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Mansonella sp. and associated *Wolbachia* endosymbionts in ring-tailed coatis (*Nasua nasua*) in periurban areas from Midwestern Brazil

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ABSTRACT

Coatis (*Nasua nasua*) are wild carnivorous well adapted to anthropized environments especially important because they act as reservoirs hosts for many arthropod-borne zoonotic pathogens. Information about filarioids from coatis and associated *Wolbachia* spp. in Brazil is scant. To investigate the diversity of filarial nematodes, blood samples (n = 100 animals) were obtained from two urban areas in midwestern Brazil and analyzed using blood smears and buffy coats and cPCR assays based on the *cox*1, 12S rRNA, 18S rRNA, *hsp*70 and *myo*HC genes for nematodes and 16S rRNA for *Wolbachia*. When analyzing coati blood smears and buffy coats, 30% and 80% of the samples presented at least one microfilaria, respectively. Twenty-five *cox*1 sequences were obtained showing 89% nucleotide identity with *Mansonella ozzardi*. Phylogenetic analyses clustered *cox*1 sequences herein obtained within the *Mansonella* spp. clade. Sequences of both *myo*HC and two *hsp*70 genes showed 99.8% nucleotide identity with *Mansonella* sp. and clustered into a clade within *Mansonella* sp., previously detected in coatis from Brazil. Two blood samples were positive for *Wolbachia*, with a 99% nucleotide identity with *Wolbachia* previously found in *Mansonella perstans, Mansonella ozzardi* and *Mansonella atelensis* and in ectoparasites of the genus *Pseudolynchia, Melophagus* and *Cimex*. The study showed a high prevalence of *Mansonella* sp. in the coati population examined, suggesting that this animal species play a role as reservoirs of a novel, yet to be described, species within the Onchocercidae family.

1. Introduction

Filarioids belonging to the Onchocercidae family can be transmitted by a plethora of arthropod hosts (e.g., mosquitoes, black flies, midges, ticks, lice and mites) to several animal species, including humans (Shelley and Coscoran, 2001; Bain et al., 2008; Michalski et al., 2010; Otranto and Dantas-Torres, 2010; Otranto et al., 2011; Capelli et al., 2018; Otranto and Deplazes, 2019; Pupić-Bakrač et al., 2021). Some of these agents have a zoonotic potential, such as *Dirofilaria immitis,* which causes heartworm disease in dogs, and *Dirofilaria repens,* which may cause a non-pathogenic subcutaneous infection in humans (Capelli et al., 2013, 2018; Pupić-Bakrač et al., 2021). While the former is the main causative agent of human dirofilariasis in the United States, the latter is more prevalent in the Old World (Dantas-Torres and Otranto, 2013). Additionally, a novel *Dirofilaria* species was recovered from the eye of a young patient in the Amazon Forest, Northern Brazil (Bain et al., 2011).

The biology and epidemiology of filarioids has been extensively investigated in domestic animals (Otranto and Dantas-Torres, 2010; Grácio et al., 2015) and, at a lesser extent, in wildlife, especially in geographical areas with a huge host biodiversity (Conga et al., 2019; Whittier et al., 2020). Wild carnivores (e.g., jackals, foxes, wolves) have been incriminated as reservoirs for *D. immitis* in different regions of the

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world (Otranto et al., 2015), contributing to the endemicity of such agent where domestic animals are under chemoprophylactic treatment (Otranto and Deplazes, 2019).

Among carnivores, procyonids have been shown to be receptive hosts for filarioids. Raccoons (*Procyon lotor*) in the USA were found infected with *D. immitis*, *Dirofilaria tenuis, Acanthocheilonema procyonis, Mansonella llewellyni, Brugia beaveri,* and *Dracunculus insignis* (Smith, 1980; Cole and Shoop, 1987; Snyder et al., 1989; Keeling et al., 1993; Harbut and Orihel, 1995; Pung et al., 1996). *Dirofilaria tenuis*, a common parasite of raccoons, was also detected in a wrist lesion of a healthy young woman (Ramirez et al., 2013).

