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Morphologic description and molecular characterization of *Trichobilharzia* species (Digenea: Schistosomatidae) infection in field-collected *Radix auricularia* (Linnaeus, 1758) in North of Iran

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Abstract

Lymnaeid snails are considered as intermediate hosts of *Trichobilharzia* species, the causal agents of avian schistosomiasis in wild and domestic birds in Iran and worldwide. The aim of the present study was to evaluate *Trichobilharzia* infection in field-collected snails of *Radix auricularia* (Linnaeus, 1758) in Guilan Province, northern Iran. The collected snails were examined for the cercarial stage of *Trichobilharzia* infection using the cercariae shedding method. Genomic DNA was extracted from collected cercariae and the soft tissue of *R. auricularia*. Cercariae were

identified by sequencing and phylogenetic analysis of the nuclear internal transcribed spacer 1 (ITS-1) region and the mitochondrial cytochrome oxidase 1 (*cox*1) gene. Snail hosts belonging to the genus *Radix* were identified based on sequencing and phylogenetic analysis of a partial fragment of the ITS-2 region of ribosomal DNA. Microscopically, *R. auricularia* snails were found to be infected with the cercarial stage of *Trichobilharzia* species (1%). Molecularly, 0.89% and 0.11% of the examined snails were found to be infected with *T. franki* and *T. regenti*, respectively. Based on the ribosomal loci, *T. franki* and *T. regenti* exhibited eight and seven haplotypes, respectively, whereas analysis of the mitochondrial gene revealed eight haplotypes in *T. franki* and two in *T. regenti*. These findings indicated *R. auricularia* served as an important role in maintaining the transmission cycle of *T. franki* and *T. regenti* in the region. Therefore, it is recommended to investigate the role of migratory waterfowl in the transmission of *Trichobilharzia* infection to native birds, as well as to implement preventive measures among the residents in the region.

Keywords: Avian schistosomiasis, *Radix auricularia*, *Trichobilharzia*, Iran

Introduction

Trichobilharzia (Digenea: Schistosomatidae) represents one of the largest genera within the family Schistosomatidae, comprising more than 40 recognized species that parasitize five orders of waterfowl and four families of freshwater snails (1). Recent studies on both definitive hosts (*Anas platyrhynchos*, *A. clypeata*, *Aythya ferina*) and intermediate hosts (*Lymnaea gedrosiana*, *L. stagnalis*, *Stagnicola palustris*, *Planorbis planorbis*) in Guilan and Mazandaran Provinces, northern Iran revealed the presence of three *Trichobilharzia* species infecting anatids and local snails (2, 3).

Freshwater snails play a pivotal role as intermediate hosts for a wide range of parasitic helminths, particularly trematodes, encompassing numerous species distributed worldwide. Among these, lymnaeid snails (Pulmonata: Basomatophora), commonly known as pond snails are of particular significance, as more than 20 species have been reported to act as intermediate hosts for 71 trematode species belonging to 13 families (4). Various freshwater snail genus within the families Lymnaeidae, Physidae, and Planorbidae are recognized as intermediate hosts of *Trichobilharzia* species. In Europe, members of the family Lymnaeidae, and in North America, both Lymnaeidae and Physidae, serve as key intermediate hosts (5).

Lymnaeid snails such as *Radix auricularia*, *L. ovata*, *L. stagnalis*, *L. peregra*, and *S. palustris* play important role in the life cycle of *T. szidati* and *T. franki* (6–8). In Iran, *Galba truncatula*, *S. palustris*, *L. schiraziensis*, and *R. auricularia* reported as intermediate hosts for *Trichobilharzia* species (9). The prevalence of infection by *Trichobilharzia* furcocercariae among different snail species ranges from 0.17% to 2.4% (10). *Radix auricularia* infection with *T. szidati* (21.56%) and *T. franki* (9.69%) was first reported from northwestern Iran by Yakhchali et al. (2016) (11).

In contrast to other regions of the world, infection of *Physa acuta* with *Trichobilharzia* furcocercariae has not yet been recorded in Iran (12). Consequently, *Physa* species appear to be unimportant or unsuitable intermediate hosts for nasal schistosomes in Iran; however, they may act as potential hosts for visceral schistosomes (3), a hypothesis that warrants further investigation. Infection with other types of furcocercariae has been observed in *P. gyrina* by Athari et al. (2006) (2).

Human cercarial dermatitis (HCD), caused by the penetration of avian schistosomes cercariae, recognized as a significant occupational and recreational disease in the Middle East, Europe, and North America (13). Guilan Province, the principal rice-growing area in northern Iran, provided a

suitable ecological environment for the transmission of avian schistosomes. Migratory waterfowl such as wild ducks, swans, and geese serve as definitive hosts and may play a major role in introducing and maintaining the infection following their seasonal migration from Europe to northern Iran (3)

Understanding the diversity of *Trichobilharzia* species, along with their intermediate and definitive hosts, is a crucial prerequisite for designing effective control programs (13). In this context, assessing *Trichobilharzia* infection in *R. auricularia* snails from northern Iran is of particular importance. Molecularly, Yakhchali et al. (2016) documented avian schistosomes' infections in *R. auricularia* and *L. gedrosiana* in the country (11). So far, molecular characterization of *Trichobilharzia* larvae isolated from *R. auricularia* has not been reported. Thus, the present study was aimed to detect and characterized *Trichobilharzia* infections in field-collected *R. auricularia* from Guilan Province, northern Iran

Materials and Methods

Study Area and Snail Collection

The province of Guilan is located in northern Iran (36°33′ to 38°27′N and 48°32′ to 50°36′E), at an altitude of approximately -27 to 300 m above sea level (ASL), with numerous water bodies and reservoirs that provide suitable habitats for freshwater snails (Fig. 1). The province experiences four seasons: winter (January to March), spring (March to June), summer (July to September), and fall (October to December), with an average annual rainfall of 1500–1900 mm and average humidity of about 80%. The study area has a semi-temperate, wet climate, with coastal plains, foothills, and forested mountains. The coastal plains along the Caspian Sea and near the foothills are primarily used for rice cultivation. After rice harvesting, from early August to late September, the rice paddies become suitable areas for raising livestock and domestic ducks, which also provide

appropriate habitats for freshwater snails (14). Field-collected lymnaeid snails were randomly sampled from reservoirs, canals, agricultural lands, and rivers in both mountainous and plain areas of Guilan Province. The snails were placed into plastic screw-capped containers and transferred alive to the Parasitology Laboratory of the School of Medicine, Guilan University of Medical Sciences. A total of 39,486 freshwater snails belonging to various genera (19,726 *R. auricularia*, 4,911 *S. palustris*, and 14,849 *P. acuta*) from different regions were collected. Snails were identified using the morphometric identification key described by Glöer (Fig. 2) (15).

