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First molecular and phylogenetic characterization of *Physaloptera clausa* in long-eared hedgehogs (*Hemiechinus auritus*) from Iran

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Abstract

Physalopteridae nematodes pose a threat to a broad spectrum of animals including amphibians, reptiles, birds and mammals. The current study was the first molecular and phylogenetic characterization of *Physaloptera clausa* parasitizing long-eared hedgehogs (*Hemiechinus auritus*) in Iran. A male road-killed *H. auritus* was collected from Shahriar City, Tehran province in May 2022 and it was subjected to necropsy. After isolating parasites, they underwent morphological analysis using a light microscope and an identification key. For molecular analysis, the genomic DNA was isolated using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. The PCR products were sequenced, the sequence data were analyzed and multiple alignments were conducted using the Clustal Omega. For phylogenetic analysis, these nucleotide sequences were aligned in MEGA 11 based on the lowest bayesian information criterion score. A cluster of parasites was found on the mucosa of the hedgehog stomach. All the nematodes were identified as *P. clausa*. The sequence obtained in this study has been submitted to GenBank® with the accession number OR088573. The phylogeny analysis revealed that the genus *Physaloptera* formed a major clade where *P. clausa* was clustered with various *Physaloptera* species, closely related to *Turgida*, *Physalopteroides* and *Skrjabinoptera* genera. Our study specified the necessity for additional taxonomic and phylogenetic research on *Physaloptera* species and related genera to fully understand the evolutionary dynamics and ecological significance of these parasites.

Introduction

Nowadays, a high attention is paid to exotic animals as pets resulting in possible transmission of zoonotic diseases. Among small mammals, hedgehogs have been considered as popular exotic pets. However, they have been shown to have a role in the eco-epidemiology of pathogens of veterinary and public health importance.[1]

Hedgehogs are broadly distributed in urban and rural areas of Iran, where keeping exotic pets is receiving a growing popularity.[2] To date, four species of hedgehogs including Brandt's hedgehog (*Hemiechinus hymelas*), East European hedgehog (*Erinaceus concolor*), desert hedgehog (*H. oethiopicus*) and the long-eared hedgehog (*H. auritus*) have been reported in Iran.[3]

Hemiechinus auritus from family Erinaceidae is one of the most familiar mammals distributed across the Middle East, Central Asia, the Cypriot island as well as northern and northeastern Africa (Libya and Egypt). The cultivated and shrubby areas, abandoned irrigation ditches, forest shelterbelts, gullies and gardens are the favorable habitats for these mammals. *H. auritus* has circannual cycle, with the highest rate in summer (a peak in July) and the least in winter.[4] They have flexible diet (insectivorous/ omnivorous) mostly feed on mollusks, earthworms and vertebrates such as snakes and frogs.[3]

The nematodes of Physalopteridae family infect amphibians, reptiles, birds and mammals. According to the global reports, some of the species of these nematodes are accounted for humans infection indicating the zoonotic significance of these parasites.[5] The nematodes of *Physaloptera* genus (Rudolphi, 1819) include more than 100 species parasitizing amphibians, reptiles, birds and mammals (definitive hosts).[6] They commonly inhabit the stomach and in the rare cases they have been found in the small intestine of the definitive hosts. The heteroxenic life cycle of these nematodes is composed of insects (e.g., beetles or roaches) as an intermediate host and occasionally snakes, lizards and anurans as paratenic hosts. The infective larval stage (L3) is transmitted to

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definitive hosts via ingestion of the infected intermediate or paratenic hosts.[7]

Physaloptera clausa (Rudolphi, 1819) feed on blood in gastric mucosa resulting in serious gastric lesions, anorexia and cachexia in hedgehogs.[8] This nematode may cause several small swollen ulcers resulting in bleeding, increased production of mucus and inflammation. In addition, it has been revealed that there are several cysts on the surface of the liver, which contained numerous *P. clausa* nematodes.[9] Furthermore, they are recognized as a possible vector for zoonotic pathogens such as *Leptospira* spp., which have little clinical importance in hedgehogs.[10] So far, there are limited molecular studies on nematodes of Physalopteridae family, especially in Iran. The current study addressed this gap in knowledge by reporting the first molecular and phylogenetic analysis of *P. clausa* parasitizing *H. auritus* in the country.

