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Original Article

Morphological and Molecular Analysis of *Enterobius vermicularis* with *Syphacia* spp.

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Abstract

Background: *Syphacia* (rodent pinworm), a common nematode in the colon of rodents, has rarely been reported in humans. The morphological identification of some pinworm species is difficult, especially in those cases where only fragments of worms are recovered. We aimed to identify isolates of *Syphacia* spp. in rodents using morphological and molecular approaches, and to compare them with human *Enterobius vermicularis*.

Methods: This study was carried out on 10 adult *Syphacia* worms collected from BALB/c mice and five adult *Enterobius vermicularis* from humans. Morphological features using existing keys and PCR-sequencing of ITS1 and 5.8s regions were applied to identify *Syphacia* spp., and its comparison with *E. vermicularis*.

Results: The total length of esophagus, the length and width of esophageal bulb, and the length and width of the right and left cephalic alae of *E. vermicularis* were larger than *Syphacia*. These parameters demonstrated significant morphological differences between *E. vermicularis* and *Syphacia*. PCR successfully produced amplicons of approximately 414 bp for *Syphacia* and 473 bp for *E. vermicularis*. The molecular method identified the Oxyurid nematodes isolated from BALB/c mice as *Syphacia obvelata*. The pairwise comparison revealed no differences in nucleotide sequences among *S. obvelata* isolates, and the sequences were identical and exhibited 100% homology.

Conclusions: This study demonstrated genetic and morphological differences between *E. vermicularis* and *S. obvelata*. Since laboratory mice (*Mus musculus*) are the specific hosts for *S. obvelata*, controlling these animals is critical to maintaining public health.

Introduction

Nematodes of the genus *Syphacia* (rodent pinworm) and *Enterobius* (human pinworm) are the most common members of the family Oxyuridae (1, 2). The two common species of *Syphacia* are *S. obvelata*, which inhabits the colon of mice, and *S. muris*, which inhabits the colon of rats (3). *E. vermicularis*, the most common nematode in humans, resides in the lumen of the large intestine. These nematodes are known as pinworms due to the female's long, pointed tail (2).

Pinworms belonging to the family Oxyuridae are nematodes with a direct life cycle. Adult females migrate nocturnally to the anus, and deposit eggs on the host perianal region. The eggs embryonate within a few hours, and infection occurs via direct ingestion of the embryonated eggs or swallowing of airborne eggs. The eggs hatch in the small intestine, and pinworm larvae migrate towards the colon and become adults (4, 5). Control of these nematodes is difficult due to their simple transmission ways (6).

Syphacia spp. are globally distributed and infect various rodent species across different regions. The prevalence of *S. obvelata* infection among rodents in Iran has been reported as 9% (7). *Syphacia* species typically cause asymptomatic infections due to their adaptation to host physiology and low pathogenicity (8). Human infection with *Syphacia* is rare, with the first case of *S. obvelata* reported in 1918 in an American child living in the Philippines (9). Rodents can serve as reservoirs or carriers of parasites due to their wide distribution and proximity to human habitats (10).

There is a significant difference in the size of adult worms of *Syphacia* spp. compared to *E. vermicularis*. The adult worm of *E. vermicularis* is larger than *Syphacia*. Adult male of *E. vermicularis* measures 2-5 mm long; adult female is 8-13 mm long. Female worms of *Syphacia* range from 3.4 to 5.8 mm in length, and male worms are smaller (1.1–1.5 mm). Bilateral cephalic alae and a prominent esophageal bulb are the most common diagnostic criteria in nematodes of the family Oxyuridae. The anterior end of *Syphacia* has small cervical alae, and the esophageal bulb is round. The posterior end of the male pinworms is bent ventrally. The male worms of *Syphacia* spp. have mamelons located at the ventral surface; however, male worms are rarely recovered because they die after mating. *Syphacia* eggs are larger than *E. vermicularis* eggs. The eggs of both are asymmetrical, the eggs of *Syphacia* being more fusiform (9, 11-12).

Morphological identification of some pinworm species is challenging, especially with incomplete specimens. To overcome these challenges, molecular tools utilizing ribosomal and mitochondrial markers are increasingly employed for accurate identification (13-16). Various studies have used molecular methods to identify *Syphacia* spp. and *E. vermicularis* (3, 15, 17-27).

