

Research Note

Morphological and molecular identification of nematodes
in the tayra *Eira barbara* from Campeche, Mexico

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Summary

The tayra *Eira barbara* is a Neotropical mustelid considered as an endangered species by Mexican environmental authorities. Despite the considerable information available on the biology and ecology of *E. barbara*, little is known about its helminth fauna. Here, we provided new records of nematodes from a road-killed tayra in Calakmul, Campeche, Mexico. The species identification of nematodes was based on morphological studies and molecular analysis of fragments of the 28S gene. The tayra specimen was infected by three nematodes: *Molineus* sp., Physalopterinae gen. sp. and *Angiostrongylus vasosum*. To our knowledge, this study is the first to report the natural infection of *E. barbara* with *Molineus* sp. and Physalopterinae gen. sp. Our study provides the first nucleotide sequences of nematodes parasitizing *E. barbara* providing a starting point against which future studies may be compared.

Keywords: *Eira barbara*; Molineidae; Physalopteridae; Angiostrongylidae; Neotropical region

Introduction

The tayra *Eira barbara* is a Neotropical mammal belonging to the family Mustelidae (Presley 2000). This species occurs from the coasts of Central Mexico to northern Argentina (Villafañe-Trujillo *et al.*, 2018) and inhabits tropical and subtropical forests, secondary forests, plantations, and human settlements (Presley, 2000). Although *E. barbara* is assigned to the category Least Concern by the International Union for Conservation of Nature, the species currently experiences population declines due to agriculture, hunting and logging (Cuarón *et al.*, 2016). In Mexico, *E. barbara* is considered as an endangered species by environmental authorities (Secretaría de Medio Ambiente y Recursos Naturales, 2010). Despite the considerable information available on the biology and ecology of *E. barbara*, little is known about its helminth fauna

(Travassos, 1917; Cameron, 1936; Machado Filho, 1950; Caballero y Caballero, 1951; Kuns & Tashian, 1954; Vicente *et al.*, 1997; Noronha *et al.*, 2002; Vieira *et al.*, 2008; Cañizales & Guerrero, 2017). To date, few studies have reported helminths of *E. barbara* and most of them have been conducted in Brazil (Machado Filho, 1950; Vicente *et al.*, 1997; Noronha *et al.*, 2002; Vieira *et al.*, 2008). The present study describes the nematodes collected from a road-killed tayra in Calakmul, Campeche, Mexico, using morphological tools and molecular analysis.

Materials and Methods

An adult male tayra was found dead on October 1, 2019, on a road in the Calakmul municipality (18°31'25.2" N, 89°43'29.7" W), Campeche State, Mexico, where it was probably hit by a ve-

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hicle. The specimen was collected and frozen until parasitological studies. The host was deposited in the Coleccion Mastozoológica (CM-1468), Campus de Ciencias Biológicas y Agropecuarias, Universidad Autónoma de Yucatán, Yucatán, México. At the laboratory, heart, lungs, gastrointestinal tract (from stomach to rectum), pancreas, liver, kidneys, and mesenteries, were dissected and examined for helminths using a stereoscopic microscope (Motic SMZ-168). Only nematodes were found and preserved in 70 % ethanol.

The nematodes were cleared, temporarily mounted in lactophenol, and subsequently identified following the keys for nematodes (Anderson *et al.*, 2009). Drawings of nematodes were made with the

aid of a drawing tube (Olympus BX50). Vouchers of nematodes were deposited in the Coleccion Nacional de Helmintos (CNHE) of the Instituto de Biología, Universidad Nacional Autónoma de México, México City, México. The measurements of nematodes were recorded in micrometres.

