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First report of *Mansonella* sp. and *Dipetalonema gracile* in the Amazonian city-dwelling threatened primate, *Saguinus bicolor*

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The pied tamarin, or *Saguinus bicolor*, is a callitrichid that inhabits Amazon Forest fragments encased within the municipalities of Manaus, Rio Preto da Eva, and Itacoatiara and their outskirts. Therefore, this primate lives in great proximity to humans, and is in critical danger of extinction, resulting from ongoing anthropogenic pressures, with habitat fragmentation being the most prominent threat. Greater conservation efforts and more studies concerning public health need to be carried out in this situation, such as the study of infectious diseases that can affect this primate, including those involving helminths. In this study, we combined necropsy, microscopy with blood smears and quick Panoptic stains, and molecular methods like nested polymerase chain reaction (PCR) targeting the internal transcribed spacer-1 (ITS-1) region, Sanger sequencing and shotgun sequencing to detect and identify filarial parasites in 71 *S. bicolor* samples. We detected 24 adult filarial worms in 6.45% of the thoracic cavities, microfilaria in 6.38% from blood smears, and filarial DNA in 28.57% positive blood samples via PCR. We identified eight of the adult worms as being from the Onchocercidae family using Sanger sequencing and one specifically as Dipetalonema gracile, using shotgun sequencing. For the positive blood samples, 70.58% of them were for

Mansonella sp., 17.64% for *Dipetalonema* sp., and 11.76% could only be identified as belonging to the Onchocercidae family. There was an event of coinfection that involved *Dipetalonema* sp. adult worm and *Mansonella* sp. microfilaria. This is the first report of the detection of *Dipetalonema gracile* and the genus *Mansonella* in *S. bicolor*, as well as an event of coinfection, pointing out this primate as a new host. It is also another step to understand the situation of filarial infections occurring in Amazonian Regions and its municipalities.

KEYWORDS

***pied tamarin*, Amazon rainforest, callitrichid, filaria, nematoda, conservation, parasitology, infectious diseases**

1 Introduction

The pied tamarin, or *Saguinus bicolor* (Primate; Callitrichidae), or even Sauim-de-coleira, Sauim-de-duas-cores, or Sauim-de-Manaus, is a neotropical primate with a distinct appearance. It has naked dark skin on its head; white fur covering its upper body; and orange brown, dark brown, or light brown colors for the fur of its lower body, from where its name derives. The adults of this species reach a body length from 28 cm to 32 cm, from 38 cm to 42 cm for the tail, and can weigh from 450 g to 550 g (1). It is a generalist species, able to survive on marginal or altered vegetation, eats fruits, small vertebrates, arthropods, eggs, nectar, and tree exudates (2, 3).

This primate is considered a critically endangered species and appears as such not only in the Official List of Endangered Brazilian Fauna, produced by the Ministry of the Environment from Brazil, but also in The International Union for Conservation of Nature (IUCN) Red List of Threatened Species (1, 4). One of the main threats for this species is the diminutive Amazonian region from where this primate is endemic, covering only approximately 7,500 km² of its territory. A great part of it overlaps with the municipality of Manaus, capital of the Amazonas State, and its metropolitan region, and also with Rio Preto da Eva and Itacoatiara. Thus, *S. bicolor* survives in forest fragments isolated inside these cities, as well as in their outskirts, being exposed to great anthropogenic pressions, namely, deforestation, degradation of its habitat through urban expansion, road kills, predation by domestic species (dogs and cats), and electrocution in the urban power network, and others (4–6).

In general, deforestation and forest fragmentation are the main threats to species worldwide, and for primates, more studies are necessary to establish the impact range of such threats. Amazonian primates are particularly threatened by climate changes connected to deforestation due to their low capacity to adapt to new climate conditions, and because many of them are frugivore, a class of animals particularly sensible to deforestation and forest fragmentation. In particular, *S. bicolor* is exposed to aggressive urban areas that form a good portion of the fragmented borders where this primate lives, and where such fragments present signs of degradation (2, 7, 8).

Additionally, there is the issue of infectious diseases involving this primate. There is not much information regarding the effects of parasites and pathogens to *S. bicolor*. Parasites in general represent an important hazard to conservation, recovery, and management programs to protect wildlife, particularly in endangered animals, and the situation of *S. bicolor* is no exception (9, 6).