Cercariae Collection and Identification

Radix auricularia snails were examined for the presence of cercarial infection using the shedding method, as described by Faltýnková et al. (2008) (16). Briefly, the collected snails were individually placed in glass Petri dishes containing 50 mL of dechlorinated tap water for 12 h under

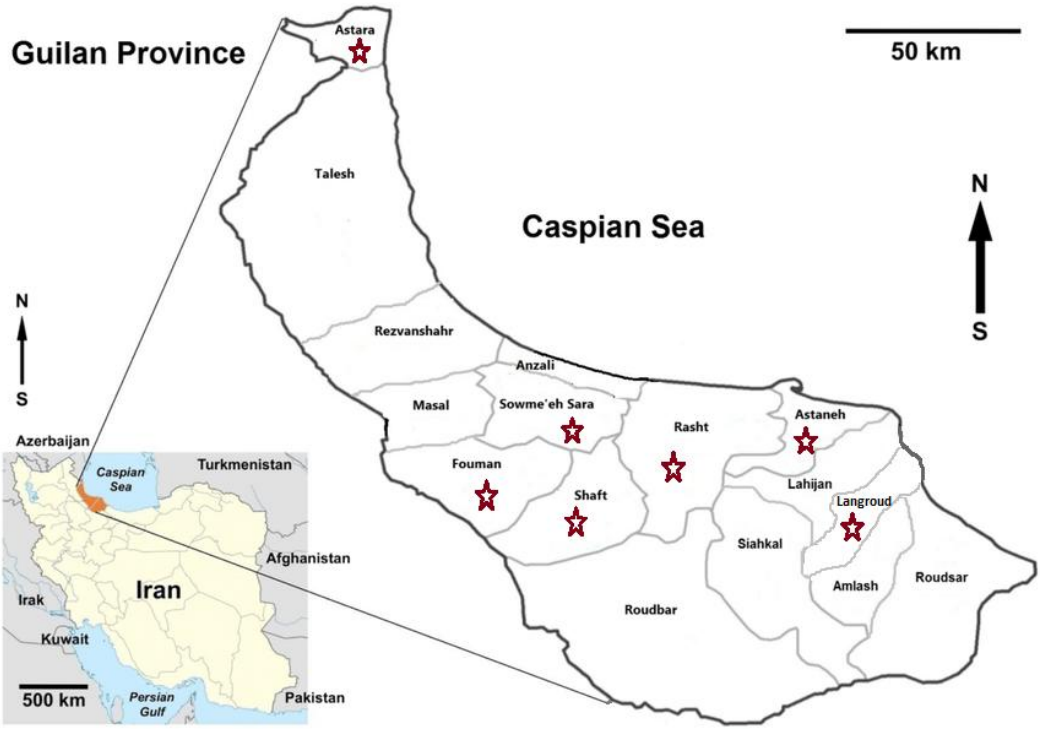

Fig. 1 Map of Iran, showing the geographical location of Guilan Province, with the stars indicating the infected districts from which *Radix auricularia* snails were collected.

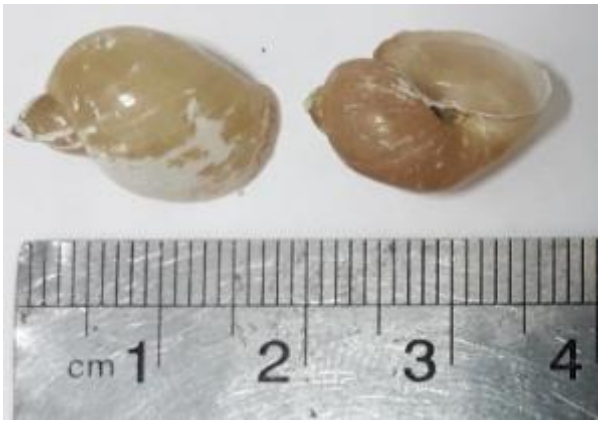

Fig. 2 Shell of *Radix auricularia* collected from Guilan province, northern Iran.

a 100-W light bulb at 20°C to induce cercarial shedding. The collected cercariae were stained with 0.5% neutral red and examined under a light microscope (BX50F4, Olympus, Tokyo, Japan) equipped with a digital camera (TrueChrome Metrics, AMADA Instrument Co., Nanjing, China). Cercariae were characterized using morphologic and morphometric examinations, as described by Podhorský et al. (2009) (17).

Molecular Procedures

DNA Extraction

Genomic DNA of *Trichobilharzia* species was extracted from collected cercariae and the soft tissue of *R. auricularia* using the instructions provided with the DNA extraction kit (Viragen, Tehran, Iran) and stored at −20°C until molecular analyses.

Polymerase Chain Reaction (PCR) Amplification of ITS-1 rDNA, ITS-2 rDNA, and *cox*1 mtDNA

Primers BD1 (5′-GTCGTAACAAGGTTTCCGTA-3′) and 4S (5′-TCTAGATGCGTTCGAARTGTCGATG-3′) were used to amplify ITS-1 rDNA fragments of *T. franki* (1,123 bp) and *T. regenti* (1,500 bp) (18). Primers Cox1_SchistoF (5′-TCTTTRGATCATAAGCG-3′) and Cox1_SchistoR (5′-TAATGCATMGGAAAAAAACA-3′)

were used to amplify a 1,250 bp fragment of the *cox*1 mtDNA gene of *T. franki* and *T. regenti* (19). In addition, primers LT1 (5′-TCGTCTGTGTGAGGGTCG-3′) and ITS1RIXO (5′-TTCTATGCTTAAATTCAGGGG-3′) were used to amplify a 500 bp fragment of the ITS-2 rDNA of *R. auricularia* (20).