Total genomic DNA was extracted from each species separately, using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Two external primers were used to amplify the D1-D3 regions of the 28S ribosomal gene; the primers were the Forward 391 (Nadler & Hudspeth, 1998) and the Reverse 536 (García-Varela & Nadler, 2005). For the PCR mix in each tube we added the following: 8.5 µl of distilled

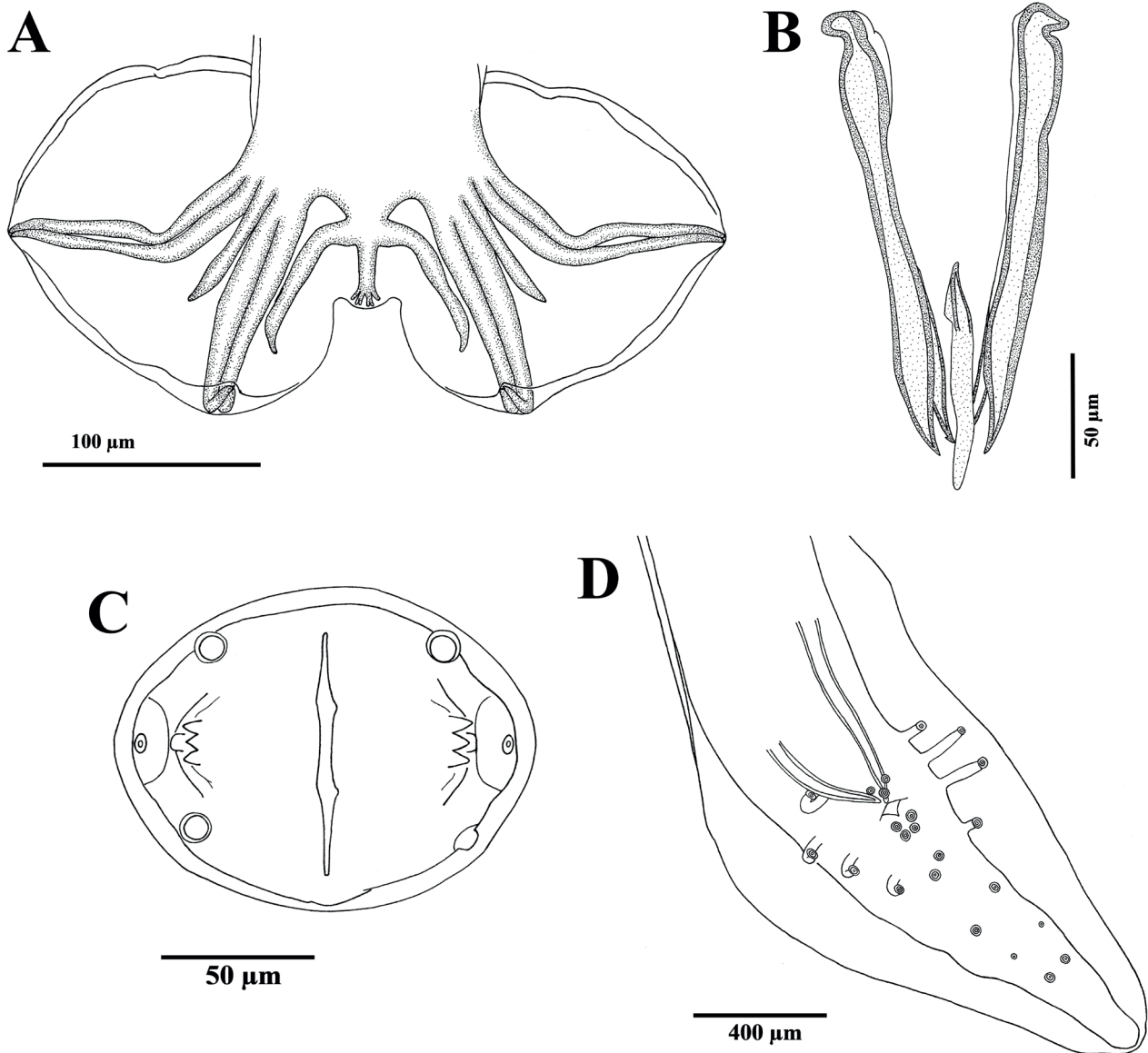


Fig. 1. A, Bursa of *Molineus* sp., ventral view. B, Spicules and gubernaculum of *Molineus* sp., ventral view. C, cephalic end of *Physalopterae* gen. sp., apical view. D, Posterior end of *Physalopterae* gen. sp., ventral view.