Ring-tailed coatis (*Nasua nasua*) are medium-sized procyonids widely distributed in South America (e.g., Brazil, Paraguay) that easily adapt from forestry to urbanized ecosystems. Such adaptability of this mammal species to both wild and anthropized environment favors parasite exchanges with other animal species (Alves-Costa et al., 2004; Emmons and Helgen, 2016). Coatis in Brazil have been found infected with *Dirofilaria incrassata*, *Dirofilaria repens,* and *Dirofilaria* sp. (Vicente et al., 1997; Guimarães et al., 2023). Additionally, at least three Onchocercids, namely *Dirofilaria* sp., *Mansonella* sp. and one Onchocercidae species yet to be characterized, have been detected in coatis from Paraná State, Southern Brazil, suggesting their role as reservoirs of zoonotic filarioids (Moraes et al., 2017; 2022). Unfortunately, microfilariae from coatis could not be identified at species level, due the limited number of sequences available in genetic databases of heat shock protein (*hps*70) and myosin heavy chain (*myo*HC) genes (Moraes et al., 2017, 2022). Conversely, many filarioids have been barcoded by using a dual approach that combines morphological key characters for adult helminths and sequencing of three molecular markers, namely 12S rRNA, cytochrome *c* oxidase subunit 1 (*cox*1) and 18S rRNA (Bain et al., 2008). Therefore, the diversity of Onchocercidae species in the Procyonidae family underpins the role played by these carnivorous as reservoirs for zoonotic filarioids.

Wolbachia endosymbionts are intracellular obligatory bacteria involved in the reproduction, development, and long-term survival of filarial nematodes (Taylor et al., 2005; Martin and Gavotte, 2010; Manoj et al., 2021). Accordingly, *Wolbachia* are vertically transmitted to the progeny through the eggs cytoplasm, being mainly associated to female nematodes reproductive system (Hoerauf et al., 2003; Bouchery et al., 2013). These endosymbionts are classified in supergroups, based on their genetic evolution in a large variety of hosts (Fenn et al., 2006; Manoj et al., 2021). *Wolbachia* spp. found in nematodes belong to the supergroups C, D (Fenn et al., 2006) and F (Casiraghi, et al., 2005; Ferri et al., 2009, 2011; Lefoulon et al., 2012).

Given the paucity of information about the life history of filarioids and associated endosymbionts parasitizing coatis from Brazil, and the possibility that they harbor species of importance for health of human and domestic animals, we characterized morphologically and molecularly blood microfilariae and assessed the prevalence of infection in coatis sampled in two urban fragments from Midwestern Brazil. Also, data were corroborated by molecular information on *Wolbachia* endosymbionts.

2. Material and methods

2.1. Blood sampling and microfilariae examination

Between March of 2018 and January of 2019, coatis were sampled every three months during 10 consecutive nights in two sampling spots [*Parque Estadual do Prosa* (PEP) (−20.44987, −54.56529)] and Brazilian Air Force Private Area (VBA) (−20.47163, −54.65405)], in Cerrado biome, located in Campo Grande city, Mato Grosso do Sul State, Midwestern Brazil. All captures and recaptures were performed by convenience, as previously described (Barreto et al., 2021; Perles et al., 2022). Briefly, coatis were captured using metal traps (1 m × 0.40 m x 0.50 m) placed arbitrarily according to the possibility of human access and availability of shadow, in order to cover most of the PEP and VBA areas. They were anesthetized with an association of Tiletamine hydrochloride and Zolazepam hydrochloride (Telazol, Zoetis® - 7 mg/kg, Intramuscularly). After chemical restraint, animals were marked with numbered colored earrings and had a microchip implanted in the subcutaneous tissue between the shoulder blades (Perles et al., 2022). Blood samples obtained from femoral vein were stored in tubes containing EDTA (Ethylenediamine tetraacetic acid) and divided for two analyses: a) microhematocrit to obtain buffy coat and blood smears stained with Diff Quick for visual detection of microfilaria and b) DNA extraction for molecular analyses. Examination of the microfilariae was performed using an optical microscopy (Leica DM-LB2). Morphological analyzes were performed by measuring the microfilariae available (i.e., from 1 to 5 specimens for each sample) (Bain et al., 2015) with Leica Las version 4.5.0 software (Leica Microsystems, Wetzlar, Germany) after Diff Quick stain followed by comparison with the scientific literature.