The PCR was performed in a 30 µL reaction mixture containing 3 µL (100 ng) of genomic DNA (diluted 1:30), 15 µL of 2× Master Mix (RED Amplicon, Denmark), 10 pmol of each primer, and 10 µL of distilled water. The temperature profile for the ITS-1 region consisted of an initial denaturation at 95 °C for 6 min, followed by 30 cycles consisting of a denaturation step at 95 °C for 45 s, an annealing step at 55 °C for 60 s, an extension step at 70 °C for 1 min, and a final extension at 72 °C for 6 min. The PCR conditions for the *cox*1 gene involved an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 52 °C for 30 s, an extension step at 72 °C for 2 min, and a final extension at 72 °C for 7 min. The thermal PCR profile for the ITS-2 region consisted of an initial denaturation step at 94 °C for 2 min, followed by 30 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 50 °C for 30 s, an extension step at 72 °C for 1 min, and a final extension at 72 °C for 7 min. Next, the PCR products were separated by electrophoresis on a 1.5% agarose gel and visualized using a UV Transilluminator (UVITEC, Cambridge, UK). Subsequently, the PCR products were sent to a domestic sequencing company (Codon Genetic Company, Tehran, Iran) for sequence determination using the Sanger method.

Phylogenetic analyses

The sequence data were assembled and edited using BioEdit and analyzed with Chromas (version 2.6.6; Technelysium Pty Ltd., Brisbane, Queensland, Australia). Sequence divergence among the isolates was calculated using MEGA-VI. The sequences obtained in the present study were aligned

and compared with the deposited ITS-1 rDNA, ITS-2 rDNA, and *cox*1 sequences in GenBank using ClustalW. Phylogenetic trees were constructed to scale using the Tamura-Nei model in MEGA-VI (21). The statistical robustness of the phylogenetic trees was assessed using bootstrap analysis with 1,000 replications. To evaluate the statistical support for branching orders, bootstrap analysis with 1,000 replicates was performed. Evolutionary distances were calculated using the maximum composite likelihood model implemented in MEGA-VI. Indices of genetic diversity including the number of haplotypes, polymorphic sites, nucleotide diversity, and haplotype diversity in the ITS-1 region and *cox*1 gene were calculated using DnaSP version 6.0 for *Trichobilharzia* species.

Results

Microscopic examination findings

Of 7,359 *R. auricularia* collected from the study areas, 74 snails (1%) were found to be infected with the cercarial stage of *Trichobilharzia* species (Fig. 3) (Table 1). *Trichobilharzia* cercariae did not detect from the collected *S. palustris* and *P. acuta* snails. Morphologically, the furcocercariae of *Trichobilharzia* were characterized by a pair of eyespots, a bifurcated tail stem at the posterior end, minute spines covering the body surface, and a spiny sheath surrounding the tail region. The organelles within the body of the cercariae were also visualized (Fig. 4). The morphometric characteristics of the identified *T. franki* and *T. regenti* are summarized in table 2. The excretory system consists of seven pairs of flame cells (2[3 + 3 + (1)]) and two pairs of ciliary patches within the collecting ducts. The cercariae possess five pairs of penetration glands: two pairs located dorsally above the acetabulum and three pairs situated towards the distal part of the body.

Table 1 The prevalence of *Trichobilharzia* infection and species diversity of identified furcocercariae in localities infected of Guilan province, northern Iran.

District (sampling location) Coordinates (latitude/longitude)	No. of examined *R. auricularia*	Microscopic examination (%)	PCR of ITS-1 rDNA region (%)	
			T. franki	*T. regenti*
Fuman (Fallahabad) (37˚16'12.0"N 49˚22'04.7"E)	464	1 (0.21)	1 (0.21)	0
Shaft (Rasteh Kenar) (37˚17'32.6"N 49˚27'04.9"E)	978	2 (0.2)	2 (0.2)	0
Someh Sara (Nokhaleh) (37˚20'55.1"N 49˚29'57.4"E)	1,174	3 (0.25)	3 (0.25)	0
Someh Sara (Kalasham) (37˚15'47.0"N 49˚24'04.7"E)	1,242	15 (1.2)	9 (0.72)	6 (0.48)
Langarud (Kumeleh) (37˚09'31.1"N 50˚09'47.1"E)	877	5 (0.57)	5 (0.57)	0
Rasht (Eshkik) (37˚20'47.0"N 49˚37'00.7"E)	543	28 (5.15)	26 (4.79)	2(0.36)
Rasht (Pirbazar) (37˚21'06.3"N 49˚32'31.6"E)	990	4 (0.4)	4 (0.4)	0
Astaneh (Lasku kalayeh) (37˚22'01.2"N 50˚01'21.3"E)	617	13 (2.1)	13 (2.1)	0
Astara (Lavandevil) (38˚17'35.2"N 48˚52'26.6"E)	474	3 (0.63)	3 (0.63)	0
Total	7,359	74 (1)	66 (0.89)	8 (0.11)

Table 2 Morphometric values of identified furcocercariae of *Trichobilharzia* species from different parts of Guilan province, northern Iran.

Morphometric characters	*T. franki* This study	*T. franki* Podhorský et al.	*T. franki* Brant & Loker	*T.regenti* this study	*T. regenti* Podhorský et al.	*T. regenti* Horák et al.
Body length	290 ± 40	316 ± 14	307	289 ± 41	333 ± 48	225 ± 32
Body width	88 ± 10	67 ± 7	ND	93 ± 9	78 ± 14	55 ± 5
Tail stem length	373 ± 34	480 ± 30	419	357 ± 25	421 ± 31	331 ± 20
Tail stem width	49 ± 5	55 ± 5	ND	50 ± 6	59 ± 9	49 ± 9
Furca length	259 ± 21	318 ± 18	234	238 ± 23	285 ± 23	206 ± 21
Total length	923 ± 79	1114 ± 51	ND	886 ± 68	1039 ± 81	759 ± 49
Head organ length	83 ± 11	75 ± 6	ND	79 ± 11	76 ± 14	77 ± 4
Head organ width	59 ± 7	53 ± 4	ND	62 ± 6	58 ± 6	44 ± 3
Anterior end to acetabulum	179 ± 24	223 ± 18	ND	187 ± 30	219 ± 38	124 ± 13
Anterior end to eye spot	124 ± 15	150 ± 10	ND	119 ± 15	145 ± 20	81 ± 4
Eye spot diameter	9 ± 1	ND	ND	9 ± 1	ND	ND
Acetabulum diameter	32 ± 6	ND	ND	34 ± 6	ND	27
Body length/total length	0.31 ± 0.03	0.28 ± 0.01	ND	0.32 ± 0.03	0.32 ± 0.03	ND
Tail stem length/total length	0.4 ± 0.02	0.43 ± 0.01	ND	0.4 ± 0.02	0.41 ± 0.02	ND
Furca length/total length	0.28 ± 0.01	0.29 ± 0.01	ND	0.26 ± 0.02	0.28 ± 0.02	ND