Adult worms of *Syphacia* spp. live in the large intestine of various rodents, and the infection of laboratory animals with these parasites has been reported (20, 28). Some parasites, such as *Syphacia*, can be transmitted from rodents to humans (9). Therefore, in rare cases where humans are accidental hosts of *Syphacia*, there is a possibility that humans will develop appendicitis with the *Syphacia* agent. Sometimes the adult worm is not wholly present on the pathology slide or not all diagnostic criteria are seen simultaneously, therefore, on the histopathological examination of appendectomy, it is possible that the cause of appendicitis was originally *Syphacia* but was mistakenly reported as *E. vermicularis*. The molecular methods have been developed and used for accurate identification and differentiation of *Syphacia* spp. from *E. vermicularis*.

We aimed to identify isolates of *Syphacia* spp. in rodents using morphological and molecular approaches and compare them with *E. vermicularis*.

Materials & Methods

Sample collection

Thirty-five BALB/c mice (*Mus musculus*) from the animal house of Shiraz University of Medical Sciences in Shiraz, southern Iran, were examined. The BALB/c mice were dissected to examine their digestive tracts for nematode parasites. Ten adult *Syphacia* worms were collected from ten infected BALB/c mice. Five adult *E. vermicularis* worms were collected from infected individuals. The adult worms were washed in physiological saline, and preserved in 70% ethanol until use.

The present study was approved by the Ethics Committee of the Shiraz University of Medical Sciences, Iran (ethic code: IR.SUMS.REC.1401.001).

Morphological studies

Nematodes were cleared in FAL (formaldehyde, alcohol, lactophenol) for morphological studies (29). Adult worms of the family Oxyuridae were identified based on the main characteristics, such as bilateral cephalic alae and a prominent esophageal bulb (11). The nematodes were photographed using a digital camera. The total length of esophagus, the length and width of esophageal bulb, and the length and width of the right and left cephalic alae of *E. vermicularis* and *Syphacia* were measured using the micrometry. The characteristics of *Syphacia* were compared with *E. vermicularis*.

Molecular studies

For molecular studies, genomic DNA was extracted from *Syphacia* and *E. vermicularis* isolates using a commercial DNA extraction kit (Yekta Tajhiz Azma, Tissue Genomic DNA Extraction Mini kit, Cat. No. FATGK001) following the manufacturer's instructions.

The internal transcribed spacer (ITS1-5.8S) region of ribosomal RNA (rRNA) was amplified by forward primer (NC5: 5'-GTAGGTGAACCTGCG-GAAGGATCATT-3' (15) and a newly designed reverse primer (New-reverse NC2: 5'-TCAATGTGTCCGCAATTCGC-3'). PCR reactions were carried out in 25 µl as follows: 12.5 µl Taq 2X Master mix (Ampliqon, Denmark), 0.5 µl of 25 Pmol of each primer, and five µl parasite genomic DNA, and then completed by 6.5 µl of distilled water. The temperature profile was one cycle of 95 ºC for 6 min (initial denaturation), then 30 cycles of 94 ºC for 30 s (denaturation), 65 ºC for 30 s (annealing), and 72 ºC for 30 s (extension), and a final extension 72 ºC for 5 min. PCR products were analyzed via gel electrophoresis and visualized under UV light.

Nucleotide sequences were compared with the reference sequences available GenBank using the BLAST system (http://www.ncbi.nlm.nih.gov/) for identification. The nucleotide sequences obtained in this study were deposited in GenBank.

Phylogenetic analysis was conducted using sequences from this study and reference sequences in GenBank using the maximum likelihood method based on the Tamura-Nei model by the Molecular evolutionary genetic analysis software (MEGA 5.0). Bootstrap analyses (1000 replicates) were carried out to determine the robustness of the finding. All information about sequences is given in Table 1.

Statistical analysis

Morphometric data were analyzed using SPSS version 23 (IBM Corp., Armonk, NY, USA). The *t*-test was performed to determine a significant difference between the means of morphometric values of isolates. A value of less than 0.05 was considered statistically significant.