2.2. Molecular assays

DNA was extracted from 200 μL of each coati blood sample using Illustra Blood Mini Kit (GE Healthcare®, USA), according to the manufacturer's instructions. To evaluate the quality of the extracted DNA and avoid false negative results, DNA samples were tested by a conventional PCR targeting the mammal *gapdh* (~400 bp GAPDH-F 5′-CCTTCATTGACCTCAACTACAT3′ and GAPDH-R 5′- CCAAAGTTGTCATGGATGACC -3′) (Birkenheuer et al., 2003). Twenty-five samples presenting microfilaria at buffy coat analysis were randomly chosen randomly and were submitted to a PCR assay using filarioid-generic primers, namely NTF (5′-TGATTGGTGGTTTTGGTAA-3′) and NTR (5′-ATAAGTACGAGTATCAATATC-3′), that amplifies a fragment (689 bp) of the mitochondrial *cox*1 gene. For further characterization, samples were submitted to PCR assays targeting the 12S rRNA (12SF 5′-CGGGAGTAAAGTTTTGTTTAACGG-3′ and 12SR 5′-CATTGACGGATGGTTTGTACCAC-3′), 18S rRNA (NC18SF1 5′-AAAGATTAAGCCATCCA-3′ and NC5BR 5′-GCAGGTTCACCTACAGAT-3′) (Casiraghi et al., 2004, 2006; Ferri et al., 2009), *hsp*70 (h70ManF 5′-TGAGACAGCTGGAGGTGTTATG-3′ and h70ManR 5′- ATCTTTCTGTGCCTCATCATCTG-3′), and *myoHC* genes (MyManF 5′-GAAGCTGAGGCTCAAGCAAT-3′ and MyManR 5′-TCTGTTTTGCTCATCGCATT-3′) (Moraes et al., 2022). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as a negative control in all PCR assays. *Thelazia callipaeda* DNA (Bezerra-Santos et al., 2022) was used as a positive control.

The presence of *Wolbachia* DNA was tested through a PCR targeting the 16S rRNA gene (Parola et al., 2000) in the same samples submitted to conventional PCR (cPCR) screening for filarioids. PCR products were subjected to 2% gel red-stained agarose gel electrophoresis and results were visualized in UV transilluminator. Amplicons were purified and sequenced in both directions using the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic Analyzer (Applied Biosystems, California, USA) in an automated sequencer (ABI-PRISM 377).

2.3. Phylogenetic analyses

Sequences obtained were compared with those available in GenBank database through BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Benson et al., 2012). For phylogenetic inferences, sequences from the present study were aligned with those retrieved from GenBank using MAFFT software version 7 (Katoh et al., 2019). The best evolutionary model was chosen under the Akaike Information Criterion (AIC) and Maximum likelihood (ML) phylogenetic analyses was performed using the iqTREE software (available at: http://iqtree.cibiv.univie.ac.at/). The phylogenetic tree edition and rooting (outgroup) were performed using TreeGraph 2.0 beta software (Stover and Muller 2010). Protein translation for *cox1* gene was performed with MEGA software (Kumar et al., 2018). Interspecific and intraspecific nucleotide divergence and the

presence of haplotypes were evaluated according criteria previously established by Ferri et al. (2009) using MEGA software version 7.0 (Kumar et al., 2018).