Materials and Methods

Sampling. A male road-killed *H. auritus* was collected from Shahriar City (35°39′35″N, 51°03′33″E), in Tehran province in May 2022. The animal weighed 692 g. To perform necropsy and parasitological investigation, the animal was transferred to the laboratory of Department of Parasitology, Tarbiat Modares University in Tehran, Tehran, Iran. The ethical approval was required and provided for this study, as stated by the Institutional Review Board No. IR.QUMS.REC.1401.

Morphological identification. For morphological diagnostic, the nematodes were isolated from the stomach and were examined microscopically. The recovered parasites were relaxed in water and fixed in 70.00% ethanol. While a number of specimens were isolated for molecular study, the others were placed on a glass slide and cleared with lactophenol for morphological study. The identification was performed using a light microscope and on the basis of an identification key.[11]

Molecular identification. The genomic DNA from the sample was isolated using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech, Ping Tung, Taiwan) following the manufacturer's guidelines. The concentration and purity of the DNA were assessed using a NanoDrop One/OneC (Thermo Scientific, Waltham, USA). Subsequently, the solutions were preserved at – 20.00 ˚C for future applications.

Amplification of *18s rRNA* gene. The genomic DNA underwent PCR amplification targeting the barcode region of the *18s rRNA* gene using primers specified by Van Steenkiste *et al.*[12] The PCR reactions of 25.00 µL were prepared, comprising 12.50 µL Taq DNA Polymerase Master Mix RED (Ampliqon, New Delhi, India), 2.00 µL of primers, 2.00 µL of DNA template and nuclease-free water to make up the total volume. The PCR cycling parameters were set on an automated thermal cycler (MyCycler; Bio-Rad, Hercules, USA) with the following program: Initial denaturation at 94.00 ˚C for 2 min; 3 cycles of denaturation at 94.00 ˚C for 40 sec, annealing at 51.00 ˚C for 40 sec, extension at 72.00 ˚C for 1 min; five touchdown cycles of denaturation at 94.00 ˚C for 40 sec, annealing at 50 to 46.00 ˚C for 40 sec (decreasing 1.00 ˚C *per* cycle), extension at 72.00 ˚C for 1 min; 35 cycles of denaturation at 94.00 ˚C for 40 sec, annealing at 45.00 ˚C for 40 sec, extension at 72.00 ˚C for 1 min and a final extension at 72.00 ˚C for 5 min. The resulting amplicon was observed on a 1.50% agarose gel stained with SYBR™ Safe DNA Gel Stain (Thermo Fisher Scientific, Waltham, USA) using UV light after electrophoresis with a Gel Doc XR + Gel Documentation System (Bio-Rad).

Polymerase chain reaction purification, gene sequencing and phylogenetic analysis. The PCR products underwent purification using the Expin™ Combo GP gel extraction kit from GeneAll (Seoul, Korea) as *per* the manufacturer's guidelines. Following purification, the samples were forwarded for sequencing on both strands to Pishgam Biotech Co. in Tehran, Iran. Chromatograms were scrutinized and validated using the Sequencher Software (version 5.4.5; Gene Codes Corp., Ann Arbor, USA) to ensure sequence quality. The sequence data from this study were deposited in GenBank® and specific accession numbers were assigned to the respective genes. Sequence alignment and similarity searches were carried out using the basic local alignment search tool within the GenBank® database at the National Center for Biotechnology Information, employing the default matrix available at www.ncbi.nlm.nih.gov/BLAST to identify matching sequences from various strains, isolates and species. The resulting sequence underwent multiple sequence alignment using Clustal Omega available at: www.ebi.ac.uk/Tools/msa/clustalo. To ensure uniformity, the selected sequences were trimmed at both ends so that they all initiated and terminated at equivalent nucleotide positions. For phylogenetic analysis, these nucleotide sequences were aligned in MEGA Software (version 11.0; Biodesign Institute, Tempe, USA) based on the lowest Bayesian Information Criterion score. The alignment was performed using the CLUSTAL W Multiple Sequence Alignment Program (UCD, Dublin, Ireland)within MEGA 11 with a gap opening penalty of 15 and a gap extension penalty of 6.66 for both pairwise and multiple alignments.[13] The phylogenetic tree was constructed employing appropriate models (maximum likelihood method and the general time reversible model with a discrete gamma distribution including invariant sites (G + I) through neighbor-joining and BioNJ algorithms. To establish the root of the tree, GenBank® sequences of Camallanidae (five genera) were utilized as outgroup species. The robustness of the tree topologies was confirmed through bootstrapping with 500 replications.[13,14]

