Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol.25 No.3 Ser. No.88 2024

IRANIAN JOURNAL OF VETERINARY RESEARCH

Short Paper

Genetic and phylogenetic evaluations of *Schistosoma turkestanicum* isolated from goats in Western Iran

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10.22099/ijvr.2024.49135.7203

(Received 17 Dec 2023; revised version 9 Jul 2024; accepted 23 Sept 2024)

Abstract

Background: Schistosomiasis, a zoonotic parasitic infection, poses significant challenges to the animal husbandry industry, leading to substantial economic losses. Despite its impact, there is limited data on the genotypes of *Schistosoma* (=*Orientobilharzia*) *turkestanicum* in Iran. **Aims**: The present study aimed to evaluate the phylogenetic relationships of *Schistosoma turkestanicum* isolated from goats by analyzing the mitochondrial cytochrome C oxidase subunit 1 (*Cox1*) gene sequence in Lorestan province, located in Western Iran. **Methods**: DNA extraction was performed on 20 male parasitic worms, and the mitochondrial *Cox1* gene was amplified using the polymerase chain reaction (PCR) process, and sent for sequencing after purification by ethanol alcohol. The sequences were trimmed using CLC Main Workbench software. Phylogenetic analysis was conducted using the Neighbor-Joining method with 1000 bootstrap replicates in MEGA6 software to assess evolutionary relationships between the *Cox1* gene sequence obtained in this study (GenBank accession No. PP627151) and various *S. turkestanicum* sequences obtained from the National Center for Biotechnology Information (NCBI). Additionally, this software was also used to plot the genetic distance matrix (nucleotide differences and similarities). **Results**: Phylogenetic analysis confirmed that the parasite isolated in this study was *S. turkestanicum*. **Conclusion**: The findings indicate that the *S. turkestanicum* lineage identified in this study is closely related to those found in Mazandaran province, Iran, as well as to African *Schistosoma* species.

Key words: Goat, Iran, Phylogeny, Polymerase chain reaction, *Schistosoma*

Introduction

Schistosoma (=*Orientobilharzia*) *turkestanicum* was first described by Skrjabin in 1913, after being discovered in the portal veins of cattle from Russian Turkestan. Adult *S. turkestanicum* worms measure between 2 and 10 mm for males and 2 to 8 mm for females (Soulsby, 1982). Schistosomiasis, a neglected anthropozoonosis, caused by this parasite, has been widely reported across China, India, Mongolia, Pakistan, Iraq, Iran, Russia, and Turkey (Kumar and de Burbure, 1986; Eslami, 1998; Li *et al*., 2008; Wang *et al*., 2009a, b).

The adult worms inhabit the portal or intestinal veins of various mammals, including cats, cattle, sheep, goats, water buffalo, horses, donkeys, mules, and camels (Kumar and de Burbure, 1986; Li *et al*., 2008; Wang *et al*., 2009a, b). Severe infections in ruminants, especially cattle and sheep, can result in significant economic losses due to reduced wool and meat production and decreased weight in sheep (Wang *et al*., 2009a). Moreover, the larvae, released from infected lymnaeid snails, may cause cercarial dermatitis in humans (Wang *et al*., 2009b; Aldhoun and Littlewood, 2012). This condition is notably prevalent along the Caspian coast of Iran (Sahba and Malek, 1979) and in several provinces of China (Li *et al*., 2008).

Numerous *Schistosoma* species have been identified in previous studies, including *S. bomfordi* (Montgomery, 1906), *S. turkestanicum* (Skrjabin, 1913), *S. turkestanicum* var. *tuberculata* (Bhalerao, 1932), *S. dattai* (Dutt and Srivastava, 1952), *S. cheni* (Hsü and Yang, 1957), and *S. harinasutai* (Kruatrachue *et al*., 1965). Traditional classifications and phylogenetic relationships within the Schistosomatidae family have been based on factors such as geographic distribution, adult parasite and egg morphology, transmission routes, and pathogenicity. However, modern molecular

techniques now allow us to evaluate these relationships based on DNA sequences (Tabaripour *et al*., 2015).