3. Results

Of the 100 coatis captured (n = 42 in PEP and n = 58 in VBA; 57 females, 43 males) 30% and 80% scored positive for microfilariae at blood smears and buffy coat, respectively.

Microfilariae were serpentine in shape had no sheath and measured 190.5 ± 14.4 μm in length and 3.29 ± 0.28 μm width. Anterior end was rounded with short head space and presented cephalic space with scattered fine nuclei (Fig. 1). The tail was long, slender, pointed with nuclei to the end. Body appeared with compact column of the nuclei purple in color, extending to the end of the tail (Fig. 1). Nuclei were sparse osmogenes in the region of the inner body with exception of two big round areas without nuclei, in the initial third of the body (about 50–55 μm from anterior end) and in the last third, before the tail (about 30–35 μm from anterior end) (Fig. 1). Unfortunately, due to limitations on the blood smear staining, it was not possible to measure distances from the nerve ring to the anterior end, distance of the excretory pore from the anterior end, and distance of the anal opening from the tip of the tail. Based on the above morphological character's specimens were morphologically identified as *Mansonella* sp.

BLAST of 25 *cox*1 sequences (603 bp) showed 88–89% nucleotide identity (100% of query coverage and 0.0 E−value) to *M. ozzardi* from human of Peru (JF412347). In addition, *cox*1 sequences revealed two different haplotypes with 15 silent mutations (Supplementary Material, Table SM1). Two *hsp*70 sequences (468 pb) showed 99.7% nucleotide identity (100% of Query Coverage and 0.0 of E−value) with *Mansonella* sp. (MW330348-49) previously detected in coatis from Paraná state, Southern Brazil. The only *myo*HC sequence showed 99.8% identity (99% of Query Coverage and 0.0 of E−value) with *Mansonella* sp. previously detected in coatis from Paraná state (MW330367). All nucleotide sequences of *cox*1 and of *hsp70* and *myoHC* were clustered in a large *Mansonella* spp. clade (Fig. 2A, B, C), with those of the latter genes grouped along with a *Mansonella* sp. previously detected in coatis from Brazil (Fig. 2B and C). No sequences were obtained for 12S rRNA and 18S rRNA genes. The two *Wolbachia* 16S rRNA sequences obtained showed >99% nucleotide identity (100% of query coverage and 1e-171 of E−value) with *Wolbachia* spp. previously detected in *M. perstans* (AY279355), *M. ozzardi* (AJ279034) *M. atelensis* (FR827940) and in *Pseudolynchia* sp. (MF461471), *Melophagus ovinus* (KY224164), *Cimex lectularis* (KU255228). Maximum likelihood phylogenetic analyses clustered the two *Wolbachia* sequences closely related to *Wolbachia* spp. previously detected in *Mansonella* spp. (Supergroup F) (Fig. 3).