Table 1: Accession numbers and additional information for sequences obtained in the present study, along with the reference sequences deposited in GenBank

Nematode species	Collection site	Host	Accession number	Reference
Syphacia obvelata				
S1	Iran/Shiraz	*Mus musculus*	PQ557684	This study
S2	Iran/Shiraz	*Mus musculus*	PQ557685	This study
S3	Iran/Shiraz	*Mus musculus*	PQ557686	This study
S4	Iran/Shiraz	*Mus musculus*	PQ557687	This study
S5	Iran/Shiraz	*Mus musculus*	PQ557688	This study
S6	Iran/Shiraz	*Mus musculus*	PQ557689	This study
S7	Iran/Shiraz	*Mus musculus*	PQ557690	This study
S8	Iran/Shiraz	*Mus musculus*	PQ557691	This study
S9	Iran/Shiraz	*Mus musculus*	PQ557692	This study
S10	Iran/Shiraz	*Mus musculus*	PQ557693	This study
SENEGAL-CB4044-WO	Senegal	*Mus musculus*	OK143548	Stewart et al. 2021 (unpublished)
SENEGAL-CB1241-WO	Senegal	*Mus musculus*	OK143549	Stewart et al. 2021 (unpublished)
POLAND-Balb/c PHMmSo1	Poland	*Mus musculus*	MF142449	Stewart et al. 2018 (2\3)
POLAND-GZMmSo	Poland	*Mus musculus*	MF142450	Stewart et al. 2018 (23)
MVC	India	*Mus musculus*	KT853017	Sundar et al. 2018 (3)
-	Taiwan	*Mus musculus*	EU263105	Parel et al. 2008 (15)
-	USA	*Mus musculus*	EF464554	Feldman et al. 2007 (30)
-	Saudi Arabia	*Mus musculus*	OQ876775	Al-Shaebi et al. 2024 (unpublished)
NOTTINGHAM-13Md01Sof	United Kingdom	*Mus domesticus*	MF142448	Stewart et al. 2018 (23)
SCOTLAND-IOM-16Mm352S	United Kingdom	*Mus musculus*	MF142451	Stewart et al. 2018 (23)
SCOTLAND_MAY-MAYBR	United Kingdom	*Mus musculus*	OK143550	Stewart et al. 2021 (unpublished)
STAFFORDSHIRE-18Md09So	United Kingdom	*Mus musculus domesticus*	OK143551	Stewart et al. 2021 (unpublished)
Syphacia nigeriana				
JERSEY-14Mg02Sp	Jersey	Myodes glareolus	OK143547	Stewart et al. 2021 (unpublished)
Syphacia frederici				
POLAND-16As-DD-24Sf	Poland	*Apodemus sylvaticus*	MF142442	Stewart et al. 2018 (23)
PORTUGAL-13As18Sf	Portugal	*Apodemus sylvaticus*	MF142444	Stewart et al. 2018 (23)
ALES-GWYN-15As01Sff	United Kingdom	*Apodemus sylvaticus*	MF142447	Stewart et al. 2018 (23)
BERKSHIRE-17As01Sf	United Kingdom	*Apodemus sylvaticus*	MF142454	Stewart et al. 2018 (23)
1436	Russia	*Apodemus uralensis*	MN652172	Gorelysheva et al. 2021 (26)
Syphacia agraria				
POLAND-18Aag04Saf2	Poland	*Apodemus agrarius*	OK143591	Stewart et al. 2021 (unpublished)
Syphacia stroma				
POLAND-GZAf488Ss	Poland	*Apodemus flavicollis*	MF142434	Stewart et al. 2018 (23)
FRANCE-12Apo11Ss	France	*Apodemus sylvaticus*	MF142436	Stewart et al. 2018 (23)
DURHAM-06As01Ss	United Kingdom	*Apodemus sylvaticus*	MF142456	Stewart et al. 2018 (23)
Syphacia muris				
-	Taiwan	*Rattus*	EU263106	Parel et al. 2008 (15)
-	USA	*Rattus*	EF464553	Feldman 2007 (30)
NETHERLANDS-15RnFF1512/25S	Netherlands	*Rattus norvegicus*	MF142453	Stewart et al. 2018 (23)
Syphacia sp.				
1393	Russia	*Microtus obscurus*	MN652171	Gorelysheva et al. 2021 (26)
MALI-07MeMDD-26	Mali	*Mastomys erythroleucus*	OK143590	Stewart et al. 2021 (unpublished)
-	Madagascar	*Rattus rattus*	MW520838	Kiene et al. 2021 (31)
Enterobius vermicularis				
O1	Iran/ Gilan	*Homo sapiens*	PQ557679	This study
O4	Iran/Shiraz	*Homo sapiens*	PQ557680	This study
O5	Iran/ Gilan	*Homo sapiens*	PQ557681	This study
O7	Iran/ Tonekabon	*Homo sapiens*	PQ557682	This study
O9	Iran/ Gilan	*Homo sapiens*	PQ557683	This study
-	Germany	*Homo sapiens*	HQ646164	Zelck et al. 2011 (32)
-	Iraq	*Homo sapiens*	MN817931	Khazaal et al. 2019 (unpublished)
Aspiculuris tetraptera				
-	Taiwan	*Mus musculus*	EU263107	Parel et al. 2008 (15)

Results

Nematodes in the family Oxyuridae are characterized by bilateral cephalic alae and a prominent esophageal bulb (Fig. 1). *Syphacia* showed small cephalic alae (69.7×14.5 µm), while *E. vermicularis* has a broad cephalic alae (200.8×54.6 µm).