water, 12.5 µl of Green GoTaq Master Mix (Promega, Madison, WI, USA), 1 µl of each primer (10 µM) and 2 µl of genomic DNA. Conditions for amplifying the 28S gene were as follows: 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, extension at 72 °C for 1 min and finally an extension at 72 °C for 10 min. The amplicons obtained in the PCR were sequenced using the external primers plus two internal primers, 503 (Stock *et al.*, 2001) and 504 (García-Varela & Nadler, 2005). Sequencing of the PCR products were carried out by Genewiz (<https://www.genewiz.com>). From the sequences obtained with each primer, consensus sequences were obtained for each nematode specimen using Geneious Pro 4.8.4® (<https://www.geneious.com>) and submitted to GenBank.

The consensus sequences were aligned with other sequences deposited in GenBank that belong to several related species of nematodes (GenBank accession numbers and species are shown on trees). The alignments were generated in ClustalW, through in <http://www.genome.jp/tools/clustalw/>, with the approach “SLOW/ACCURATE” and weight matrix “CLUSTALW (for DNA)”. The nucleotide substitution model was estimated with jModelTest v2 (Darriba *et al.*, 2012). Maximum Likelihood method (ML) was implemented in RAxML v. 7.0.4 (Stamatakis, 2006). Analysis was performed with 1,000 repetitions Bootstrap. The ML trees were visualized in FigTree v.1.4.3 (Rambaut, 2007). Molecular variation of 28S data sets was estimated using uncorrected p distances in MEGA v.6.

Ethical Approval and/or Informed Consent

All applicable national and institutional guidelines for the care and use of animals were followed.

Results and Discussion

Three nematode taxa were identified in *E. barbara* from Calakmul, Campeche, Mexico: *Molineus* sp. (CNHE 9709, accession numbers MW853689 and MW853690) (Molineidae), Physalopterinae gen. sp. (Physalopteridae) (CNHE 9708, accession number MW853691) and *Angiostrongylus vasosum* (Baillet, 1866) (Angiostrongylidae) (accession number MW853688). To our knowledge, this study is the first to report the natural infection of *E. barbara* with *Molineus* sp. and Physalopterinae gen. sp. in Mexico, while *A. vasorum* is the only helminth species reported previously in this host (Caballero y Caballero, 1951; García-Prieto *et al.*, 2012).

Three complete and two incomplete specimens of *Molineus* sp. were collected from the intestines of *E. barbara*. Two male specimens collected from Calakmul presented morphological characteristics of the genus *Molineus* (Cameron, 1936; Marroquín-Muciño *et al.*, 2017). They have 26 cuticular ridges in the synlophes at midbody and symmetrical bursa with pattern of type of 2-1-2. Rays 2 and 3 are equal, run parallel, and reach bursal margin. Rays 4 are short with their extremities nearer to those of rays 5 than those

of rays 3. Rays 5 and 6 are equal, run parallel, and reach bursal margin. Rays 8 arise from the base of the dorsal ray. Dorsal ray divided into 2 short primary branches, each one of these, in turn, forks again into rays 9 and 10, with rays 10 distinctly bifid (Fig. 1.A). Spicules complex, base of handle with thickenings, equal in size (178–185 long), blade divided approximately at 1/3 distance from proximal end into external and internal processes. Internal process slender, ending in hammer shape while external process terminating in point (Fig. 1.B). Gubernaculum 95–100 long (Fig. 1.B).