Acetabulum to anterior end/body length ratio	0.61 ± 0.05	0.71 ± 0.05	ND	0.64 ± 0.06	0.66 ± 0.05	ND
Eye to anterior end/body length ratio	0.42 ± 0.03	0.48 ± 0.02	ND	0.41 ± 0.03	0.44 ± 0.03	ND
Head organ width and length ratio	0.71 ± 0.04	0.71 ± 0.12	ND	0.78 ± 0.04	0.79 ± 0.15	ND
Body width and length ratio	0.3 ± 0.02	0.21 ± 0.02	ND	0.32 ± 0.02	0.24 ± 0.07	ND
Tail stem width and length ratio	0.13 ± 0.01	0.11 ± 0.01	ND	0.14 ± 0.01	0.14 ± 0.02	ND

ND= No data

Fig. 3 Furcocercariae of *Trichobilharzia franki* obtained from 20×.

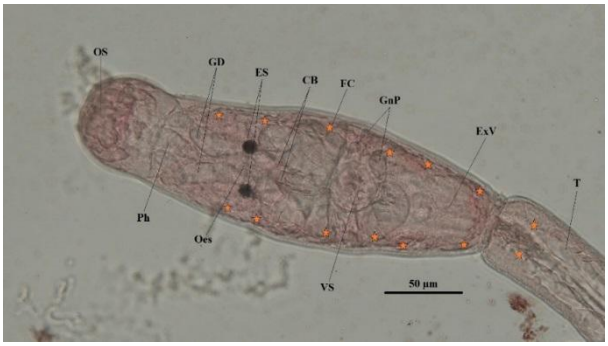

Fig. 4 Furcocercariae of *Trichobilharzia franki*. The stars indicate the location of flame cells, 2 [3+3+ (1)] =14, which has been marked based on the live video of furcocercariae using an Olympus Microscope equipped with a digital camera (TrueChrome Metrics, China). OS (Oral Sucker); Ph

(Pharynx); GD (Glands ducts); ES (Eye Spots); Oes (Oesophagus); CB (Caecal Bifurcation); FC (Flame Cell); GnP (Genital Primordium); VS (Ventral Sucker); ExV (Excretory Vesicle); T (Tail).

Molecular Findings

Based on PCR analysis of the ITS-1 rDNA region, 66 (0.89%) and 8 (0.11%) of the 7,359 identified *R. auricularia* snails were found to be infected with *T. franki* and *T. regenti*, respectively (Table 1). Sequences of the ITS-1 rDNA region (accession numbers: OP821154–OP821219 for *T. franki* and OP821220–OP821227 for *T. regenti*), the ITS-2 rDNA region (accession numbers: OP868734–OP868745 for *R. auricularia*), and the *cox*1 mtDNA gene (accession numbers: OP740584–OP740646 for *T. franki* and OP740647–OP740655 for *T. regenti*) were deposited in the GenBank® database.

In the ITS-1 rDNA region, the intraspecific divergence among isolates of *T. franki* and *T. regenti* ranged from 0–0.8% and 0–1.1%, respectively. For the *cox*1 mtDNA gene, intraspecific divergence was 0–0.7% for both *T. franki* and *T. regenti*. The intraspecific variation in the ITS-1 rDNA region among haplotypes of *T. franki* and *T. regenti* ranged from 0.1–0.8% and 0.2–1.1%, respectively, while in the *cox*1 mtDNA gene it ranged from 0.2–0.7% for both species. Comparisons of ITS-1 rDNA sequences with other isolates deposited in GenBank® revealed intraspecific divergence of 0–4.5% for *T. franki* and 0–2.9% for *T. regenti*. Similarly, comparisons of *cox*1 mtDNA sequences with GenBank® isolates showed intraspecific divergence of 0–0.9% for *T. franki* and 0–3.3% for *T. regenti*.

Haplotype analysis of the ITS-1 rDNA region revealed that 66 isolates of *T. franki* comprised eight haplotypes, with 12 polymorphic sites. Of those, 54 isolates belonged to a single haplotype, while the remaining isolates were distributed across the other seven haplotypes (Table 3). In *T.*

regenti, eight isolates represented seven haplotypes with 22 polymorphic sites; one haplotype included two isolates, while the remaining six consisted of a single isolate each (Table 4).

Table 3 Haplotype grouping for *Trichobilharzia franki* isolates based on ITS-1 and *cox*1 accession number of isolates in each group.

Gene	Haplotype	Number of isolates	Accession number
ITS-1	1	1	OP821154
	2	6	OP821155, OP821164, OP821169, OP821186, OP821196, OP821202
	3	54	OP821156, OP821157, OP821158, OP821159, OP821160, OP821161, OP821162, OP821163, OP821165, OP821166, OP821167, OP821168, OP821170, OP821171, OP821172, OP821174, OP821175, OP821176, OP821177, OP821178, OP821179, OP821180, OP821181, OP821182, OP821183, OP821184, OP821185, OP821187, OP821189, OP821190, OP821191, OP821192, OP821194, OP821195, OP821197, OP821198, OP821199, OP821200, OP821201, OP821203, OP821204, OP821205, OP821206, OP821207, OP821209, OP821211, OP821212, OP821213, OP821214, OP821215, OP821216, OP821217, OP821218, OP821219
	4	1	OP821173
	5	1	OP821188