With regard to this article, the focus is on filarial parasites. Filariasis is the general name given to the parasitic infection caused by filarial worms (Nematoda; Spirurida; Filaroidea; Onchocercidae) of medical and veterinary importance. They have a great variety of hosts, like reptiles, birds, and mammals such as non-human primates, humans, rodents, ungulates, canids, and felids. These filarial parasites have to be transmitted by hematophagous insects, some of which are anthropophilic. They can also occur in various continents, being distributed mainly in tropical and equatorial regions, as well as in temperate and coastal areas. The infections caused by filaria are of great importance to tropical communities, where some of them are considered neglected diseases and are a matter of public health (9–14). In the Amazon Region, mansonellosis, caused by *Mansonella ozzardi* and *M. perstans* in this region, and onchocerciasis, caused by *Onchocerca volvulus*, – stand out for the major threat they represent for vulnerable populations. For primates in the same area, it has been reported that filarial worms of the genus *Dipetalonema* and the species *Mansonella mariae* have been infecting a variety of these animals, including the *Saguinus* sp. However, the whole situation is still scarcely studied (13–20).

As such, the aim of this study is to report, by all accounts for the first time, the presence of *Mansonella mariae* and *Dipetalonema gracile* in *Saguinus bicolor*, detected using necropsy, blood smears, nested PCR, Sanger sequencing, and NGS, as efforts towards nature conservation by casting some light on the situation of infectious diseases for this threatened primate.

2 Materials and methods

2.1 Collecting samples

Necropsies were carried out on carcasses of *S. bicolor* stored inside freezers, at −20°C, at the Projeto Sauim-de-Coleira laboratory, located in the Federal University of Amazonas. In the

process, thoracic and abdominal cavities, as well as the subcutaneous space in the arms of the animals were inspected to search for adult filaria. When those were found, they were collected and preserved in phosphate-buffered saline solution inside 2-mL or 15-mL microtubes. Blood samples were also obtained from the said cavities of some of the carcasses, and were stored in 3-mL ethylenediaminetetraacetic acid (EDTA) tubes, placed inside coolers with ice packs, and transported to the laboratory located at the Leônidas e Maria Deane Institute/Fiocruz Amazônia Building (ILMD), to be stored in refrigerators, at 4°C.

As for the living animals, they were sampled at the Wild Animals Screening Center (CETAS), located inside the Superintendence of the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA). Blood was collected, stored, and transported in the same way as described above.

The samples were processed at the Leônidas e Maria Deane Institute/Fiocruz Amazônia ILMD, the Oswaldo Cruz Institute, Fiocruz (IOC/Fiocruz), at the Laboratory of Molecular Genetics of Microorganisms, Rio de Janeiro, and at the pathology laboratory of Federal Rural University of Amazonia (UFRA).

The *S. bicolor* were from various neighborhoods from all over Manaus, its outskirts and state and federal highways still inside its territory, and also from the outskirts and urban area of Rio Preto da Eva.

2.2 Morphological analysis

Nematodes collected were washed in saline solution, fixed in heated acidified formal alcohol (AFA), and later preserved in 70% ethanol. The specimens were clarified with 50% Aman lactophenol and temporarily mounted between slide and coverslip for the observation of morphological characteristics under light microscope. Morphological analysis was conducted with the help of researchers from the Federal Rural University of Amazonia (UFRA), and the taxonomic keys used for the identification were described in the literature (21, 22).

2.3 Microfilaria search

The blood samples were prepared in blood smears using microscope glass slides, and then stained by way of Panoptic fast staining. When ready, they were visualized using optical microscopes (40× and 100× objective lenses). The taxonomic keys used for the identification were described in the literature (23, 24).

2.4 Molecular analysis

Dneasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) was used to extract DNA from the blood samples and the adult filarial worms, following the manufacturer's protocol.