The esophagus length, esophageal bulb dimensions, and cephalic alae measurements of *E. vermicularis* were significantly larger than those of *Syphacia* (Table 2). These parameters demonstrated significant morphological differences between *E. vermicularis* and *Syphacia* (P. value ≤ 0.05).

All Oxyurid nematodes isolated from BALB/c mice were morphologically identified as *Syphacia*.

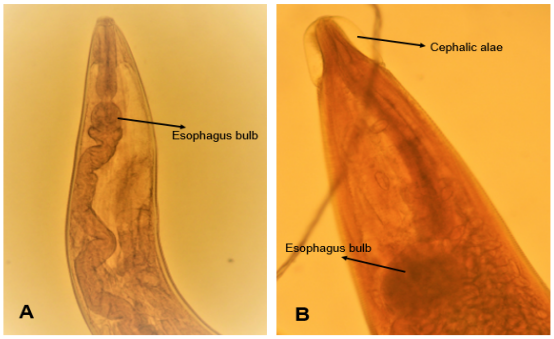

Fig. 1: Esophageal bulb of *Syphacia* (10X) (A), *Enterobius vermicularis* (10X) (B)

Table 2: Comparison of *Syphacia* and *Enterobius vermicularis* based on the size of the esophagus, esophageal bulb, and cephalic alae

Parameters (µm)	*Syphacia* Mean ± S.D*	*Enterobius vermicularis* Mean ± S.D	*P.* value
Esophagus length	392.92± 11.64	848.25 ± 13.78	0.000
Esophageal bulb length	144.30 ± 4.50	210.60 ± 0.00	0.000
Esophageal bulb width	109.20 ± 6.36	202.80 ± 0.00	0.000
Right cephalic alae length	68.92 ± 10.50	218.40 ± 44.12	0.002
Right cephalic alae width	15.20 ± 3.46	54.60 ± 22.06	0.016
Left cephalic alae length	70.52 ± 6.59	183.30 ± 16.54	0.000
Left cephalic alae width	13.77 ± 3.24	54.60 ± 22.06	0.014

* Standard deviation

Molecular analysis of the ITS1-5.8S ribosomal RNA region confirmed the identification of *Syphacia* spp. PCR successfully produced amplicons of approximately 414 bp for *Syphacia* and 473 bp for *E. vermicularis* (Fig. 2).

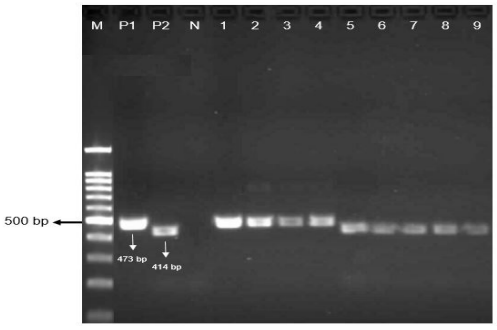

Fig. 2: Agarose gel electrophoresis of ITS-PCR products from *Enterobius vermicularis* (1-4) and *Syphacia* (5-9) isolates, M: 100 bp DNA Marker, P1: positive control of *Enterobius vermicularis*, P2: positive control of *Syphacia*, N: negative control

After sequence analysis, the edited sequences were compared with the reference sequences available GenBank using the BLAST system, and the Oxyurid nematodes isolated from BALB/c mice were identified as *S. obvelata.*

The ITS sequences of *E. vermicularis* and *S. obvelata* from this study were deposited in GenBank with the accession numbers in Table 1.

A phylogenetic tree was constructed with sequences obtained in this study and the reference sequences available in the GenBank (Table 1) using MEGA 5.0 software. The phylogenetic tree indicated that five isolates of *E. vermicularis* obtained in this study were taxonomically grouped into one haplotype. The phylogenetic tree also showed that 10 isolates of *S. obvelata* obtained from mice based on ITS sequences were similar. Therefore, one haplotype was identified (Fig. 3). Intra-species variation of *S. obvelata* sequences obtained in this study with the reference sequences available in the GenBank was reported to be 0-0.6%.