	6	1	OP821193
	7	1	OP821208
	8	1	OP821210
*cox*1	1	43	OP740584, OP740587, OP740588, OP740589, OP740592, OP740593, OP740594, OP740595, OP740597, OP740598, OP740600, OP740601, OP740604, OP740608, OP740609, OP740610, OP740612, OP740613, OP740614, OP740615, OP740617, OP740620, OP740621, OP740622, OP740624, OP740626, OP740627, OP740628, OP740629, OP740631, OP740632, OP740633, OP740635, OP740636, OP740637, OP740638, OP740639, OP740641, OP740642, OP740643, OP740644, OP740645, OP740646
	2	3	OP740586, OP740607, OP740618
	3	2	OP740590, OP740591
	4	2	OP740596, OP740640
	5	2	OP740605, OP740606
	6	1	OP740616
	7	1	OP740619
	8	1	OP740634

In the *cox*1 mtDNA gene, haplotype analysis revealed eight haplotypes with nine polymorphic regions among 55 isolates of *T. franki*. Of these, 43 isolates belonged to a single haplotype, while the remaining isolates were distributed across the other seven haplotypes (Table 3). In *T. regenti*,

two haplotypes with two polymorphic regions were observed among eight isolates, each haplotype comprising four isolates (Table 4).

Table 4 Haplotype grouping for *Trichobilharzia regenti* isolates based on ITS-1 and *cox*1 accession number of isolates in each group.

Gene	Haplotype	Number of isolates	Accession number
ITS-1	1	1	OP821220
	2	1	OP821221
	3	1	OP821222
	4	1	OP821223
	5	2	OP821224, OP821227
	6	1	OP821225
	7	1	OP821226
*cox*1	1	4	OP740647, OP740648, OP740649, OP740654
	2	5	OP740650, OP740651, OP740652, OP740653, OP740655

Phylogenetic analysis of the ITS-1 rDNA region, with a bootstrap value of 97% (Fig. 5), showed that the haplotypes of *T. franki* clustered with reference sequences from *T. franki* isolates obtained from *A. platyrhynchos* in Iran (MH410291, MH410293); *R. auricularia* from Italy (MK053632), Switzerland (AJ312041), the Czech Republic (AF356845, AY713969), the United Kingdom (KJ775868, KJ775869), and Poland (AY713964); as well as *L. peregra* from France (HM131180, HM131184) and Iceland (FJ469805).

Fig. 5 Phylogenetic tree of furcocercariae of *Trichobilharzia franki* obtained in this study and reference sequences of avian schistosomes retrieved from the GenBank® based on ITS-1 rDNA nucleotide sequences. The tree was rooted using *Trichobilharzia regenti* (accession no. EF094535) as the out-group. The scale shows the number of nucleotide substitutions per site between DNA sequences.

Phylogenetic reconstruction based on partial ITS-1 rDNA sequences showed that the haplotypes of *T. regenti* clustered with reference sequences, with a bootstrap value of 99% (Fig. 6), containing *T. regenti* isolates obtained from *A. platyrhynchos* in Iran (MH198311–MH198314, MH410290), the Czech Republic (GU233740, EF094538), Poland (EF094535), Switzerland (AJ312049), and France (HM439494). They also clustered with *A. clypeata* from the Czech Republic (EF094540), *Cygnus olor* from France (HM439497), *A. fuligula* from Poland (EF094534), *Mergus merganser*

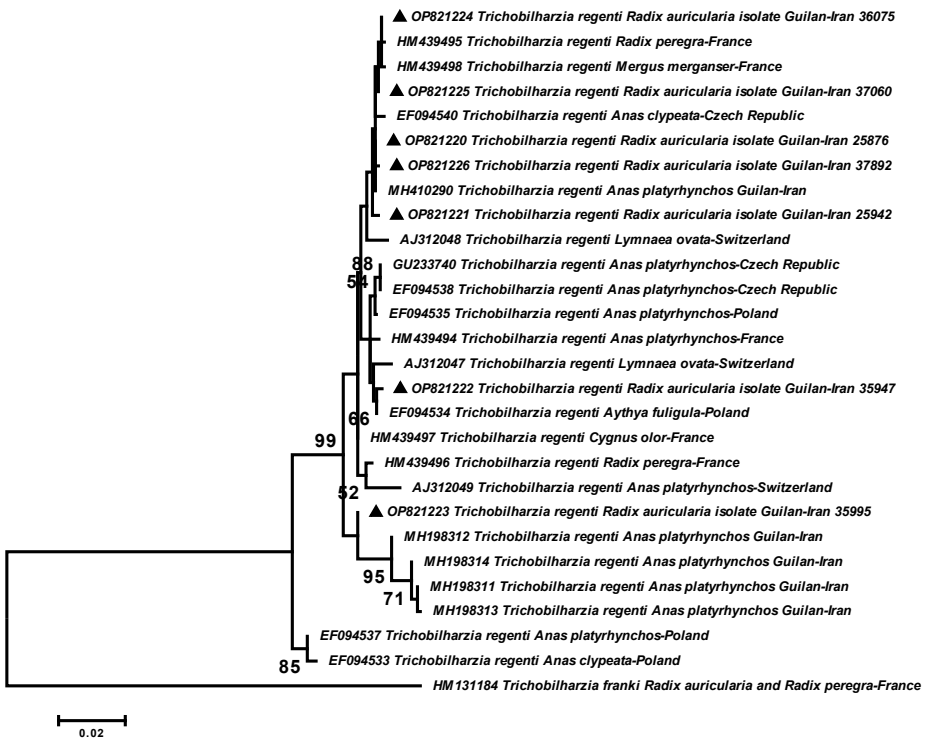

Fig. 6 Phylogenetic tree of furcocercariae of *Trichobilharzia regenti* obtained in this study and reference sequences of avian schistosomes retrieved from the GenBank® based on ITS-1 rDNA nucleotide sequences. The tree was rooted using *Trichobilharzia franki* (accession no. HM131184) as the out-group.

from France (HM439498), and isolates from *R. peregra* in France (HM439495, HM439496) and *L. ovata* from Switzerland (AJ312047, AJ312048). In contrast, isolates of *T. regenti* from Poland obtained from *A. platyrhynchos* (EF094537) and *A. clypeata* (EF094533) formed a sister group to this cluster.