The 28S sequences of two specimens of *Molineus* sp. had a length of 708 and 1000 bp. The genetic data obtained from our specimens had 90.6 % sequence similarity to a sequence of *Perostrostrongylus falciformis* (Schlegel, 1933) on GenBank (KY365435). The alignment for this nematode had a length of 1101 bp and the nucleotides frequencies were as follows: A = 0.297787, C = 0.171575, G = 0.264384 and T = 0.266254. The ML tree had a value of ln = -7165.961205 (Fig. 2). In the phylogenetic tree, our sequences of *Molineus* were grouped with a sequence of *P. falciformis* of the family Filaroididae (Bootstrap support value = 70). In turn, this species were grouped within a clade that contained other nematodes of the families Filaroididae and Pseudaliidae, such as *Parafilaroides decorus* Dougherty & Herman, 1947, *Filaroides martis* (Werner, 1782), *Pseudalius inflexus* (Rudolphi, 1808) and *Torynurus convolutus* (Kühn, 1829) with low bootstrap support value (24). The genetic differences between our sequences were null. Although other species of Molineidae have already been sequenced, our sequences had not similarities greater than 60 %, and were not considered in the phylogenetic analyses. This result indicates that it is necessary to review in more detail the taxonomy and classification of molineid nematodes, as reported by other works. Recently, molecular data provided by de Oliveira Simões *et al.* (2019) demonstrated that Molineidae does not constitute a monophyletic group but further analysis, which include additional taxa and genetic markers, should be conducted to elucidated the phylogenetic relationships within Molineidae. Additionally, it would be possible that no other sequences of *Molineus* have been submitted to the GenBank. Clearly, more genetic information is needed for this genus.

Up to now, nine species of the genus *Molineus* have been described from native terrestrial Neotropical carnivores: *Molineus felineus* Cameron, 1923, *Molineus barbaris* Cameron, 1923, *Molineus major* Cameron, 1936, *Molineus pardalis* Cameron, 1936, *Molineus paraensis* Travassos, 1937, *Molineus nasuae* Lent & Freitas, 1938, *Molineus barbatus* Chandler, 1942, *Molineus brachyurus* Costa & Freitas, 1967, and *Molineus lotoris* Marroquín-Muciño, Osorio-Sarabia, García-Prieto & Mata-López, 2017 (Cameron, 1923, 1936; Travassos, 1937; Lent & Freitas, 1938; Chandler, 1942; Costa & Freitas, 1967; Marroquín-Muciño *et al.*, 2017). According to the taxonomic key proposed by Marroquín-Muciño *et al.* (2017) which includes the species within *Molineus* distributed worldwide, species can be separated based on