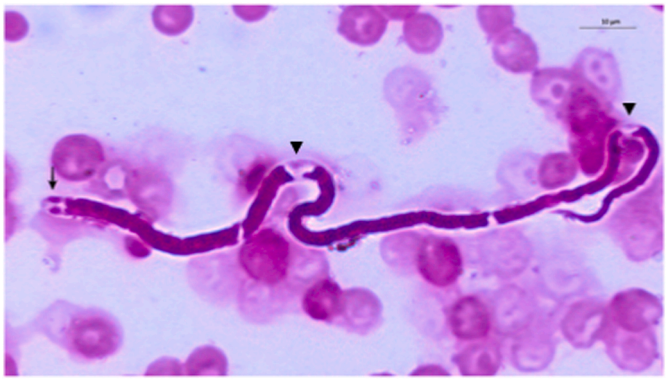

Fig. 1. Microfilaria of *Mansonella* sp. from a coati (*Nasua nasua*) blood smear stained with Diffy Quicky. Body of the microfilariae with compact column of the nuclei purple in color, sparse osmogenes in the region of the inner body with exception of two big round areas without nuclei (arrow head), with anterior end rounded with short head space and cephalic space with scattered fine nuclei (arrow). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Coatis herein examined were infected with blood circulating *Mansonella* sp. microfilariae, suggesting that this animal species is a host for this onchocercid of potential zoonotic interest. Indeed, *Mansonella* includes a variety of species infecting a large number of different mammal hosts, such as carnivores, ungulates, sciurids, and primates (Anderson, 2000). Of those, *M. perstans*, *M. ozzardi* and *M. streptocerca* have a zoonotic potential (Mediannikov and Ranque 2018), being reported in South and Central America (i.e., *M. perstans* and *M. ozzardi*) (Lima et al., 2016; Mediannikov and Ranque 2018) and in Africa (i.e., *M. perstans* and *M. streptocerca*) (Simonsen et al., 2011; Mediannikov and Ranque, 2018).

Though morphological and molecular information are essential for proper diagnosis at species level, the microfilariae were morphologically identified until genus level (*Mansonella* sp) in the present study. The only species from this genus found in Procyonidae is *Mansonella llewellyni*, which was first described in subcutaneous tissue of a raccoon (*Procyon lotor*) in Maryland, USA (Price, 1962). This species was also found in different regions of USA in raccoons (Telford and Forrester, 1991; Pung et al., 1996), with microfilariae (290 μm in length, with long tail ending in delicate button-hook curve without nuclei) (Price, 1962) differing morphologically from those found herein in coatis. Furthermore, molecular analyses performed in the present study confirmed the identification at genus level as *Mansonella* sp., clustering the obtained sequences with *M. ozzardi* and *M. perstans*, both of zoonotic concern. Even though the biological life cycle of this filarioid from coatis is not known, it could be transmitted by Ceratopogonidae and Simuliidae arthropods, being the latter intermediate hosts for *Mansonella* genus (Shelley and Coscoran, 2001). The presence of coatis in urban areas may favor pathogens transmission and exchange to humans and domestic animals.

Herein, *Wolbachia* 16S rRNA sequences associated with the new detected *Mansonella* sp. from coatis showed to belong to the supergroup F. *Wolbachia* endosymbionts have been strictly associated with filarioids but not with other nematode groups (Bordenstein et al., 2003; Taylor et al., 2013). These endosymbionts seem to be host-specific, with each genotype associated with a specific filarioid species (Taylor et al., 2013). For instance, Laidoudi et al. (2020) detected *Mansonella*-associated *Wolbachia* 16S rRNA sequences that clustered into the supergroup F in a blood sample from a red howler monkey (*Allouata macconnelli*) in French Guiana.

Data suggest that the use of two or more distinct molecular markers is necessary for an accurate taxonomical identification of filarioids (Bain et al., 2008). Herein we provide the first sequencing of *cox*1 gene, the most used molecular marker for molecular taxonomy (Bain et al., 2008), from procyonid-associated filarioids. The phylogenetic inferences based on both *hsp*70 and *myo*HC genes associated with the low genetic divergence found in BLAST analysis (0.2–0.3%) indicates that *Mansonella* species detected in coatis sampled in the state of Mato Grosso do Sul corresponds to that one previously reported in coatis from Paraná state (Moraes et al., 2022).

Coatis tend to form large familiar groups and easily adapt in urbanized areas, mainly as a consequence of natural habitat encroachment, land use changes, deforestation and urbanization. Indeed, coatis are often seen searching for food in garbage and walking on the streets and climbing on house walls, near to dogs and cats, in both studied areas (PEP and VBA). The close contact between coatis and humans and domestic animals may favor the exchange of zoonotic pathogens. Therefore, the molecular surveillance of filarioids in coatis from urban areas is much needed. Future studies are necessary to describe this putative novel *Mansonella* species through morphological description and molecular characterization of adult nematodes. The search for adults of this putative novel *Mansonella* sp. in coatis' subcutaneous tissues and