As for amplifying the filarial DNA of the adult worms and the blood samples, a nested PCR was carried out, with the target being the first internal transcribed spacer (ITS1) region, as described by Tang et al. (16). The specificities are described in Table 1. For the first reaction, the PCR mixture was 5 µL of each of the product of the DNA extractions, 10 µL of 5X Green GoTaq® Flexi Reaction Buffer (5X), 4 µL of $MgCl_2$ (at a concentration of 25 mM in 1.5 mL), 1 µL of the Deoxynucleotide triphosphates (DNTP) set (40 mM in 1000 µL), 0.2 µL of each primer (Table 1), 0.1 µL of GoTaq® Flexi DNA Polymerase (5 µ/µL), and 29.5 µL of ultra-pure water for each sample. The conditions for the thermal cycler were 7 min at 94°C, 40 cycles of 20 s at 94°C, 20 s at 60°C, and 30 s at 72°C.

The second mixture comprised 2 µL of the production of the first mixture, 10 µL of the buffer, 1 µL of the DNTP set, 4 µL of $MgCl_2$, 0.1 µL of each primer (Table 1), 0.1 µL GoTaq® Flexi DNA Polymerase, and 32.6 µL of ultra-pure water. For this second reaction, the conditions at the thermal cycler were 35 cycles of 20 s at 94°C, 20 s at 50°C, 20 s at 72°C and 10 min at 72°C. The concentrations of each product remained the same.

The size of the products of the second reaction was then estimated by agarose gel electrophoresis, using tris acetate–EDTA buffer (TAE buffer), 3 µL of ethidium bromide (500 µg/mL, in water), and 3 µL of 100bp DNA ladder, and was then electrophoresed for 1 h at 100 V in the system. Positive samples were then purified using QIAquick PCR Purification Kit (QIAGEN), following the manufacturer's instructions.

Sanger sequencing and next generation sequencing (NGS) were performed together with researchers from the Laboratory of Molecular Genetics of Microorganisms, Oswaldo Cruz Institute, Fiocruz (IOC/Fiocruz), Rio de Janeiro. NGS was specifically applied to the sample of one single adult filarial worm, taken from an *S. bicolor* carcass positive for the presence of 20 adult filarial worms in its thoracic cavity. The chromatograms from Sanger sequencing were then read using the *Chromas Lite* and *Chromas* programs, to single out a fragment of the sequence. The chosen fragments were then submitted to GenBank, using BLASTn and following the configurations: standard database, Onchocercidae taxid, and "somewhat similar" sequences. This was done to compare with the sequences stored in this database, to conclude the identification in terms of species, genus, or family. Only ≥93% matches with 100% query response were considered as an acceptable result, to identify the species.

Additionally, from the filarial worm submitted to shotgun sequencing, a phylogenetic study with a multi locus sequencing typing (MLST) approach was derived. Moreover, a barcoding analysis

TABLE 1 List of primers used for the detection and identification of filarial parasites in *Saguinus bicolor*.

Primers applied for the detection and identification of filarial parasites in *Saguinus bicolor*		
Target	Primers	Fragment size
First internal transcribed spacer (ITS-1)	JM-F-0021 Tan : GTGCTGTAACCATTACCGAAAGG JM-U-0022 Tan : CGCAGCTAGCTGCGTTCTTCATCG ITS1-F Tan: GGTGAACCTGCGGAAGGATC ITS-FBIS Tan: GGTGAACCTGCRGMWGGATCA JM-F-0022-R Tan: TGCTTATTAAGTCTACTTAA	1st reaction: ~ 710pb 2nd reaction: ~ 305–312pb

for the gene cytochrome c oxidase subunit 1 gene (CO1) and a phylogenomic analysis for *Wolbachia* sp. were carried out (25–27).

2.5 Statistical analysis

To calculate the percentages of the results, the Microsoft Excel program was used. Also, to verify the normality of the distribution of the data acquired, the Shapiro–Wilk test was performed, and for the verification of statistical significance of the data, Student's t-test was applied in case normality of distribution was observed and Wilcoxon or Mann – Whitney tests otherwise. All these tests were conducted using the Past software (version 4.13).

2.6 Ethical aspects

Living animals were sampled with approval by the Comissão de Ética no Uso de Animais da Universidade Federal do Amazonas (CEUA/UFAM) (Authorization N. 017/2020) (Figure S1). This study also has a license with the Sistema de Autorização e Informação em Biodiversidade (SISBio), provided by the Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (code: 0671530320200723) (Figure S2). This study was also registered in the Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado (SisGen), under access no. A0A1A95 (Figures S3, S4); and in the Sequence Read Archive (SRA) (reads of *Dipetalonema* Sp.: SRR21859278; the mitochondrial genome of *Dipetalonema* sp.: NC 066230.1; and the ribosomal gene of *Dipetalonema* sp.: MZ727043).