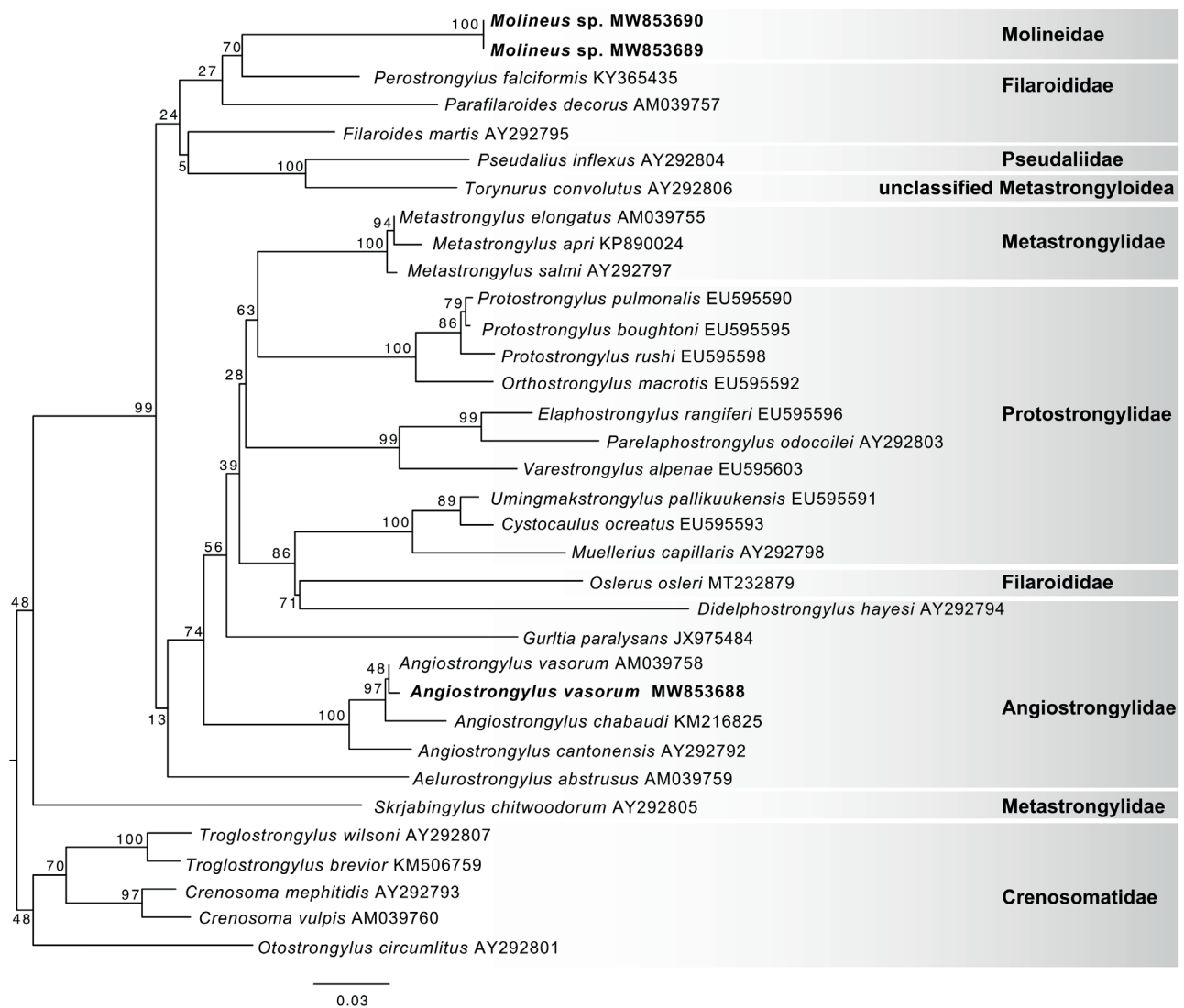


Fig. 2. Maximum likelihood tree of nematodes of the order Strongylida found in *E. barbara* constructed on partial large subunit ribosomal gene (28S). Likelihood = -7165.961205. The names of the species in bold type belong to the specimens sequenced in this study. In front of each species name is the GenBank accession number of the sequences used for phylogenetic analysis.

male characters, such as number and form of terminal process of spicules, length and form of spicules, synlophe ridges, and length and form of the dorsal ray. Our specimens can be differentiated from four species (i. e. *M. barbaris*, *M. pardalis*, *M. major*, and *M. nasuae*) by having 2 instead of 3 terminal processes in the spicules. In addition, the spicules of specimens from Campeche are longer (178–185) than those of *M. barbaris* (<100), *M. pardalis* (75) and *M. major* (130). Among the remaining five species with 2 terminal processes in the spicules, *M. barbatus*, *M. lotoris* and *M. felineus* have shorter spicules than our specimens (90–100, 99–115 and 120, vs 178–185, respectively) while *M. brachyurus* has longer spicules (205–217). Moreover, *M. paraensis* and *M. lotor* possess a smaller number of ridges in the synlophe than our

specimens (14 and 17, vs 26, respectively). It may be possible that these differences represent morphological variations or they may allow the description of a new species. Further morphological examination of more specimens, particularly males, and molecular analyses (including additional genes such as COX-1 and ITS) of several *Molineus* species are necessary to reach a definitive identification.

Two complete and one incomplete nematodes were found in the stomach of *E. barbara*. The observed characteristics of two complete males agree with morphological descriptions of members of the subfamily Physalopterinae (Bain *et al.*, 2013). The specimens are 24995–45468 long. The anterior end is dome-shaped, composed of two semicircular and convex pseudolips that laterally

surround the oral opening. Each pseudolip has a pair of papillae, marginally and dorsoventrally located, and an amphid is located on a porous-like circumscribed region (Fig. 1.C). Internal margin of lips present three internal lateral teeth and external then is projected a single tooth (Fig. 1.C). Posterior end is ventrally curved, with lateral alae, cuticle presents prominent cuticular striations. Twenty one papillae plus a pair of phasmids are present (Fig. 1.D): four pairs of pedunculated papillae, located in externolateral region of the lateral alae; three papillae just anterior to cloacal aperture, the central papilla is larger than the laterals and placed closer to the cloacal aperture; five postcloacal papillae pairs. Among the post-cloacal papillae, the first two pairs are located just posterior of the cloaca; the third and fourth pairs are asymmetrical, with the left papillae placed higher than the right; the pair of phasmid are located before the fifth pair of papillae. Spicules are different in size, the right is 380–590 long and the left 640–990 long.