The present study aimed to determine the *Cox1* gene sequence of *S. turkestanicum* isolated from goats in Lorestan province, as reported for the first time by Nayebzadeh *et al*. (2021), and to compare its phylogenetic pattern with those of other schistosome species available in genomic databases. By analyzing the mitochondrial *Cox1* gene sequence, we explored the phylogenetic relationships of *S. turkestanicum* within the Schistosomatidae family.

Materials and Methods

Sample collection

The present study focused on the genetic and phylogenetic identification of *S. turkestanicum*, building upon Nayebzadeh *et al*.'s (2021) report of the first occurrence of *S. turkestanicum* in Lorestan province. To do so, *S. turkestanicum* worms were isolated from the mesenteric veins of goats slaughtered in Azna slaughterhouse, Lorestan province. The worms were morphologically identified using specific identification keys (Soulsby, 1982). Following identification, the worms were preserved in 70% ethanol and stored in the parasitology laboratory at Lorestan University until DNA extraction.

DNA extraction

DNA was extracted from a pool of 20 male *S. turkestanicum* worms using a DNA extraction kit (Favorgen, Taiwan), according to the manufacturer's protocol. The quality and quantity of the extracted DNA were assessed via 1% agarose gel electrophoresis and a NanoDrop spectrophotometer, respectively.

PCR assay

To amplify a 1125 bp fragment of the *Cox1* gene (mitochondrial DNA of *S. turkestanicum*), a forward primer (5´-GGC GGG TTT ATA GGT TTA G-3´) and a modified reverse primer (5´-CTT ATT TAA TGA ATA ACC AAC TAT A-3´) (Li, 2008) were used. PCR was carried out in a 25 µL reaction volume containing 2 µL of DNA sample (100 ng), 12.5 µL of Master Mix (Yekta Tajhiz Azma, Iran), 1 µL of each primer (10 nM), and 8.5 µL of double-distilled water. Thermal cycle used in this PCR process was carried out in BioRad (USA) thermal cycler machine using the protocol performed with pre-denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The final extension step was done at 72°C for 5 min. The accuracy of PCR was confirmed by electrophoresis on a 1.5% agarose gel using 0.5X TBE buffer and visualized using a gel documentation system.

Ethanol purification

Following PCR confirmation, the volume of PCR products was increased up to 200 µL, then purified using the ethanol purification method. The products were dissolved in 1200 µL of 96% ethanol and stored at -20°C for 5 min. The samples were then centrifuged at 7,827 g for 5 min, and the supernatant was discarded. In the next step, 200 µL of 70% ethanol was added to the remaining sediment. After thorough pipetting, the sediment was fully dissolved in ethanol and stored at 4°C for 8 min. The solution was centrifuged at 15,339 g for 5 min, and the supernatant was removed. Finally, the microtubes were incubated at 65°C for 5 min, and the pellet formed at the bottom of the tube was dissolved in 30 µL of distilled water. A sample volume of 30 µL, containing 100 ng/µL of DNA, was sent to Bioneer Company (South Korea) for sequencing.

Investigation of genetic genealogy and diversity

The nucleotide sequences of the *Cox1* gene from the mitochondrial DNA of various *Schistosoma* species, including *Schistosoma turkestanicum* isolate OT1 of *Cox1* (KC456231, KC456232, KC456233, KC456234), *Schistosoma bovis* (AY157212), *Schistosoma intercalatum* (AY157208), *Schistosoma leiperi* (AY157207), *Schistosoma haematobium* (AY157209), *Schistosoma mattheei* (AY157211), *Schistosoma margrebowiei* (AY157206), *Schistosoma rodhaini* (AY157202), *Orientobilharzia turkestanicum* (AY157200), *Schistosoma malayensis* (AY157198), *Schistosoma mekongi* (AY157199), *Gigantobilharzia huronensis* (AY157188), *Trichobilharzia regenti* (AY157190), *Dendritobilharzia pulverulenta* (AY157187), *Bilharziella polonica* (AY157186), *Austrobilharzia variglandis* (AY157196), and *Orientobilharzia canaliculata* (AY157194) were retrieved from the NCBI database. These sequences, along with the nucleotide sequence extracted in this study, were edited using CLC Main Workbench software (v.5) and aligned via the ClustalW method in MEGA6 software. A phylogenetic tree was subsequently constructed using the Neighbor-Joining method with 1,000 bootstrap replicates in MEGA6 software. To plot the genetic distance matrix of the *Cox1* gene sequence from this study in comparison with other *Cox1* sequences collected from the NCBI, the number of base substitutions per site was calculated using the Maximum Composite Likelihood model and pairwise distance calculation procedure for each comparison in MEGA6 software.