Phylogenetic analysis of the *cox*1 gene in the present study showed that the haplotypes of *T. franki* clustered with reference sequences from previous studies, with 99% statistical support (Fig. 7). Additionally, the haplotypes of *T. regenti* formed a single branch with isolates from Iran and other countries, supported by a bootstrap value of 99% (Fig. 7).

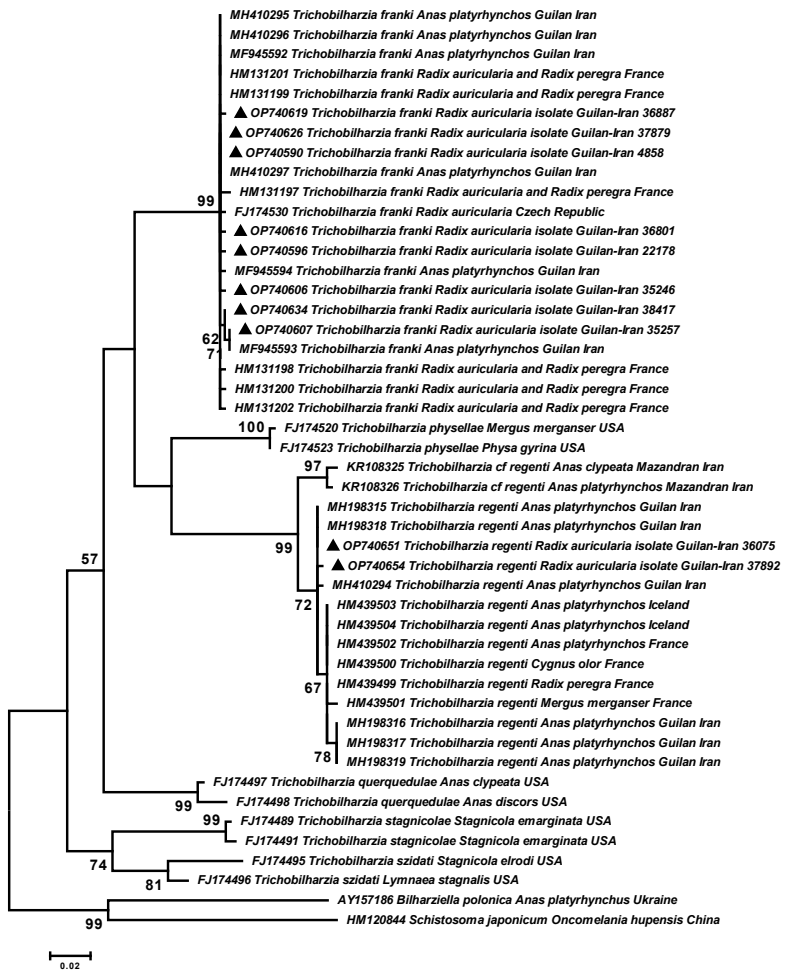

Fig. 7 Phylogenetic tree of furcocercariae of *Trichobilharzia* species obtained in this study and reference sequences of avian schistosomes retrieved from the GenBank® based on *cox*1 mtDNA nucleotide sequences. The tree was rooted using *Bilharziella polonica* (accession no. AY157186) and *Schistosoma japonicum* (accession no. HM10844) as the out-group.

Phylogenetic tree of *R. auricularia* based on ITS-2 rDNA region (Fig. 8) showed with a bootstrap value of 99% that the *R. auricularia* isolates from this study clustered with sequences from Spain (AJ319628), Italy (MK053633), Denmark (KP271016), France (HQ003224), the Czech Republic (GU574307), and Russia (OM240701).

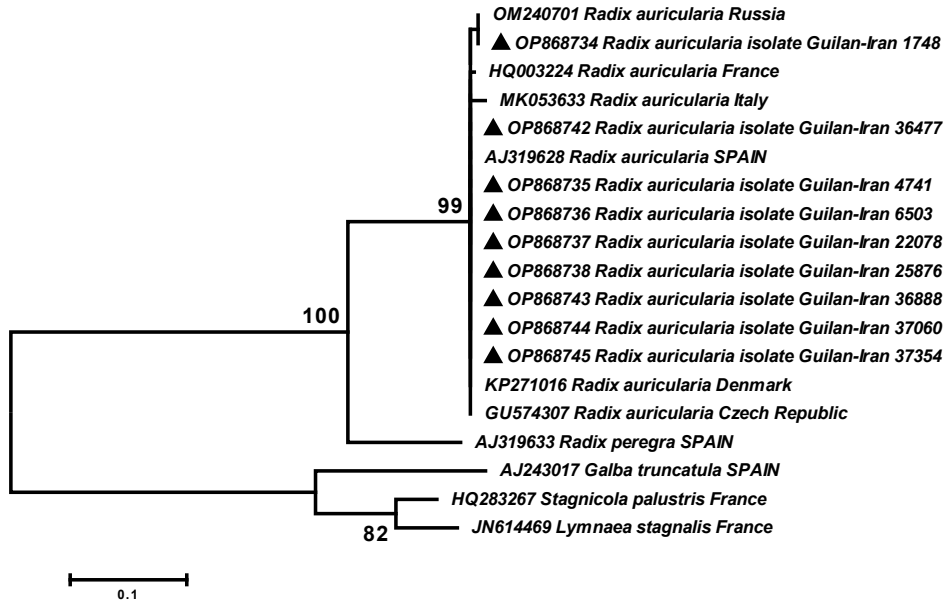

Fig. 8 Phylogenetic tree of *Radix auricularia* obtained in this study and reference sequences retrieved from the GenBank® based on ITS-2 rDNA nucleotide sequences. The tree was rooted using *Radix peregra* (accession no. AJ319633), *Galba truncatula* (accession no. AJ243017), *Stagnicola palustris* (accession no. HQ283267), and *Lymnaea stagnalis* (accession no. JN14469) as the out-group.

Discussion

Furcocercariae of bird schistosomes, particularly *Trichobilharzia* species are the most common cause of HCD in northern and southern Iran (22). *Ornithobilharzia turkestanicum* also reported as another causative agent of human cercarial dermatitis around the Caspian Sea and northwestern Iran (23, 24). Snails of the families Lymnaeidae, Physidae, and Planorbidae serve as the main intermediate hosts of bird schistosomes, while other freshwater snail families, including Ampullariidae, Pleuroceridae, Valvatidae, Littorinidae, and Hydrobiidae, have a lesser role in transmission (25).