Moreover, this study was supported by the Fundação de Amparo à Pesquisa do Estado o Amazonas (FAPEAM), under the program, UNIVERSAL AMAZONAS (EDITAL No. 006/2019).

3 Results

3.1 Prevalence of infection

The study was conducted with 71 *S. bicolor* in total, through necropsies (62 carcasses) and sampling of living animals (10 primates). It is necessary to add here that one of the animals was sampled both while it was still alive and via necropsy.

From the necropsies, it was possible to sample whole blood from 46 carcasses.

In the necropsies, 6.45% (4/62) were positive for the presence of adult filarial worms (Shapiro–Wilk: 1.9079E-15; Wilcoxon: 0.125). A total of 24 adult filarial worms were found, where 20 of the parasites were from a single carcass, 2 from another distinct carcass, and 1 parasite each from 2 other carcasses. These filarial worms were all found in the thoracic cavities. No filarial worm was found in any other part of the body.

From the 56 blood samples, 47 microscope slides were prepared, from which 6.38% (3/47) were positive for the presence of microfilaria (Shapiro–Wilk: 1.305E-13; Wilcoxon: 0.25). The molecular analysis was carried out with all the 56 blood samples, showing that 28.57% (16/56) were positive for the presence of filarial DNA and 71.42% (40/56) were negative (Shapiro–Wilk:1.822E-11; Wilcoxon: p-value ¾ 0.05) (Figures 1, 2).

The percentage of negatives in both the necropsy and in the molecular analysis was 76.19% (32/42) (Shapiro–Wilk: 1.091E-10; Wilcoxon: p-value ¾ 0.05). For the sampling in living *S. bicolor*, 20% (2/10) were positive in the molecular analysis, while 80% (8/10) were negative (Shapiro–Wilk: 4.672E-06; Wilcoxon: 0.5).

Considering all the positives, independent of the diagnostic method applied or the vital signs of the animals, the prevalence of infection was 23.94% (17/71) in the *S. bicolor* samples and 76.06% (54/71) had no filarial infection (Shapiro–Wilk: 9.7884E-14; Mann-Whitney: 6.185E-10).

3.2 Morphological and molecular identification

With the exception of 10 adult filarial worms that were too deteriorated to be morphologically identified, 14 of them were identified as *Dipetalonema gracile*, using the taxonomic keys provided (21, 22) (Figure 3). For their morphology, the female specimens exhibited *vulva posterior* at the muscular esophagus and sinuous *vagina vera*; and the male specimens exhibited, in the posterior portion of their bodies, cloacal papillae and post cloacal bands arranged in two rows, with the right shorter than the left. The left spicule was subdivided into the proximal handle, membranous alae, and flagellum and then, with simple right spicule.

For the microfilaria, two of them were identified as *Mansonella*. sp., using the taxonomic keys from the literature (23, 24), presenting unsheathed and slender bodies, as well as fully nucleated tails (Figure 4).

As a whole, the molecular analysis found 70.58% (12/17) of *Mansonella* sp. among the positive animals in this study (Shapiro–Wilk: 6.499E-06; Wilcoxon: 0.0004); 17.64% (3/17) of *Dipetalonema gracile* (Shapiro–Wilk: 7.306E-07; Wilcoxon: 0.25); and 11.76% (2/17) of positives that could only be identified as belonging to the Onchocercidae family (Shapiro–wilk: 1.586E-07; Wilcoxon: 0.05). Additionally, there was an event of coinfection by *Dipetalonema gracile* adult worm and *Mansonella* sp. microfilaria for one of the *S. bicolor*.

The results of Sanger sequencing showed the presence of *Mansonella* sp. in 75% (12/16) of the blood samples (Shapiro–Wilk: 0.5272E-06; Wilcoxon: 0.0004); *Dipetalonema gracile* in 6.25% (1/16) of the blood samples(Shapiro–Wilk: 4.553E-08; Wilcoxon: 1); and 1 of the 16 positives (6.25%) could only be identified as belonging to the Onchocercidae family in a GenBank search (Shapiro–Wilk: 4.553E-08; Wilcoxon: 1). For 12.5% (2/16), no identification could be obtained from GenBank (Shapiro–Wilk: 3.408E-07; Wilcoxon: 0.5). Sanger and NGS data analysis were carried out by ACPV and CHAC.

