR+ G model) of ITS sequences of *Clinostomum* spp. the numbers indicate the bootstrap support values from 1000 replicates, R (bold letter) correspond four sequences each of a species of fish used in the current study.

TABLE 3. The list of fish species infected with *Clinostomum complanatum* metacercaria in Iran, along with their family and localities in Iran

Host family	Host species	locality	reference
Cyprinidae	anonymous	West Azerbaijan	Rasouli & Pourghasem, 2016
Cyprinodontinae	*Aphanius dispar*	Hormuzgan	Gholami *et al.* 2011
Cyprinodontinae	*Aphanius sophiae,*	Tehran	Hosseini, 1987
Cyprinidae	*Carassius auratus,*		
Cyprinidae	*Barbus* sp.		
Cyprinidae	*Alburnoides bipunctatus*	Mazandaran	Pazooki & Mansourian, 2012
Cyprinidae	*Chalcalburnus chalcoides*	Guilan	Maleki & Malek, 2006
Cyprinidae	*Squalius cephalus*		
Gobiidae	*Neogobius fluviatilis*		
Cobitidae	*Cobitis taenia*		
Cyprinidae	*Capoeta Capoeta gracilis*	Mazandaran	Malek & Mobedi, 2001
Cyprinidae	*Pseudoraspora parva*		Ghazifard *et al.* 2010

Clinostomum complanatum has a complex life cycle. The presence of this parasite in a region depends on the availability of three host's species i.e. snails, fishes and birds, simultaneously. A lack of this parasite in the Gheshlagh reservoir reflects unsuitable region for the completion of the parasite life cycle. The reservoir is a very large water body with no vegetation around without the snails and birds to complete the parasites life cycle. The pattern observed here, is similar to the study that was conducted by Wang *et al.* (2017), which demonstrated that the dams usually do not possess the fishes infected with metacercariae. Instead, the rivers are more suitable for digenean trematode to compete their life cycle. Instead, the Gheshlagh River possesses a moderate climate and extensive vegetation around that provide a suitable environment for presence of the hosts.

The specimens found here, are smaller in comparison with previously reported specimens of *C. complanatum* (Caffara *et al.*, 2011; Li *et al.*, 2018; Simsek *et al.*, 2018). According to Caffara *et al.* (2014a), the traits in metacercariae can be affected by differences in the hosts and developmental stages. For example, the genital complex in the present study is located in the posterior end of middle third of the body. Such situation is similar with what is seen in metacercariae of *C. complanatum* by Gholami *et al.* (2011) and *C. tilapiae* by Caffara *et al.* (2017). Therefore, the genital complex position in metacercariae cannot be a reliable character in delaminating clinostomid species. The anterior testes here, is displaced by cirrus sac, typical to *C. complanatum* (Caffara *et al.*, 2011). The frontal view of the genital pore in SEM is lancoelate in shape and is located approximately in the middle of the body (Fig. 4D). The cuticular spines were observed on the tegument (Fig. 4F) which also have been reported in *C. complanatum* (Caffara *et al.*, 2011), *C. marginatum* and *C. attenuatum* (Caffara *et al.*, 2014b).

Molecular data have shown very useful in evaluating the taxonomic status of parasites, detecting cryptic species, phenotypic plasticity and linking larval forms to the adults (Perez-Pron De Leon *et al.*, 2016). The metacercariae taken of fish exhibit relatively the morphological characters near to the adult specimens. For this reason, the metacercariae have been described several times morphologically (Caffara *et al.*, 2011; Caffara *et al.*, 2014a; Wang *et al.*, 2017; Li *et al.*, 2018; Simsek *et al.*, 2018). In these cases, DNA sequences support the morphological description. ITS1 and ITS2 markers are used for species delamination for the most digenean families (Nolan & Cribb, 2005). The molecular identification using the ITS gene region here, determined the true identity of the metacercariae from the Gheshlagh River as *C. complanatum*.

The metacercariae of *C. complanatum* have been reported in several fish from Iran. There is no detailed study on the morphological and molecular characterizations of this species in Iran so far; only a few studies have addressed to the pathogenicity and ecological aspects of the metacercariae (Malek & Mobedi, 2001; Gholami *et al.*, 2011). The only report of *C. complanatum* as adult was by Shamsi *et al.* (2013), which reported this species in the four bird species in the North of Iran. This study is the first morphological and molecular characterization on *C. complanatum* metacercariae in freshwater fish of Iran. A total of 257 freshwater fish reported from Iran (Jouladeh-Roudbar *et al.*, 2015), with 11 species as host for *C. complanatum*. The potential hosts may be increased with more parasitological studies. The understanding of the biology and distribution of *C. complanatum* require the sampling efforts from all hosts i.e. snails as first intermediate host, freshwater fish as second intermediate host and fish-eating birds as final host at more geographic regions.

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