Results

Morphological analysis. During necropsy and precise observation of gastrointestinal tracts, stomach revealed an intensive infection with medium to large-sized nematode parasites, which all were attached to the mucosa of the fundus (Fig. 1). The mucosa was thickened, highly congested and there were erosions occurred in sites of parasitic attachment. All nematodes recovered from the stomach of the examined hedgehog found to be *P. clausa* based on morphological features observed in light microscope examination and on the basis of identification key (Fig. 1). The species morphological description was previously reported.[9] The helminths were light pinkish and robust. The cuticle was tick with slight transverse striations (in both male and female) and it was reflected over the lips shaping a large cephalic collarette. The buccal cavity was cylindrical and thick-walled and it was surrounded with large-sized and paired triangular-shaped lateral pseudolabia. The females were 31.00 - 48.00 mm long and 1.30 - 1.50 mm wide. The eggs were oval-shaped, 38.00 × 52.00 µm in size and contained embryos when deposited. The males were 22.00 - 27.00 mm long and 0.81 - 1.30 mm wide with dissimilar and subequal spicules. There was large caudal ala in front of the cloaca with presence of cuticular ridges in posterior end of the male.

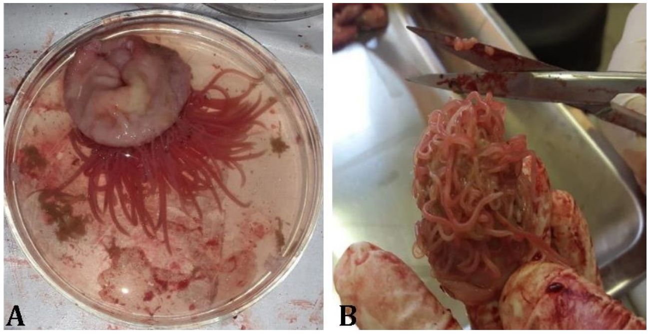

Fig. 1. A) The stomach of long-eared hedgehog (*Hemiechinus auritus*) parasitized with **B)** *Physaloptera clausa* nematodes attached to the mucosa of the fundus.

Polymerase chain reaction, sequence and phylogenetic analysis. In order to characterize *P. clausa*, 18S rDNA was amplified using barcoding primers targeted against specific region of DNA and the purified PCR products were sent for custom sequencing. The nucleotide sequences (18S rDNA) of *P. clausa* were found to be of good quality after analysis in Sequencher software. An amplicon of approx. 543 bp was amplified by PCR using primers targeting the barcode region of the *18S rDNA* gene. The nucleotide sequence homology was recorded in the range of 96.00 - 100% during BLAST analysis. The PCR products were purified and sent for custom sequencing. The sequence obtained in this study has been submitted to GenBank® under the accession number OR088573.

A comparison of the *P. clausa* sequence with other Spiruromorpha nematodes in GenBank® resulted in an automatically generated phylogenetic tree showing that the order consists of ten families: *Acuariidae, Diplotriaenidae, Onchocercidae, Habronematidae, Spirocercidae, Thelaziidae, Gongylonematidae, Cystidicolidae, Rhabdochonidae,* and *Camallanidae*. The analysis grouped all taxa except for outgroup (five genera of Camallanidae) into three main clades (Fig. 2). Physalopteridae was identified as the sister group to the Diplotriaenidae and Spirocercidae families with strong nodal support for this relationship. The topologies also indicated that the genus *Physaloptera* formed a major clade where *P. clausa* was clustered with various *Physaloptera* species closely related to *Turgida*, *Physalopteroides*, and *Skrjabinoptera* genera (Fig. 2).

Discussion

This study represented the first molecular evidence of *P. clausa* in Iran. This genus has previously been reported from three hedgehog species in Iran including *E. europaeus*, *E. concolor*, and *H. auritus* (Table 1). However, these reports were conducted only based on morphological features.

According to the global reports, *P. clausa* was isolated from the stomach of *E. deabatus,* Algerian hedgehogs (*Atelerix algirus*) and Amur hedgehog (*Erinaceus amurensis*).[8,19] The larval stage of this parasite is mostly prevalent in a wide variety of reptiles, frequently found in lizards. A study in Russia revealed that European viper (*Vipera berus*) is a new host for juvenile of *P. clausa*.[20]

There are few studies on *P. clausa* over the world. Despite the fact that the identification of this species in definitive hosts is not complicated, the control of infection caused by these parasites is difficult to manage, as there are a variety of species serve as intermediate hosts as well as paratenic hosts. Furthermore, there are multiple limitations regarding the study of specimens in habitats of both definitive and intermediate hosts. Besides, it is difficult to capture live animals for purposes such as parasitological investigation and identification of the source of infection as well as susceptible species.