Results

DNA extraction from *S. turkestanicum* parasitic worms was successful, and amplification of the *Cox1* gene, producing a 1125 bp fragment, was performed using specific primers. The appearance of a single, distinct band on a 1% agarose gel without non-specific products confirmed successful amplification and primer specificity (Fig. 1). PCR was repeated in larger volumes (200 µL) to obtain sufficient product for subsequent analyses.

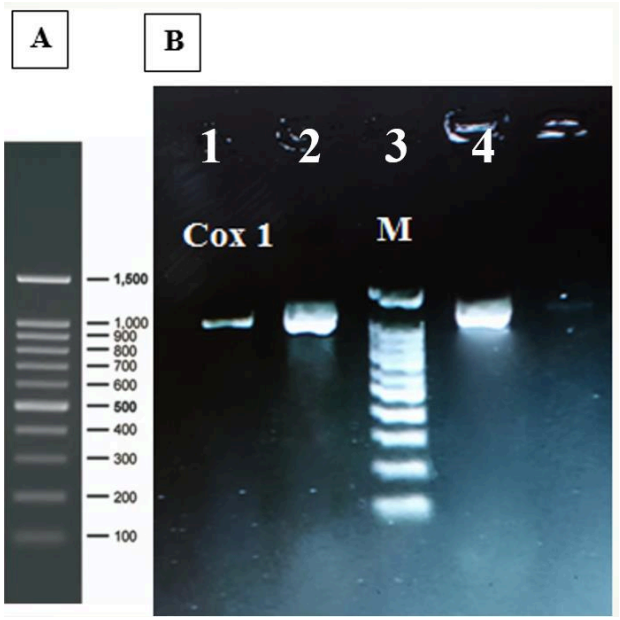

Fig. 1: (**A**) 100 bp DNA ladder map, and (**B**) electrophoresis results of the amplification of the *Cox1* gene from *Schistosoma turkestanicum* (accession No. PP627151) with a length of 1125 bp, visible in Lanes 1, 2, and 4. Lane M: Represents 100 bp size marker

Genetic and phylogenetic evaluations

The PCR products were successfully sequenced in both the sense and antisense directions. Post-sequencing, the quality of the nucleotide sequences was assessed using CLC Main Workbench (v.5). Low-quality bases at the beginning and end of the sequences were trimmed, and the remaining high-quality portion was used for alignment with other sequences retrieved from the NCBI database. A phylogenetic tree was generated using MEGA6 software, based on a common region of 828 base pairs shared among the sequences (Fig. 2).

Analysis of the nucleotide sequence of the *Cox1* gene from *S. turkestanicum* revealed the shortest genetic distance to *S. turkestanicum* isolates from Mazandaran province, Iran (KC456231, KC456232, KC456233, and KC456234), as well as *Orientobilharzia turkestanicum* (AY157200). The *S. turkestanicum* sequence isolated from goats in Lorestan province is represented by the accession number PP627151. The outgroup species, represented by the accession number MN228593.1, was used to root the phylogenetic tree (Figs. 3 and 4). According to the phylogenetic tree, sequences with greater genetic (or nucleotide) similarity appear as closely linked branches. The lineage of the sequence extracted in this study (PP627151) closely matched *Schistosoma* parasites identified in Mazandaran province (KC456231, KC456232, KC456233, KC456234), and *O. turkestanicum* (AY157200).

Discussion

Schistosomiasis is an endemic parasitic disease found in various regions of Iran. *Schistosoma turkestanicum*, the causal agent of the disease, has been reported in provinces such as Khuzestan (Massoud, 1973), Isfahan (Ghadirian and Hoghooghi, 1973), Mazandaran (Jooybar and Babolsar cities) (Hosseini *et al*., 1997), and Fars (Abdigoudarzi and Karimi, 2020). During a 1993 epidemic in Babolsar, the disease caused substantial economic losses in sheep farming (Hosseini *et al*., 1997).