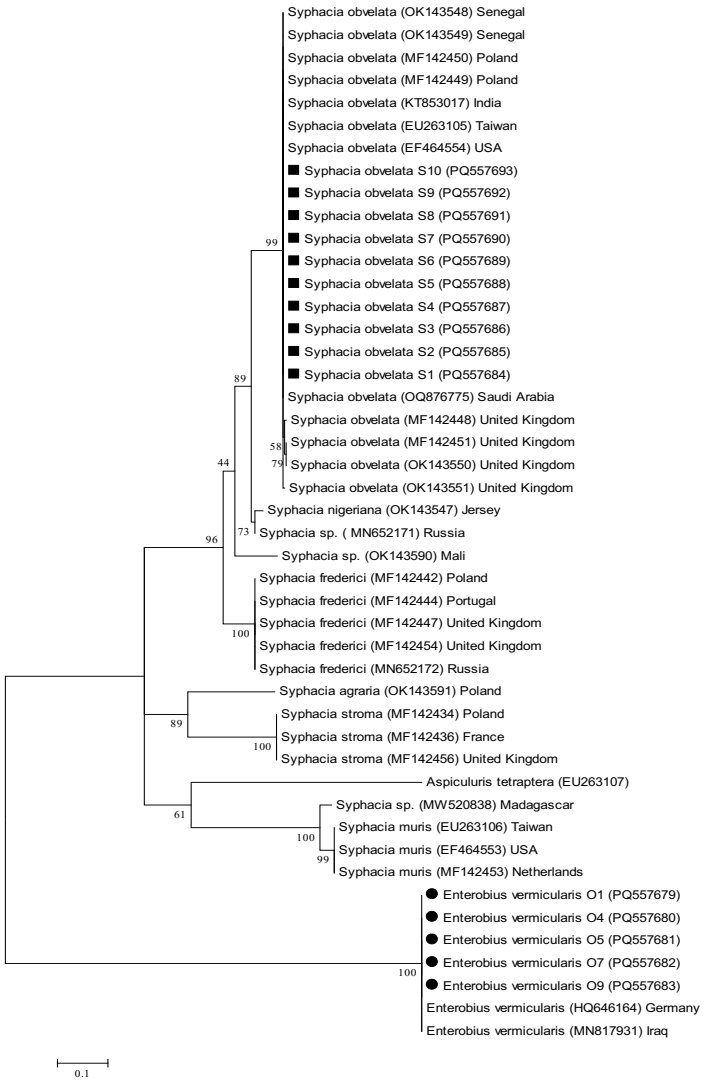

Fig. 3: Phylogenetic tree of ITS sequences of *Enterobius vermicularis* and *Syphacia obvelata* isolates obtained in this study and reference sequences available in Genbank using the Maximum Likelihood method. *Aspiculuris tetraptera* (Accession number: EU263107) is considered as the outgroup

Discussion

Syphacia spp. are the most common pinworms infecting rodents worldwide and can occasionally infect humans (20, 33), and a human case infected with *S. obvelata* has been reported in the Philippines (9). *E. vermicularis,* a human large intestine nematode, is sometimes detected in ectopic sites such as the appendix, kidney, uterus, urinary tract, female genital tract, eye/s, and subcutaneous nodule. Accurate identification of ectopic oxyuriasis agents is critical for diagnosis and treatment (34, 35). In this study, morphological and molecular techniques were applied for the identification and differentiation of *Syphacia* from *E. vermicularis*.

In the present study, only female worms of *Syphacia* were recovered from laboratory mice, because male worms of *Syphacia* die soon after mating and are often not found in infections (20). The findings of our study regarding the size of female *S. obvelata* (3-5 mm in length) were consistent with the results of other studies (36, 37). Abdel-Gaber isolated *S. obvelata* from the laboratory mice *Mus musculus* in Egypt, and the female worm of *S. obvelata* measured 2.93–4.65 mm long (20). The female worm of *S. obvelata* had a small cephalic alae and a round esophageal bulb. The tail was long and pointed, and the uterus was filled with the eggs. In general, the morphological features of *S. obvelata* isolated in this study were similar to that of *S. obvelata* isolated from *M. musculus* in different studies with few differences in measurements of the various body parts such as esophagus length and esophageal bulb length (36, 37). Based on morphological criteria, *S. obvelata* differs from other *Syphacia* species (38, 39).