Compared to mitochondrial genes, the *18S rRNA* gene evolves at a slower rate, making it a valuable tool for distinguishing between samples at higher taxonomic levels. It is a commonly used molecular marker in eukaryotic diversity research. In this study, the 18S rDNA was employed for molecular and phylogenetic characterization of *P. clausa* isolated from *H. auritus* in Iran.

In this study, the *18S rDNA* gene was used for molecular and phylogenetic characterization of *P. clausa* isolated from *H. auritus*. This gene evolves slowly and is suitable for resolving relationships at higher taxonomic levels. The amplified ~600 bp sequence showed high

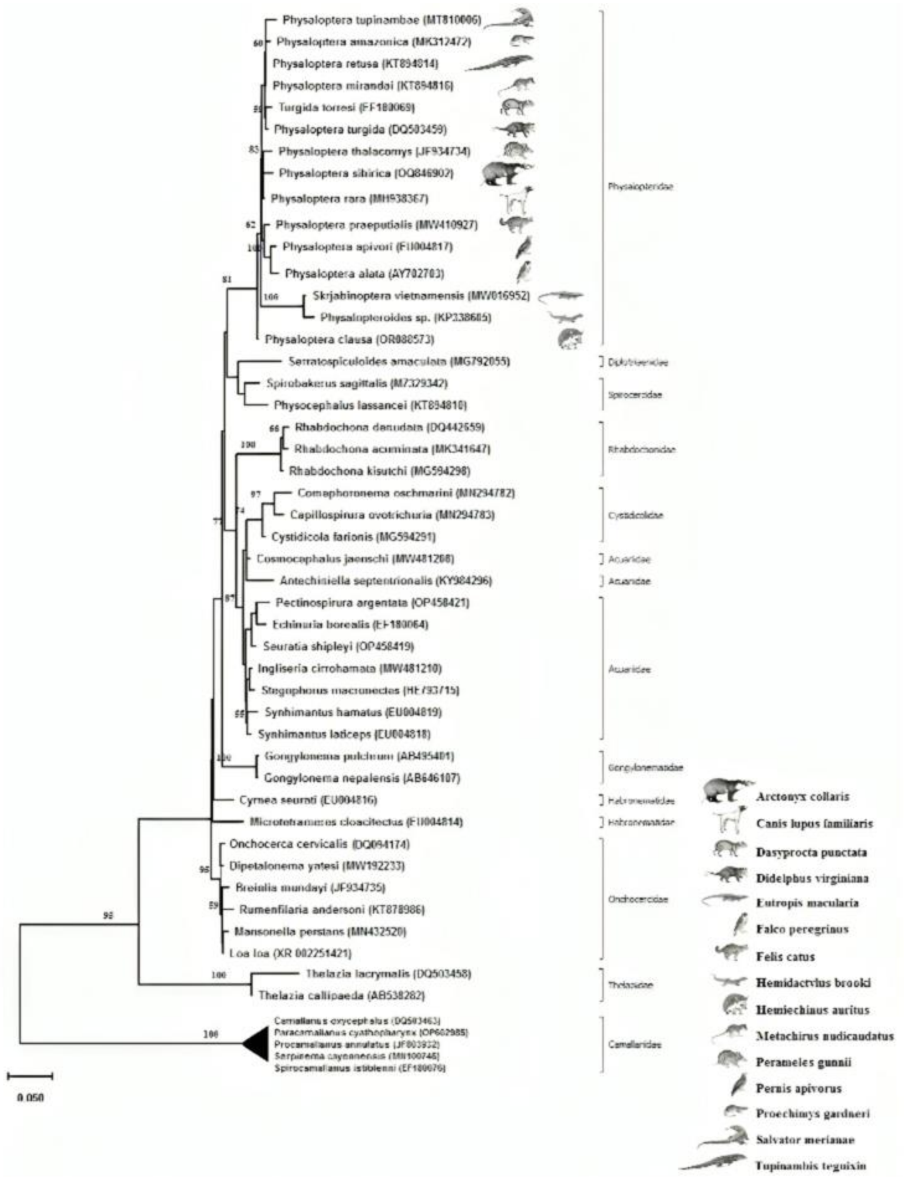

Fig. 2. The evolutionary history of 18s rRNA was inferred using the Maximum Likelihood method and General Time Reversible + G + I model. The analysis involved 53 nucleotide sequences with a total of 1928 positions in the final dataset. To ensure the accuracy of the results, 500 bootstrap replicates were performed, and the tree displayed represents the highest log likelihood value (-10753.11). The percentage of trees in which the associated taxa clustered together is shown above the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The evolutionary analyses were conducted using MEGA11.