For the 24 adult filarial worms, DNA were extracted and amplified for 14 parasites (Figures 5, 6). Using Sanger sequencing and comparison through GenBank, eight were established as being from the Onchocercidae family and another five could not be identified at a molecular level. Through NGS, one of them was identified as *Dipetalonema gracile*.

10.3389/fitd.2023.1080218

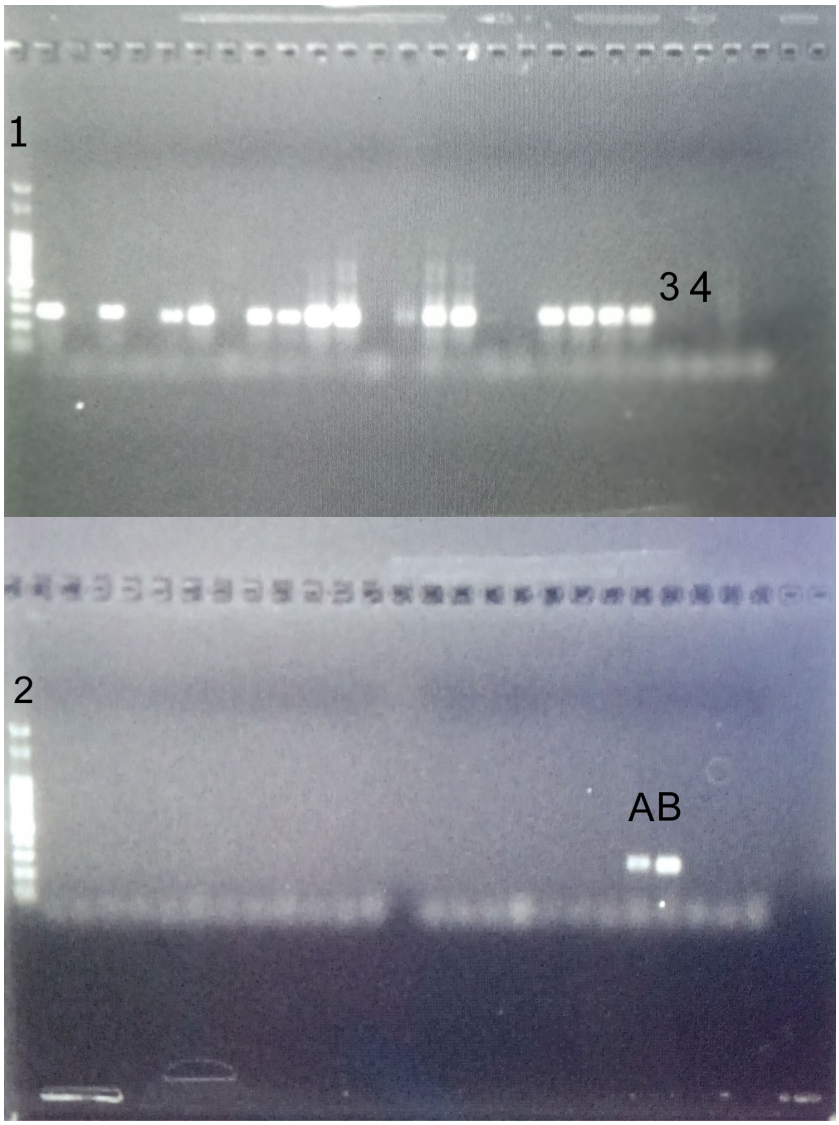

FIGURE 1
Electrophoresis gel with results of the Nested-PCRs conducted to confirm the presence of filarial DNA in blood samples from *Saguinus bicolor*. Showing: numbers 1 and 2 corresponding to the DNA Ladder; 3 and 4 corresponding to the negative control; letter A and B corresponding to positive controls.

4 Discussion

The *S. bicolor* is an endangered primate that is important for conservation, and thus, for public health, considered an umbrella species (5). In this study, we sampled both living and dead specimens of *S. bicolor*. We also combined different types of diagnostic methods for a more integrated approach, where it was difficult to obtain biological samples from such animals (28).