Consistent morphological and morphometric differences distinguish pinworms of mice (*S. obvelata*) from those of humans (*E. vermicularis*). The cephalic alae of *S. obvelata* are small, whereas those of *E. vermicularis* are larger and more distinct (33). In the present study, the cephalic alae of female *E. vermicularis* worms obtained from humans were slightly broader than that of the cephalic alae of *S. obvelata* obtained from mice. In general, the total length of esophagus, the length and width of esophageal bulb, and the length and width of the right and left cephalic alae of *E. vermicularis* were larger than *Syphacia,* which may be related to the more complex interactions of *E. vermicularis* with the human host immune system. In study of Wu et al., microscopic examination of stool demonstrated adult *E. vermicularis* with cephalic alae and a prominent, muscular, esophageal bulb (40).

Since morphological identification of some nematodes is challenging, molecular methods using ribosomal and mitochondrial markers are employed (24, 41). The ribosomal markers such as ITS regions are usually conserved, and used as effective genetic markers for the identification of helminths (42, 43). The mitochondrial genes can be used for studies of genetic variability, systematic and phylogenetic relationship analyses of nematodes (43). Tavan et al. characterized the genotypes of *E. vermicularis* using the PCR-sequencing method of the mitochondrial cytochrome C oxidase subunit 1 (*cox1*) gene (24). In this study, we employed a molecular method using ITS and 5.8s regions for the identification and differentiation of *S. obvelata* and *E. vermicularis*. PCR revealed a band at approximately 414 bp, indicating that the worm belongs to *Syphacia spp.,* and amplification at approximately 473 bp belonging to *E. vermicularis*. Molecular methods using the nuclear ribosomal region have been employed to differentiate organisms that are morphologically similar (43).

In this study, the pinworms isolated from mice were identified as *Syphacia obvelata* based on morphological characteristics, polymerase chain reaction, and sequencing. Tanideh et al. isolated *S. obvelata* from BALB/c mice, and the high frequency of this nematode in labora-

tory mice *Mus musculus* showed the spread of these helminths in colonies kept under conventional conditions (28). In another study, infection with *S. obvelata* was recorded in the caecum and colon of the laboratory mice (44). *S. obvelata* is a common gastrointestinal parasite of the house mouse *M. musculus* with high prevalence in laboratory animals (45). The presence of *S. obvelata* in colonies of laboratory mice is justified by the life cycle of this nematode, which causes infection in a larger number of mice in short periods.

In the present study, the ITS sequence of *S. obvelata* isolated from mice was similar and had a 100% homology with *S. obvelata* isolated in Senegal (Accession nos. OK143548-OK143549), Poland (Accession nos. MF142449- MF142450), India (Accession no. KT853017), Taiwan (Accession no. EU263105), USA (Accession no. EF464554) and Saudi Arabia (Accession no. OQ876775). Therefore, there is no correlation between the homogeny of these isolates and their geographical origin. ITS sequence of *S. obvelata* showed two differences from the *S. obvelata* sequence from United Kingdom (Accession nos. MF142451, OK143550). Those mutations were all transversions at positions 15 and 49. In addition, there was a transversion from A to G base at position 99 on the *S. obvelata* sequence from the United Kingdom (Accession no. OK143551). ITS sequence of *S. obvelata* had two differences at positions 15 and 227 to the *S. obvelata* sequence from United Kingdom (Accession no. MF142448). Intraspecies sequence differences among *S. obvelata* obtained in this study with the reference sequences available in the GenBank was 0-0.6%.

Phylogenetic analysis indicated that five isolates of *E. vermicularis* obtained in this study were taxonomically grouped into one haplotype. The ITS sequences of one haplotype (Accession nos. MF142449- MF142450) were identical with *E. vermicularis* isolated from humans in Germany (Accession no. HQ646164) and Iraq (Accession no. MN817931). In general, the ribosomal sequences are less variable than mitochondrial sequences, and genetic diversity is critical for the survival and adaptability of a parasite.

Conclusion

This study demonstrated significant genetic and morphological differences between *E. vermicularis* and *S. obvelata*. Morphological analyses showed that *E. vermicularis* exhibited larger esophageal and cephalic alae measurements compared to *S. obvelata*, reflecting host-specific adaptations. Molecular analysis using ITS sequences confirmed the identification of *S. obvelata* in laboratory mice and *E. vermicularis* in humans, highlighting the importance of integrating molecular tools with morphological methods for accurate nematode identification.

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Conflict of interest

The authors declare that there is no conflict of interest.

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