In recent studies, a high prevalence of adult *T. regenti* and *T. franki* helminths reported in domestic ducks in Guilan Province; however, no studies have investigated their intermediate hosts

in this region (14, 26). In this study, *R. auricularia* snail, identified for the first as an intermediate host of *T. regenti* and *T. franki* in North of Iran. Likewise, the role of *R. auricularia* in transmission of *T. franki* reported in other regions of the world (7). *Radix auricularia* snail is highly adapted to permanent, still water bodies, such as rice fields, small streams, and agricultural canals in the lowlands of Guilan, at altitudes ranging from –27 to 100 m ASL. *Radix auricularia* also reported as an intermediate host of *Fasciola gigantica* in areas where *T. regenti* and *T. franki* were prevalent (27). Additionally, *Trichobilharzia* furcocercariae infections reported in *L. gedrosiana* (0.08%), *S. palustris* (0.06%), and *L. stagnalis* (0.04%) in neighboring province of Mazandaran, located east of Guilan (2). Karamian et al. (2011) noted that *Melanoides tuberculata* was another intermediate host of avian schistosomes in Iran (28). Global reports indicated prevalence in snail intermediate hosts of *Trichobilharzia* species ranges from 0.05% to 52.4% (29). In current study, the prevalence of *T. regenti* cercariae in *R. auricularia* was 0.11%, lower than the 2% reported by Horák et al. (1998) (30). To our knowledge, this represented the only report of *R. auricularia* infection with *T. regenti* in Europe. In the Far East, prevalence of *T. regenti* in *R. rubiginosa* was 1.2% (31). In contrast, *T. franki* was more prevalent in *R. auricularia* (0.89%), exceeding the prevalence reported in the same species by Ferte et al. (2005) (0.05%) and in *L. pregra* by Jouet et al. (2008) (0.07%) (32, 33). In Iceland, the prevalence of *T. franki* furcocercariae infection in *L. peregra* was 1.4% (34), whereas in Germany, it ranged from 11% to 25% in *R. auricularia* (7). In Italy, Cipriani et al. (2011) and De Liberato et al. (2019) reported prevalence of *T. franki* infection in *R. auricularia* as 9.6% and 1.2%, respectively (20, 35). The reported prevalence were considerably higher than those observed in the present study. Low infection rates of trematode larval stages in the first intermediate snail hosts appeared to be a common finding, as some snails may not have reached the cercarial shedding stage at the time of sampling. Nevertheless, the long lifespan of

snails, coupled with their ability to shed thousands of cercariae daily, allows the infection of many definitive hosts, thereby sustaining the parasite's life cycle (36). Interestingly, *Trichobilharzia* species show a strong host specificity for snails, driven by the host-finding behavior of their miracidia and the limited range of suitable intermediate hosts (35). Differences in the prevalence of *Trichobilharzia* larval stages in intermediate hosts may be also influenced by multiple factors, including water temperature, alkalinity, salinity, eutrophication, snail size, population density, immunological susceptibility, physiological suitability, bird population dynamics, seasonality, and diagnostic methods (29, 37-39). In this wirk, the main factors affecting cercarial prevalence were: (a) sampling across all seasons, (b) collection from diverse water sources (rivers, agricultural canals, rice fields, and ponds), and (c) variation in the abundance of definitive avian hosts at the sampling sites.

Sampling was conducted year-round to cover all seasons. In some months, no cercariae were detected in the intermediate host snails. Consequently, the reported prevalence represented an annual average, which was generally lower than those observed in some endemic areas worldwide. The results also indicated that the distribution of snail intermediate hosts was not uniform across different water sources. The highest prevalence, along with the greatest abundance of intermediate and definitive avian hosts (both domestic and wild), was recorded in rice fields and areas near human settlements, thereby increasing the risk of human exposure to cercarial dermatitis.

Physa acuta snail is a globally invasive freshwater snail that has dispersed from North America to all continents except Antarctica (40–42). *Trichobilharzia* infections in *Physa* species have been predominantly reported from their native range in North America, with only sporadic records from other regions, possibly due to differences in ecological conditions or host–parasite compatibility. Nevertheless, additional investigations on *Physa* snails from various regions are required to better

elucidate their role in the transmission of *Trichobilharzia* species worldwide (2, 3). In contrast, *S. palustris* appears to have a broader geographical involvement in *Trichobilharzia* transmission across different regions. In current investigation, there was no *Trichobilharzia* cercariae infection in *P. acuta* similar to Athari et al. (2006) and Ghohardehi et al. (2013) in the neighboring province of Mazandaran, Iran (2, 3). These findings collectively suggested that *Physa* snails appeared unlikely to play a significant role in the transmission of *Trichobilharzia* species in endemic regions of northern Iran. None of the 4,911 *S. palustris* snails examined in this work was also found to be infected with *Trichobilharzia* cercariae. While, Athari et al. (2006) and Gohardehi et al. (2013) previously reported low infection rates of 0.06% (3/3,934) and 4.3% (2/53) for *Trichobilharzia* species in Mazandaran Province. Therefore, *S. palustris* could potentially serve as an intermediate host for *Trichobilharzia* spp., under suitable ecological conditions in northern Iran.

The morphology of the excretory system is one of the most important criteria for cercarial identification. All *Trichobilharzia* species share the same flame cells formula, 2[3+3+(1)] =14, except *T. corvi* (2[3+2+(1)] =12) and *T. arcuata* (2[3+4+(1)] =16) (43, 44). The flame cell formula observed in this study was identical to those reported for most *Trichobilharzia* species. In total, 14 flame cells were observed in cercariae of *T. franki* and *T. regenti*. One pair of flame cells was located in the upper part of the tail, close to the posterior end of the cercarial body; three pairs were situated posterior and three pairs anterior to the ventral sucker.