Table 1. Main characteristics of *Physaloptera clausa* in different province of Iran, based on the results reported in the literature.

Species	Year	Provinces	Diagnostic methods	Sample size	Percentage	Male/Female	References
E. concolor	2013	Mazandaran	Necropsy and morphological detection	10	40.00	4/6	2
H. auritus	2016	Sistan & Baluchestan	Necropsy and morphological detection	50	40.00	35/15	3
E. europaeus	2013	West Azerbaijan	Necropsy, morphological detection and scanning electron microscopy	12	-	6/6	9
E. concolor	2014	East Azerbaijan	Necropsy and morphological detection	42	36.84	-	15
Not specified	2014	Mazandaran	Necropsy and morphological detection	9	44.40	3/6	16
E. europaeus	2015	West Azerbaijan	Necropsy and morphological detection	44	93.00	23/21	17
E. europaeus	2015	West Azerbaijan	Necropsy, morphological detection and transmission electron microscopy	12	-	6/6	18

homology with existing *Physaloptera* sequences in GenBank®. Phylogenetic analysis confirmed the clustering of *P. clausa* within the Physalopteridae family with strong bootstrap support, supporting previous reports on the position of physalopterids among related nematodes.[21-25]

The phylogenetic clustering observed in our analysis, where *P. clausa* grouped tightly with other *Physaloptera* species from various hosts and regions, suggested that this nematode exhibited a relatively broad host range and potentially low host specificity. This was consistent with previous studies reporting *P. clausa* in multiple hedgehog species as well as reptiles and amphibians, indicating ecological flexibility in its host use.[9] The strong clustering of isolates across different geographical origins may reflect wide geographic dispersion, possibly facilitated by the movement of intermediate or paratenic hosts such as reptiles.

Moreover, the close phylogenetic relationships among isolates from distant regions raise the possibility of either recent spread or ancient, conserved lineages with global distribution. These findings supported the hypothesis that *P. clausa* had a complex life cycle involving a range of hosts and habitats, which might help maintain genetic continuity across different ecosystems. However, more molecular data from diverse host species and locations are needed to draw firm conclusions on its biogeographic patterns and host adaptation.

Although the *18S* gene provided useful insights, it has limitations in resolving close evolutionary relationships. We acknowledged that using additional markers, such as ITS1/2, 28S, or mitochondrial genes (e.g., cyclooxygenase 1), could offer higher resolution and clarify the phylogenetic position more accurately. However, due to limited resources and the scarcity of reference sequences for these markers in GenBank® for *Physaloptera* spp., we chose 18S rDNA as the most suitable candidate for this preliminary investigation. Future studies should consider a Multilocus Sequence Typing (MLST) to improve resolution and address taxonomic uncertainties.

Currently, public databases lack comprehensive and well-annotated sequences of physalopterid species, especially from mitochondrial and genomic regions, which limits the construction of robust phylogenetic trees. Some available sequences are unidentified at the genus or species level, adding further uncertainty.

Choosing appropriate molecular markers is critical for accurately reconstructing evolutionary relationships. Mitochondrial genes evolve faster and are often used for species-level differentiation, while 18S is more stable and suited to deeper phylogenetic relationships. Integrating additional markers with intermediate evolutionary rates, such as the ITS region, would provide a more comprehensive understanding of *Physaloptera* diversity and its evolutionary dynamics.

In conclusion, this study provided the first molecular and phylogenetic characterization of *P. clausa* from *H. auritus* in Iran. The 18S rDNA-based analysis confirmed the identification of *P. clausa* and revealed its phylogenetic clustering within the *Physalopteridae* family. The observed phylogenetic relationships suggested a broad host range and wide geographic distribution for *P. clausa*, reflecting its ecological flexibility and the complex nature of its life cycle. Despite using a single genetic marker, our findings offered valuable insight into the evolutionary position of this parasite. However, further studies incorporating multiple genetic loci (e.g., ITS, Cyclooxygenase 1 1, 28S) and sampling across diverse hosts and regions are essential to resolve taxonomic ambiguities and better understand the evolutionary dynamics and ecological implications of *Physaloptera* species.

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Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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