According to the statistical tests applied, there is no statistical significance of the number of positive and negative animals found. This may be because of the limited number of samples.

These findings correspond to what is reported in the literature, where the presence of *Mansonella mariae* and *Dipetalonema* spp. in *Saguinus imperator* is described, in the Amazonian region (19). The presence of *D. freitasi* and *D. gracile* in *Lagothrix poeppiigii*, *Pithecia monachus monachus*, and *Cacajao calvus ucayalii* is also described, in the Amazonian region (22, 28). Events of coinfection of filaria parasites, as detected in this study, were also described in human beings in the peri-urban area of a Brazilian Amazon municipality, São Gabriel da Cachoeira, where *M. ozzardi* and *M. perstans* were detected together (29). Additionally, in the same location, there was molecular detection of *Mansonella mariae* in *Simulium oyapockense*, an anthropophilic vector that also feeds on blood from primates, showing some possibility of zoonotic risk (30).

However, it is not possible to determine what species from the genus *Mansonella* was found. The search done using the database from GenBank pointed a bigger similarity with *Mansonella mariae*,

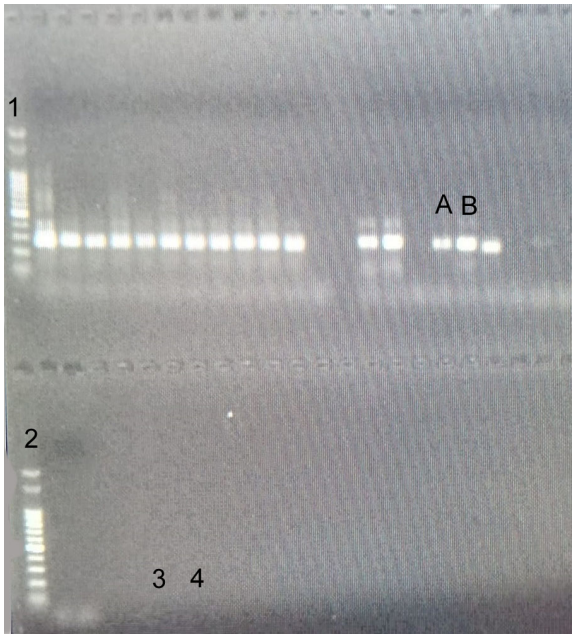

FIGURE 2
Electrophoresis gel with the results of Nested-PCR conducted to confirm the presence of filarial DNA in blood samples from *Saguinus bicolor*. Showing: numbers 1 and 2 corresponding to the DNA ladder; 3 and 4 to negative control; letters A and B corresponding to positive control.

and a much lower match with other parasites of the same genus, including *Mansonella ozzardi* and *Mansonella perstans*, that are the other *Mansonella* sp. found in the Amazon region. Due to the fact that ITS is a spacer DNA that is highly variable, and while the comparison between the sequences from this study with the ones stored in GenBank pointed to a ≥98% match with *Mansonella mariae* sequences, the query was lower than 100%.

Consequently, there is a possibility that the *Mansonella* sp. found is from a species that is not referenced yet in the GenBank database and has not been pointed out in the Amazon region. More studies are necessary to establish this, such as the construction of phylogenic trees, using methods like maximum likelihood estimations.

In addition, the shotgun sequencing of the *Dipetalonema gracile* adult worm that recovered a complete mitochondrial DNA genome and most parts of ribosomal DNA locus, carried out as a part of this research by our partners, in this project, can greatly increase the genetic database of filarial parasites, and with this, further help the identification of filarial parasites (25). Moreover, these results can help elucidate the evolutionary connections within the Onchocercidae family, since understanding the evolutionary history of filarial parasites can be quite challenging, with this being especially true for the Amazon region, where there are records of errors in the identification of filariae of the genus *Mansonella* for decades, an issue where more specialized molecular methods may help (26, 29, 31, 32).