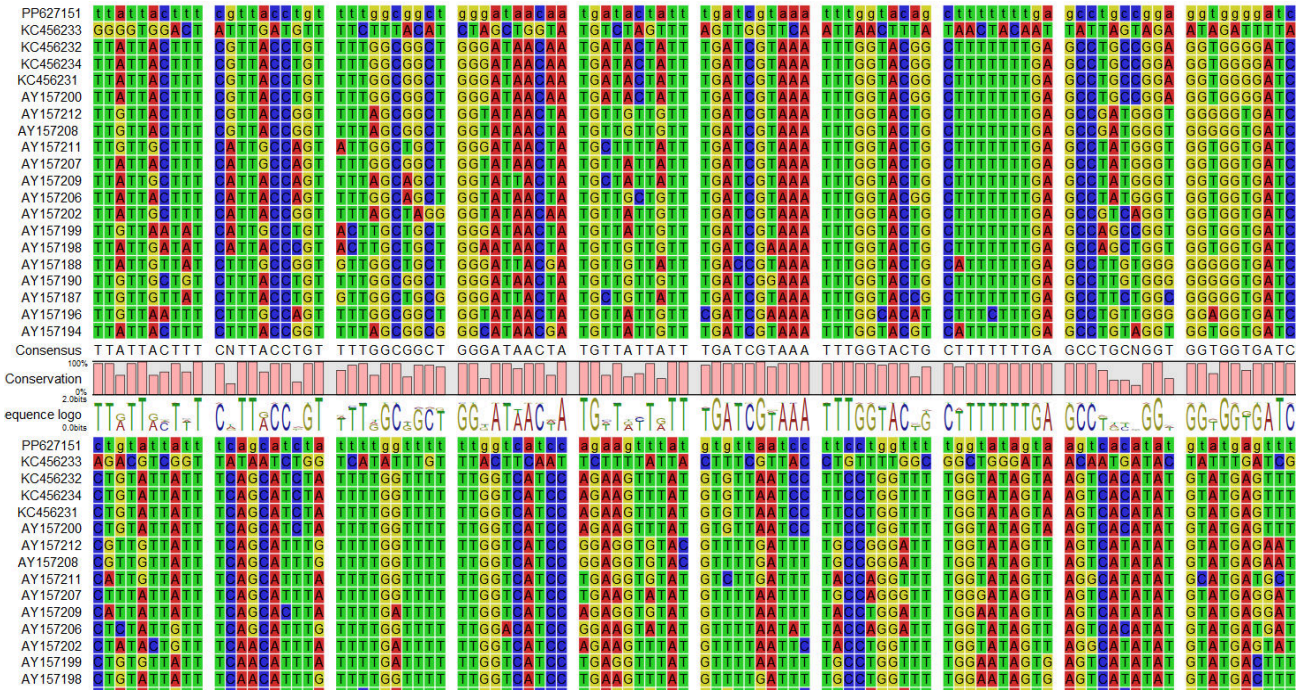

Fig. 2: Alignment of a portion of the *Cox1* gene sequence from *Schistosoma turkestanicum* (accession No. PP627151) with other *Cox1* gene sequences retrieved from the NCBI database