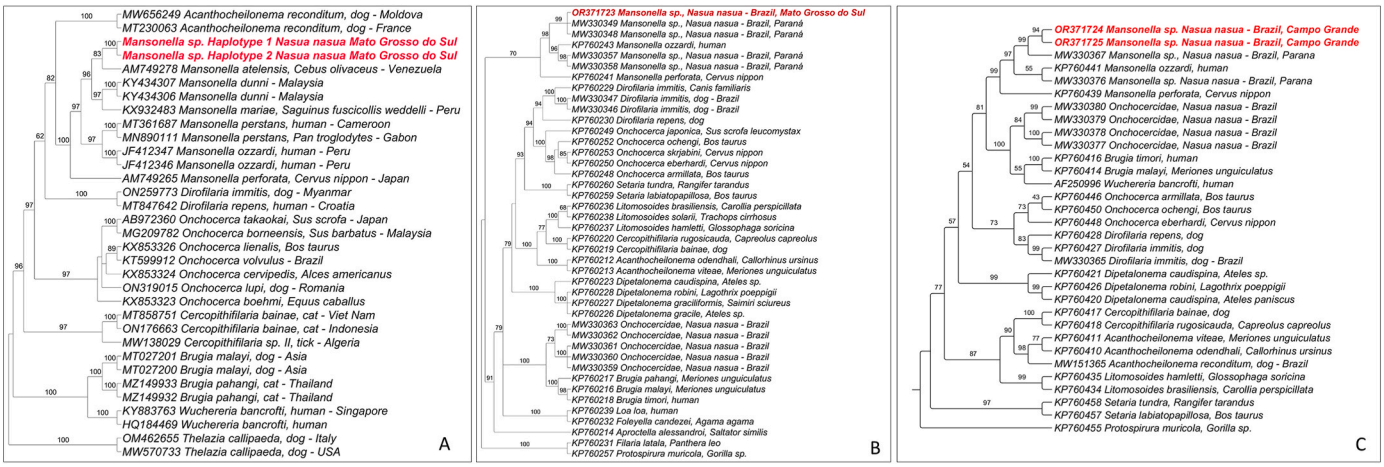

Fig. 2. Phylogenetic tree based on Maximum likelihood method from filarioids detected in blood samples from coatis (*Nasua nasua*) from Campo Grande city, Mato Grosso do Sul state, Brazil. A) *cox1* gene, *Thelazia callipaeda* was used as outgroup (alignment of 681 bp and TIM3+G as evolutionary model); accession numbers from the sequences detected at the present study: OQ261670-OQ261693; B) *myoHC* gene, *Filaria latala* and *Protospirura muricola* were used as outgroups (alignment of 480 pb and HKY + G as evolutionary model); C) *hsp70* gene, *Protospirura muricola* was used as outgroup (alignment of 545 pb and TN + G as evolutionary model).