The morphology of our specimens closely resembles to the genera *Physaloptera* and *Turgida* due to the following morphological features: Caudal alae broad, three internal lateral pairs of teeth, absence of submedian pairs of teeth, and four pairs of pedunculate papillae (Bain *et al.*, 2013). The genus *Physaloptera* has been reported in mammals (marsupials, myrmecophagans, rodents and carnivores), birds, squamates, and anurans around the world, while *Turgida* occurs in marsupials, caviomorph rodents, and primates in the Neotropical region (Bain *et al.*, 2013). These two closely related genera can be differentiated by the number of uteri, two to four in *Physaloptera* and more than four in *Turgida* (Anderson *et al.*, 2009). Unfortunately, no females were found in this study to place our specimens into a specific genus. Considering *Physaloptera* species described in carnivores from the Americas, only *Physaloptera maxillaris* Molin, 1860 and *Physaloptera rara* Hall & Wigdor, 1918 have unequal spicules with similar spicule length compared to those of our specimens but only the specimens from Mexico possess the third and fourth postcloacal papillae pairs asymmetrical. However, variations in the arrangement of caudal papillae have been reported in some species, such as *Physaloptera brevivaginata* Seurat 1917 (Esteban *et al.*, 1995) from *Myotis blythii* in Spain and *Physaloptera clausa* Rudolphi, 1819 from *Atelerix algirus* in Morocco (Seurat, 1917), from *Erinaceus europeus* in Europe (Ortlepp, 1922), and from *Urocyon cinereo-argenteus* in Mexico (Caballero y Caballero & Peregrina, 1938). Among *Turgida* species, *Turgida torresi* shows a similar arrangement of caudal papillae compared with our specimens, however, *T. torresi* Travassos, 1920 possesses the fourth postcloacal papillae pairs symmetrical and subequal spicules (Travassos, 1920).

The sequence of Physalopterinae gen. sp. had a length of 1159 bp. BLAST analysis showed similarities of 91.7 and 90.3 % with sequences of *Physaloptera* sp. (MG808041) and *T. torresi* (KY990020), respectively. The alignment for this data set had a length of 1434 bp and the nucleotides frequencies were as follows: A = 0.276853, C = 0.193091, G = 0.265001 and T = 0.265054. The ML tree had a value of ln = -9676.672333 (Fig. 3). The result of the

phylogenetic analysis showed that our specimen was the sister species of the clade formed by *Physaloptera* sp. and *T. torresi* with high Bootstrap support value (100). The species of this clade belong to Physalopteridae, and therefore we obtained a monophyletic group of this family in our analysis. In turn, this clade was the sister group of *Cylicospirura petrowi* (Sadykhov, 1957) (KM434335), a member species of Spirocercidae (Bootstrap support value= 24). Several sequences of the cytochrome c oxidase subunit 1 (COI) gene for different Physalopteridae species are available within GenBank, however, we could not successfully amplify this gene for our specimen and therefore we could not make a comparison for a more accurate identification. Further morphological examination of more specimens, particularly females, and the generation of more sequences from different species allows us to achieve a definitive identification of this nematode.