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1. Schistosoma Turkestanicum (PP627151)		0.003	0.004	0.003	0.004	1.689	1.729	1.615	1.619	1.831	1.774	1.858	1.705	1.593	1.716	0.004	1.699	2.045	1.949	1.753	4.478
2. Schistosoma Turkestanicum (KC456232)	0.006		0.002	0.000	0.002	1.686	1.727	1.613	1.616	1.828	1.772	1.855	1.702	1.590	1.713	0.002	1.697	2.042	1.947	1.751	4.475
3. Schistosoma Turkestanicum (KC456233)	0.009	0.003		0.002	0.003	1.686	1.727	1.613	1.616	1.828	1.772	1.855	1.702	1.590	1.713	0.003	1.697	2.042	1.947	1.750	4.474
4. Schistosoma Turkestanicum (KC456234)	0.006	0.000	0.003		0.002	1.686	1.727	1.613	1.616	1.828	1.772	1.855	1.702	1.590	1.713	0.002	1.697	2.042	1.947	1.751	4.475
5. Schistosoma Turkestanicum (KC456231)	0.009	0.003	0.006	0.003		1.686	1.727	1.613	1.616	1.828	1.772	1.855	1.702	1.590	1.713	0.000	1.697	2.042	1.947	1.751	4.475
6. Schistosoma Bovis (AY157212)	0.621	0.603	0.609	0.603	0.597		0.041	0.138	0.235	3.747	3.023	1.360	1.817	1.695	1.821	1.686	1.672	1.942	1.882	1.815	4.372
7. Schistosoma Intercalatum(AY157208)	0.665	0.646	0.652	0.646	0.640	0.117		0.162	0.088	0.477	3.157	1.372	1.851	1.755	1.877	1.727	1.668	1.917	1.876	1.815	4.478
8. Schistosoma Leiperi (AY157207)	0.600	0.582	0.588	0.582	0.576	0.185	0.176		1.423	1.055	0.271	1.367	1.821	1.624	1.844	1.613	1.537	1.996	1.638	1.795	4.507
9. Schistosoma Haematobium (AY157209)	0.613	0.594	0.600	0.594	0.588	0.216	0.217	0.169		6.024	2.514	1.357	1.896	1.756	1.967	1.616	1.629	1.869	1.807	1.770	4.420
10. Schistosoma Mattheei (AY157211)	0.731	0.711	0.717	0.711	0.705	0.256	0.273	0.249	0.317		2.121	1.587	1.721	1.614	1.793	1.828	1.875	1.902	1.846	1.960	4.448
11. Schistosoma Margrebowiei (AY157206)	0.692	0.673	0.679	0.673	0.667	0.273	0.270	0.237	0.292	0.316		1.459	1.770	1.639	1.776	1.772	1.668	1.982	1.716	1.683	4.692
12. Schistosoma Rodhaini (AY157202)	0.741	0.722	0.728	0.722	0.716	0.483	0.515	0.472	0.461	0.557	0.524		1.607	1.875	1.684	1.855	1.810	2.250	2.002	1.737	4.835
13. Schistosoma Mekongi (AY157199)	0.633	0.614	0.614	0.614	0.609	0.652	0.698	0.657	0.690	0.621	0.653	0.621		1.440	0.026	1.702	1.451	1.542	1.718	1.547	4.651
14. Gigantobilharzia Huronensis (AY157188)	0.681	0.662	0.662	0.662	0.657	0.671	0.686	0.673	0.684	0.648	0.670	0.757	0.618		1.473	1.590	16.764	1.512	1.580	1.491	4.359
15. Schistosoma Malayensis (AY157198)	0.628	0.609	0.609	0.609	0.604	0.655	0.682	0.682	0.736	0.655	0.630	0.652	0.115	0.585		1.713	18.566	1.545	1.690	1.591	4.469
16. Orientobilharzia Turkestanicum(AY157200)	0.009	0.003	0.006	0.003	0.000	0.597	0.640	0.576	0.588	0.705	0.667	0.716	0.609	0.657	0.604		1.697	2.042	1.947	1.751	4.475
17. Trichobilharzia Regenti (AY157190)	0.729	0.710	0.710	0.710	0.716	0.640	0.631	0.614	0.699	0.739	0.637	0.709	0.558	0.440	0.505	0.716		1.613	1.395	1.417	4.599
18. Dendritobilharzia Pulverulenta (AY157187)	0.831	0.811	0.811	0.811	0.811	0.802	0.774	0.831	0.782	0.798	0.794	0.903	0.597	0.561	0.547	0.811	0.577		1.904	1.586	4.083
19. Austrobilharzia variglandis (AY157196)	0.816	0.796	0.790	0.796	0.790	0.799	0.782	0.655	0.775	0.792	0.719	0.831	0.649	0.684	0.652	0.790	0.524	0.746		1.103	3.916
20. Orientobilharzia Canaliculata (AY157194)	0.735	0.716	0.722	0.716	0.722	0.710	0.696	0.713	0.721	0.786	0.651	0.674	0.593	0.628	0.612	0.722	0.541	0.614	0.448		4.507
21. Mus musculus Cox1 gene (MN228593.1)	1.862	1.841	1.837	1.841	1.841	1.827	1.871	1.870	1.815	1.887	1.936	1.925	1.921	1.815	1.869	1.841	1.911	1.759	1.703	1.862	