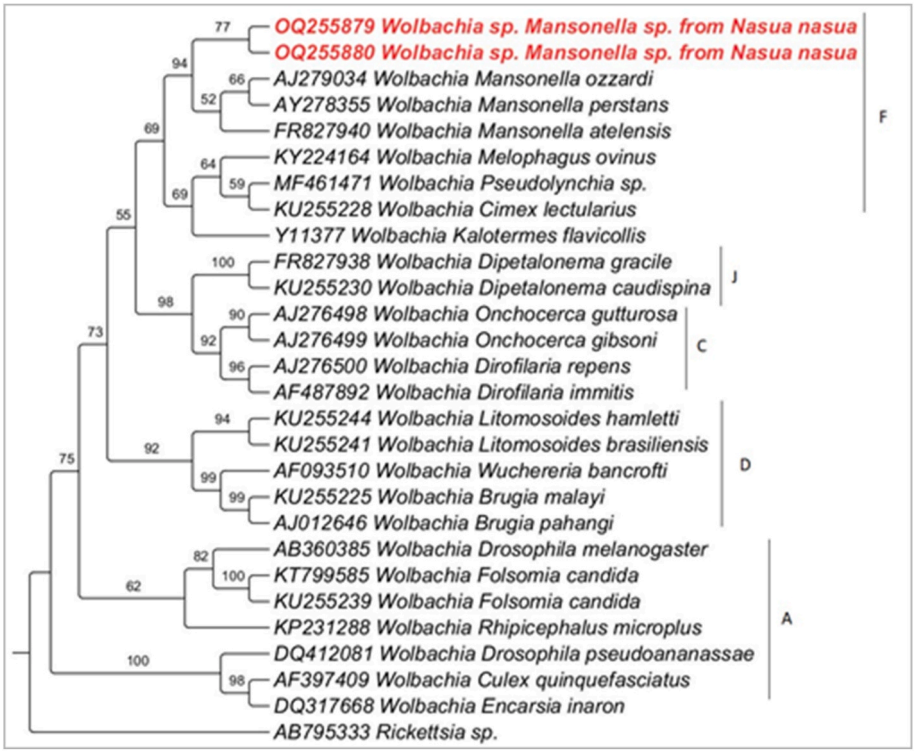

Fig. 3. Phylogenetic tree based on Maximum likelihood method from 16S rRNA of *Wolbachia* sp. detected in blood samples from coatis (*Nasua nasua*) from Campo Grande city, Mato Grosso do Sul state, Brazil. *Rickettsia* sp. was used as outgroup. Analyses were performed based on an alignment of 337 bp and K2P + G4 as evolutionary model.

peritoneal cavity is needed in order to shed light on the real identity of this putative novel filarioid. Finally, the molecular screening of dipterans in the studied region for the presence of filarioid DNA will contribute to the understanding of the biological cycles of coati-associated filarioids.

5. Conclusions

Coatis may play a role as reservoirs of a novel yet to be described *Mansonella* species, that might show a potential zoonotic risk. Considering the significance of *Mansonella* genus, further studies are needed to identify its pathogenicity and to elucidate its biological life cycle.

Ethical statement

All experimental procedures were approved by the "Instituto Chico Mendes de Biodiversidade" (ICMBio) (SISBIO 49662-8) and by the Ethics Committee on Animal Use of the School of Agricultural and Veterinary Sciences, UNESP (CEUA FCAV/UNESP 06731/19), Ethics Committee on Animal Use of the Universidade Católica Dom Bosco (CEUA UCDB 001/2018) and Air Force Cooperation Agreement (Nº01/GAP-CG/2018).

Data availability statement

The datasets generated and analyzed during the current study are available in the NCBI – GenBank – Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession numbers: *Wolbachia* sp. 16SrRNA (OQ255879-OQ25588); *Mansonella* sp. *cox1* (OQ261670-OQ261693); *hsp70* (OR371724- OR371725); *myoHC* (OR371723).

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Author contributions

Conceptualization, L.P., D.O., H.M.H., M.R.A; **Methodology**, L.P. W.T.G.B., G.C.M., R.P.L., J.A.M-R., C.E.O.; formal analysis, L.P., D.O., M. R.A.; **Investigation**, L.P., D.O., W.T.G.B., G.C.M., R.P.L., J.A.M-R., H.M. H., C.E.O., R.Z.M., M.R.A.; **Resources**, D.O., H.M.H., M.R.A.; **Data curation**, L.P., D.O., H.M.R., C.E.O., M.R.A.; **Writing—original draft preparation**, L.P., D.O., M.R.A.; **Writing—review and editing**, L.P., D. O., W.T.G.B., G.C.M., R.P.L., J.A.M-R., H.M.H., C.E.O., R.Z.M., M.R.A.; **Supervision**, D.O., R.Z.M., M.R.A.; Funding **acquisition**, D.O., H.M.H., M.R.A. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2023.08.002.

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