In this study, the total length of *T. franki* cercariae (923 ± 79 µm) was slightly greater than that of *T. regenti* (886 ± 67 µm). When compared with previous studies, both *T. franki* and *T. regenti* cercariae were smaller than those reported by Horák (17), which documented lengths of 1,114 µm for *T. franki* and 1,039 µm for *T. regenti*. Interestingly, Horák et al. (1998) reported a total length of 759 µm for *T. regenti*, considerably smaller than the measurements observed in the present

study (30). These findings indicated notable intraspecific variation in cercarial size, likely influenced by environmental conditions, host-related variables, or methodological differences in measurement. Although the size of certain *Trichobilharzia* cercariae has historically been used to define new species (Szidat, 1942) (45), recent studies have shown that multiple factors including snail size, temperature fluctuations, cercarial shrinkage, and the use of fixatives can significantly influence schistosomes cercarial measurements (7). As a result, morphological dimensions that were traditionally relied upon for species identification and description have proven to be less reliable in avian schistosomes (43, 46).

The loci of the ITS-1 rDNA and *cox*1 mtDNA gene have proven to be reliable molecular markers for assessing sequence divergence among avian schistosomes (47). In the present study, molecular findings demonstrated the presence of both *T. franki* and *T. regenti* in field-collected *R. auricularia*. The haplotype diversity and number of polymorphic sites in the ITS-1 rDNA region (*T. franki* = 0.326; *T. regenti* = 0.964) were higher than those in the *cox*1 mtDNA gene (*T. franki* = 0.388; *T. regenti* = 0.556). These results are consistent with previous reports (14, 47).

Phylogenetic analysis of the ITS1 locus revealed that the *T. franki* haplotypes identified in the present study clustered within a large group with strong support (97% bootstrap), together with isolates obtained from *A. platyrhynchus* in Iran, *R. auricularia* from Italy, Switzerland, the Czech Republic, the UK, Poland, and *L. plegora* from France and Iceland. Haplotype 2 exhibited 100% similarity to the Iranian isolate (MH410293) from *A. platyrhynchus*, whereas haplotype 3 was 100% identical to both the Italian isolate from *R. auricularia* (MK053632) and the Iranian isolate from *A. platyrhynchus* (MH410291). Haplotype 5 showed 100% similarity with *T. franki* (AJ312041) from *R. auricularia* in Switzerland.

The phylogenetic tree based on the ITS1 locus indicated that our haplotypes of *T. regenti* identified in the present study were placed in a large cluster with a 99% bootstrap value, together with *T. regenti* isolates from *A. platyrhynchus* in Iran, the Czech Republic, Poland, Switzerland, and France. Additionally, *T. regenti* isolates from *A. clypeata* in the Czech Republic, *C. olor*, *A. fuligula*, *M. merganser*, and *L. plegora* from France, as well as *L. ovata* from Switzerland, were also included in the same cluster. In contrast, *T. regenti* isolates obtained from *A. platyrhynchus* and *A. clypeata* in Poland showed a sister relationship with this large cluster. In our study, haplotype 1 exhibited 100% similarity to the Iranian isolate (MH410290) obtained from *A. platyrhynchus*.

Based on the phylogenetic tree analysis of the *cox*1 gene, the *T. franki* haplotypes identified in the present study clustered within the *T. franki* group with a bootstrap value of 99%. This cluster included isolates obtained from *A. platyrhynchus* in northern Iran, *R. auricularia* in France and the Czech Republic, and *L. plegora* in France. Haplotype 1 showed 100% similarity to the Iranian isolate (MH410296) obtained from *A. platyrhynchus*, whereas haplotype 2 was 100% identical to the Iranian isolate (MF945593) obtained from the same host species.

Phylogenetic analysis of the *cox*1 gene revealed that the *T. regenti* haplotypes identified in the present study clustered within a large group containing isolates from *A. platyrhynchus* in northern Iran, France, and Iceland, as well as from *C. olor* and *M. merganser* in France. They also clustered with isolates collected from *L. plegora* in France, indicating a broad geographic distribution of genetically similar haplotypes. Haplotype 1 exhibited 99% identity with both the Iranian isolate (MH410294) from *A. platyrhynchus* and the French isolate (HM439499) from *L. plegora*, whereas haplotype 2 was 100% identical to the Iranian isolate (MH198315) from *A. platyrhynchus*. The clustering patterns observed in this study indicate that genetically similar haplotypes are broadly

distributed among different host species and across diverse geographic regions. Several novel haplotypes were also detected, which have not been reported in previous studies.

Conclusions

From the results of this study, *R. auricularia* was found to be infected with furcocercariae of *T. franki* and *T. regenti* in northern Iran. The genetic analyses corroborated previous reports, demonstrating that populations of *T. franki* and *T. regenti* from Iran were genetically similar to those identified in Europe. This finding suggested that these schistosomes were likely dispersed via migratory duck hosts, thereby facilitating gene flow among populations associated with compatible snail hosts in Iran. These results highlighted the need for further researches on the role of migratory waterfowl in transmitting *Trichobilharzia* infections to native birds and humans, as well as the integration of this knowledge into regional infection prevention and control strategies in northern Iran

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Declarations

Ethics approval

Ethical approval for this study was granted by the Research Ethics Committee of Urmia University (IR.URMIA.REC.1400.002).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest to disclose.

Statements

We want to declare that the work described is original research that has not been published previously and is not under consideration for publication elsewhere. All the authors listed wish to be considered for publication in the Journal of BMC Veterinary Research. All authors (Armin Aligolzadeh Kenarsari, Mohammad Yakhchali, Keyhan Ashrafi Fashi, Meysam Sharifdini) have approved the paper for publication. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The authors utilized AI for language editing to enhance technical clarity and readability. The authors thoroughly reviewed, revised, and approved all AI-assisted text, and they maintain full responsibility for the accuracy and integrity of the final published content.

Data Availability

The nucleotide sequences generated in this study have been deposited in GenBank under accession numbers of sequences of ITS-1 rDNA region (OP821154–OP821219 for *T. franki* and OP821220–OP821227 for *T. regenti*), ITS-2 rDNA region (OP868734–OP868745 for *R. auricularia*), and *cox*1 mtDNA gene (OP740584–OP740646 for *T. franki* and OP740647–OP740655 for *T. regenti*). Other data generated or analyzed during this study are fully provided within the manuscript and available from the corresponding author upon request.

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