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-
The specimen was collected and frozen until parasitological studies. The host was deposited in the Coleccion Mastozoologica (CM-1468), Campus de Ciencias Biologicas y Agropecuarias, Universidad Autonoma de Yucatan, Yucatan, Mexico. 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, temporally 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 Biologia, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico. 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 water.

Fig. 1. A, Bursa of Molineus sp., ventral view. B, Spicules and gubernaculum of Molineus sp., ventral view. C, cephalic end of Physalopterinae gen. sp., apical view. D, Posterior end of Physalopterinae 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.geneiwiz.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 vasorum* (Baillet, 1866) (Angiostrogylineidae) (accession number MW853688). To our knowledge, this study is the first to report the natural infection of *E. barbara* with *Molineus* sp. and Physalopteridae 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; Marroquin-Muciño et al., 2017). They have 26 cuticular ridges in the synlophe 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 *Perostrongylus falciformis* (Schlegel, 1933) on GenBank (KY365435). The alignment for this nematode had a length of 1101 bp and the nucleotide 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 Filarioidea (Bootstrap support value = 70). In turn, this species were grouped within a clad that contained other nematodes of the families Filarioidea and Pseudalidae, such as *Paraflaroides decorus* Dougherty & Herman, 1947, *Filarioidea 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.

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. lotor* 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 semi-circular 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 circumscissed region (Fig. 1.C). Internal margin of lips present three internal lateral teeth and external then is project-ed 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 external lateral 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 postcloacal 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 peduncu-late papillae (Bain et al., 2013). The genus Physaloptera has been reported in mammals (marsupials, myrmecophageans, 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 (And-erson 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 Moline, 1860 and Physaloptera rara Hall & Wigdor, 1918 have unequal spicules with similar spicule length compared to those of our specimens but only the spec-imens from Mexico possess the third and fourth postcloacal papil-laes asymmetrical. However, variations in the arrangement of caudal papillae have been reported in some species, such as Phy-saloptera 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 arrange-ment of caudal papillae compared with our specimens, however, T. torresi Travassos, 1920 possesses the fourth postcloacal papillae pairs symmetrical and subequale 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 = 0.276883, 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 be-long to Physalopteridae, and therefore we obtained a monophylet-ic group of this family in our analysis. In turn, this clade was the sis-ter group of Cylicospirura petrowi (Sadykov, 1957) (KM434335), a member species of Spirocercidae (Bootstrap support value= 24).

Several sequences of the cytchrome 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 suita-ble 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 se-queanced species, since it obtained a 99.20 % identity with A. vaso-rum (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 iden-tity 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 %. Unfortu-nately, 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 similaris) as intermediate hosts (Anderson 2000). In addition, several paratenic hosts such as amphibians, reptiles, ro-dents and birds have been reported for both nematode species
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.

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