Unfortunately, a few fragments of nematodes were found in the pulmonary arteries in the *E. barbara* specimen to present suitable morphological characteristics for identification. However, one fragment of the nematode was sequenced with the 28S gene, and the sequence obtained had a length of 1159 bp. The result of the BLAST analysis confirmed the taxonomic identity of our sequenced species, since it obtained a 99.20 % identity with *A. vasorum* (AM039758). Additionally, our sequence obtained high values of the percentage of identity with other species of the same genus: 96.49 % with *Angiostrongylus chabaudi* Biocca, 1957 (KM216825) and 95.46 % with *Angiostrongylus cantonensis* (Chen, 1935) (AY292792). The phylogenetic analysis where the taxonomic identity and phylogenetic position of our *A. vasorum* specimen were explored with the same data set that was used for *Molineus*, since they both belong to Strongylida, and therefore, the values of the length of the aligned matrix, as well as the nucleotide frequencies and the likelihood of the phylogenetic tree are the same as described above (Fig. 2). Our sequence was grouped with the other sequenced specimen of *A. vasorum* that was obtained from *Vulpes vulpes* (Bootstrap support value= 48). In turn, *A. vasorum* was nested in a monophyletic clade that also grouped the other two species of the genus represented in our analysis with high Bootstrap support value (100). The genetic difference between the compared specimens of *A. vasorum* was only 0.82 %. Unfortunately, the lack of adequate material to explore the morphology of these nematodes did not allow us to ascertain the existence of phenotypic differences that support the genetic differences found in the 28S gene.

Physalopterinae gen. sp. and *A. vasorum* have indirect life cycles (heteroxenous species). Various insects, such as cockroaches (*Blattella germanica*) and crickets (*Gryllus pennsylvanicus*), have been reported as intermediate hosts of physalopterines (Cawthorn & Anderson 1976; Anderson 2000). The life cycle of *A. vasorum* involves gastropods (e.g., *Arion ater*, *Biomphalaria glabrata*, *Bradybaena similis*) as intermediate hosts (Anderson 2000). In addition, several paratenic hosts such as amphibians, reptiles, rodents and birds have been reported for both nematode species