Fig. 3: Pairwise distance estimation matrix for the *Cox1* gene of *Schistosoma turkestanicum* (accession No. PP627151) and *Cox1* sequences of other species from the NCBI database. The number of base substitutions per site between sequences is shown. Standard error estimates are displayed above the diagonal, obtained by a bootstrap procedure (1000 replicates). Analyses were conducted using the Maximum Composite Likelihood model

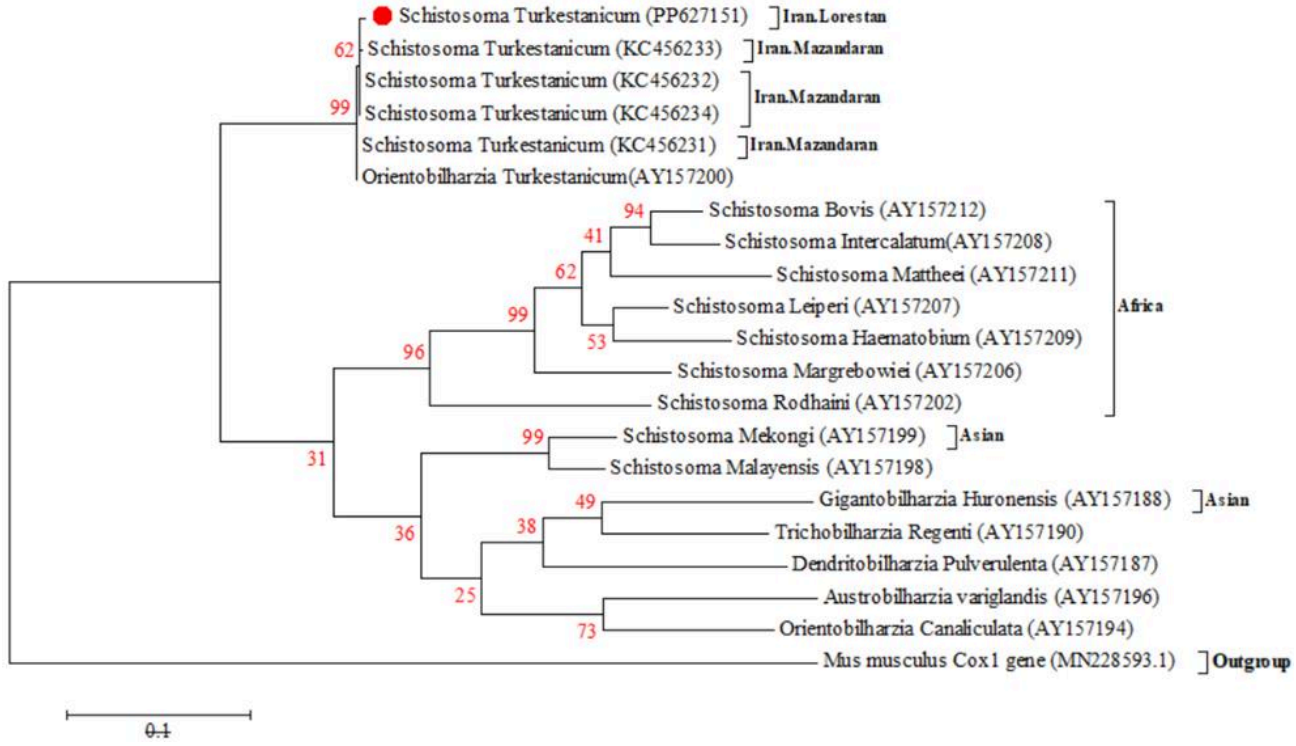

Fig. 4: Phylogenetic tree of the *Cox1* gene from *Schistosoma turkestanicum* (accession No. PP627151) constructed using the Neighbor-Joining statistical method with 1000 bootstrap replicates. Accession No. MN228593.1 corresponds to the outgroup species