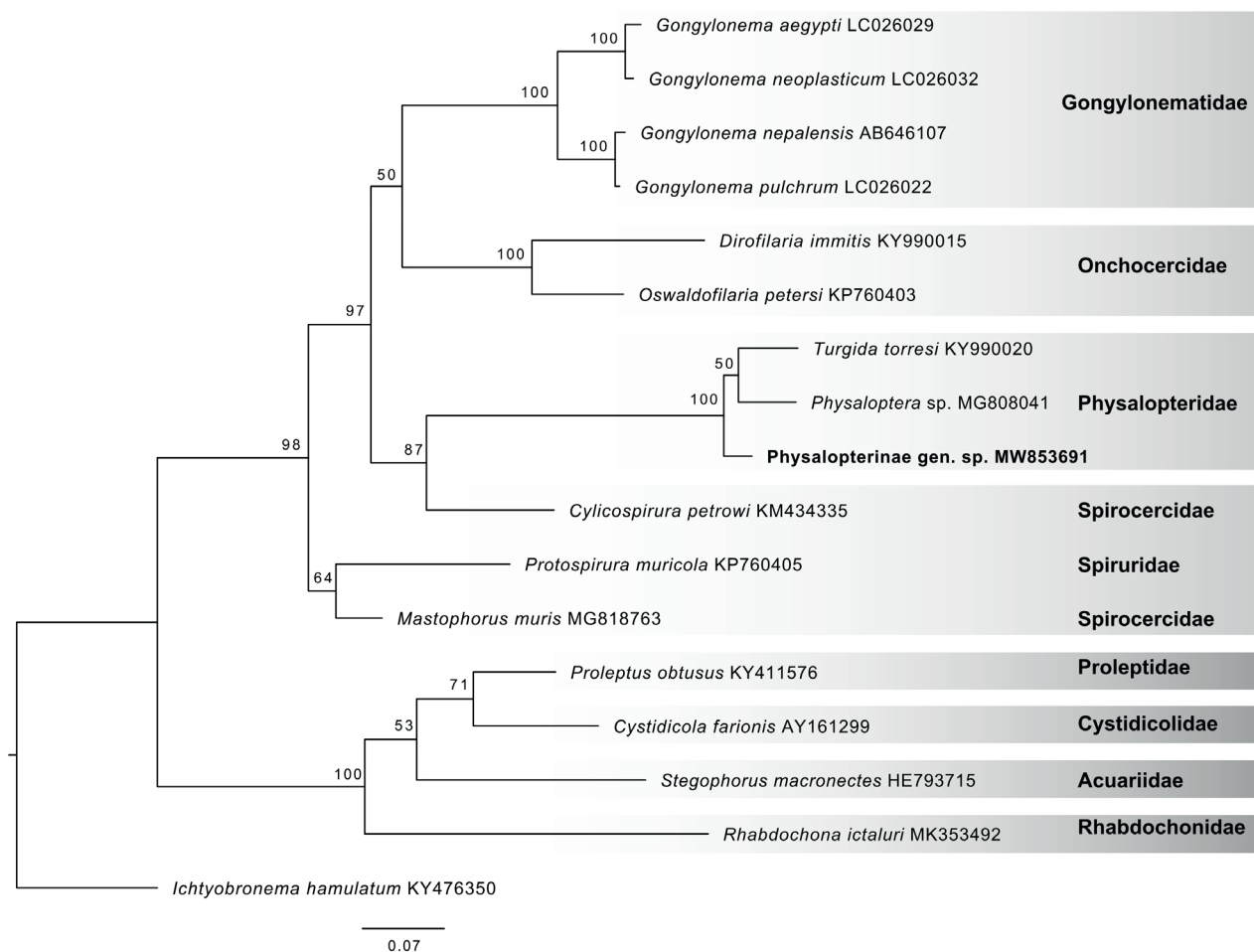


Fig. 3. Maximum likelihood tree of the nematode of the subfamily Physalopterinae found in *E. barbara* constructed on partial large subunit ribosomal gene (28S). Likelihood = -9676.672333. The names of the species in bold type belong to the specimens sequenced in this study. In front of each species name is the GenBank accession number of the sequences used for phylogenetic analysis.

(Cawthorn & Anderson 1976; Anderson 2000). Many carnivores acquire nematodes frequently through the ingestion of paratenic hosts (Anderson 2000). *Eira barbara* is an opportunistic omnivore that consumes fruits, honey, insects, and small vertebrates (Presley 2000). Studies on scats of tayras reported that their diets include several rodent species, which may act as potential paratenic hosts for heteroxenous nematodes. The third nematode species identified in this study, *Molineus* sp., has a direct life cycle (monoxenous nematode).

Our study increases to 14 the number of helminth taxa reported in *E. barbara*. Previously 12 taxa had been reported from *E. barbara*: the acanthocephalans *Pachysentis gethi* (Machado Filho, 1950) from Brazil (Machado Filho, 1950) and Venezuela (Cañizales & Guerrero, 2017) and *Prosthenorchis elegans* (Diesing, 1851) from Brazil (Travassos, 1917), and the nematodes *Toxascaris leonina* (Linstow, 1902) from Brazil (Noronha *et al.*, 2002), *Toxascaris* sp. from Brazil (Vieira *et al.*, 2008), *Dirofilaria spectans* Freitas &

Lent, 1949 from Brazil (Noronha *et al.*, 2002), *Dyoctophyma renale* (Goeze, 1782) from Mexico (Kuns & Tashian, 1954), *Filaria carvalhoi* Freitas & Lent, 1937 from Brazil (Vieira *et al.*, 2008), *A. vasorum* from Mexico (Caballero y Caballero, 1951), *Angiostrongylus* sp. from Brazil (Vieira *et al.*, 2008), *Physaloptera* sp. from Brazil (Vieira *et al.*, 2008), *M. barbaris* from Trinidad and Tobago (Cameron, 1936) and Brazil (Vicente *et al.*, 1997), and *M. major* from Trinidad and Tobago (Cameron, 1936) and Brazil (Vicente *et al.*, 1997).

Overall, three helminth taxa were identified from *E. barbara* in this study: *Molineus* sp., Physalopterinae gen. sp. and *A. vasorum*. The molecular data are the first to be obtained for the helminths of *E. barbara*. Given the conservation status of *E. barbara* in Mexico, it would be advisable to conduct further helminthological studies based on faecal samples or road-killed specimens, incorporating molecular analysis to increase the knowledge of the helminths of this endangered species.

Conflict of Interest

Authors state no conflict of interest.

Acknowledgement

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