Previous studies indicated the absence of *S. turkestanicum* and its intermediate hosts in Lorestan province (Mansourian, 1992). However, Mirfendereski *et al*. (2016) reported the presence of intermediate host snails in Lorestan, and in 2021, Nayebzadeh *et al*. confirmed the presence of *S. turkestanicum* in the region for the first time. A study by Karimi *et al*. (2004) on snails from Shadegan, Khuzestan, found that 8% of the snails (n=1,321) collected from six different regions were infected with trematode larvae.

Among mitochondrial genes, the *Cox1* gene is one of the most conserved protein-coding genes in animals (Folmer *et al*., 1994) and is widely used in evolutionary studies, serving as an excellent phylogenetic marker (Miya and Nishida, 2000). Certain regions of the *Cox1* gene are highly conserved, enabling the design of universal primers for a variety of species (Folmer *et al*., 1994). Additionally, the variable regions of the *Cox1* gene allow for species differentiation (Hebert *et al*., 2003).

Previous phylogenetic studies have grouped *Orientobilharzia* species with the genus *Schistosoma* based on nuclear and mitochondrial gene sequences (Snyder and Loker, 2000; Attwood *et al*., 2002; Lockyer *et al*., 2003; Brant and Loker, 2005; Webster *et al*., 2006; Li *et al*., 2008; Wang *et al*., 2009b). These findings suggested that *Orientobilharzia* should be classified within *Schistosoma*, despite traditional classifications

that positioned *Orientobilharzia* separately in the phylogenetic tree. However, such studies often rely on the sequences of one or several genes, without considering gene arrangement. This selective approach in gene position and length can lead to errors in analyzing genetic changes and relationships among parasites (Wang *et al*., 2011).

To date, only a few studies have examined *S. turkestanicum* genotypes in sheep worldwide. Li *et al*. (2008) used mitochondrial *Cox1* and *Nad1* genes to study the genetic structure and phylogenetic relationships of *Schistosoma* variants in cattle, sheep, and Kashmir goats.

In 2015, Tabaripour *et al*. analyzed the sequences of *Cox1* and *Nad1* genes and found that *S. turkestanicum* from Mazandaran province was phylogenetically closer to African *Schistosoma* species than to Asian ones.

A study conducted by the Razi Vaccine and Serum Research Institute of Iran identified *S. turkestanicum* cercaria using a nested-PCR method in snails as intermediate hosts (Motamedi *et al*., 2008). In this study, we used the mitochondrial *Cox1* gene for the molecular characterization of *S. turkestanicum*. Our phylogenetic analysis showed that the *S. turkestanicum* parasites in this study were genetically closer to African *Schistosoma* species than to Asian ones. Additionally, the *Cox1* gene of *S. turkestanicum* from Lorestan province exhibited the shortest genetic distance (nucleotide differences) and highest similarity to *S. turkestanicum* from Mazandaran province and *S. bovis*.

As molecular genetics and systematics continue to advance, methods of species identification and classification are frequently revised as new comparative data become available (Tibayrenc, 2006).

The comparison of the *Cox1* gene sequences from *S. turkestanicum* parasites investigated in this study with corresponding sequences from GenBank (NCBI) revealed nucleotide-level variations. These differences may result in altered protein profiles and functions, potentially leading to distinct geographic categorizations of the parasite. Furthermore, these nucleotide variations may contribute to grouping similar organisms into the same category based on genetic sequences. The phylogenetic analysis, based on *Cox1* gene diversity, demonstrated that the lineage of *S. turkestanicum* from Lorestan province, reported for the first time by Nayebzadeh *et al*. (2021), is closely related to *S. turkestanicum* from Mazandaran province and African *Schistosoma* species from other regions.

Acknowledgement

This study has been supported financially by the Office of Vice-Chancellor for Research, Lorestan University, Iran (grant No. 1400302111401).

Conflict of interest

The authors declare no conflict of interests.

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