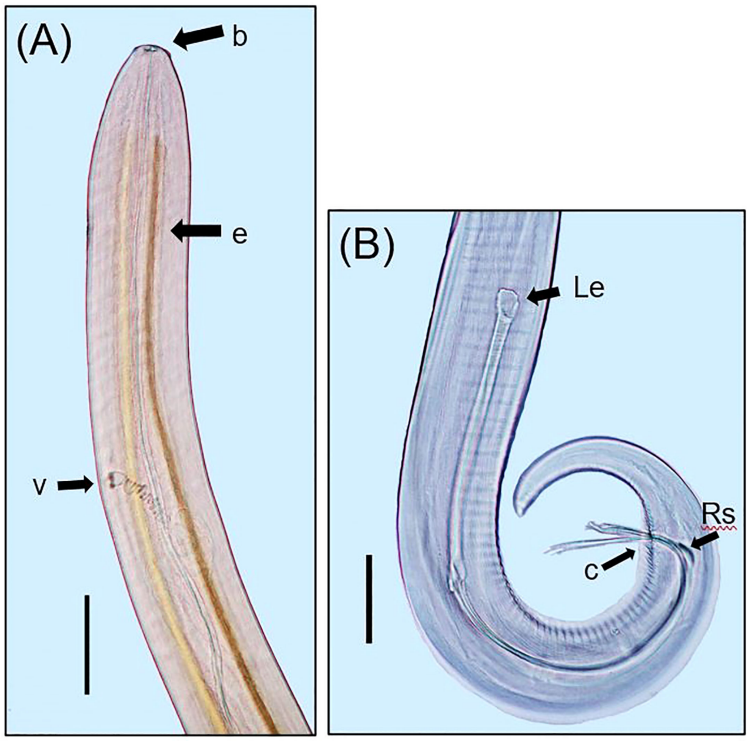

FIGURE 3
Morphology of adult *Dipetalonema* sp. **(A)** anterior region of the female, b: oral opening, e: Esophagus, v: vulvar opening and sinuous vagina. Bar: 20 µm. **(B)** anterior region of the male, showing reproductive organs and rough area: Le: left spicule, Rs: right spicule, c: cloacal opening and cloacal papillae. Bar: 20 µm.

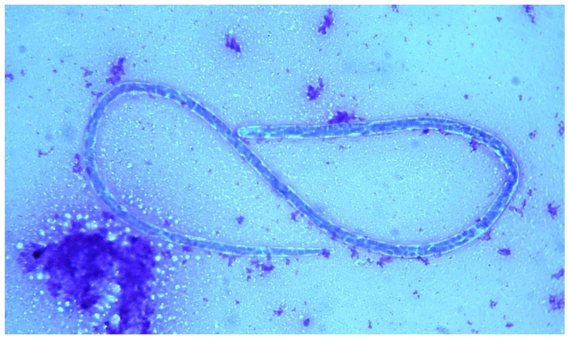

FIGURE 4
Mansonella sp. microfilaria visualized under an optical microscope (100x objective).

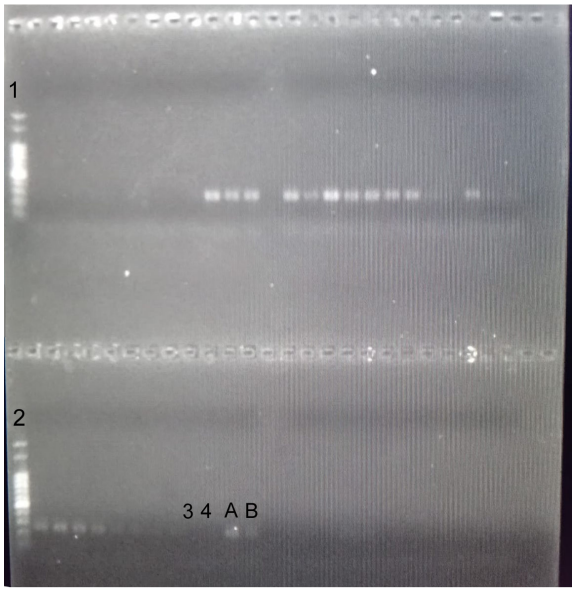

FIGURE 5
Electrophoresis gel with the results of Nested-PCR conducted to amplify the DNA of filarial parasites found in carcasses of *Saguinus bicolor*. Showing: numbers 1 and 2 corresponding to the DNA ladder; 3 and 4 corresponding to negative control; letter A and B, to positive control.

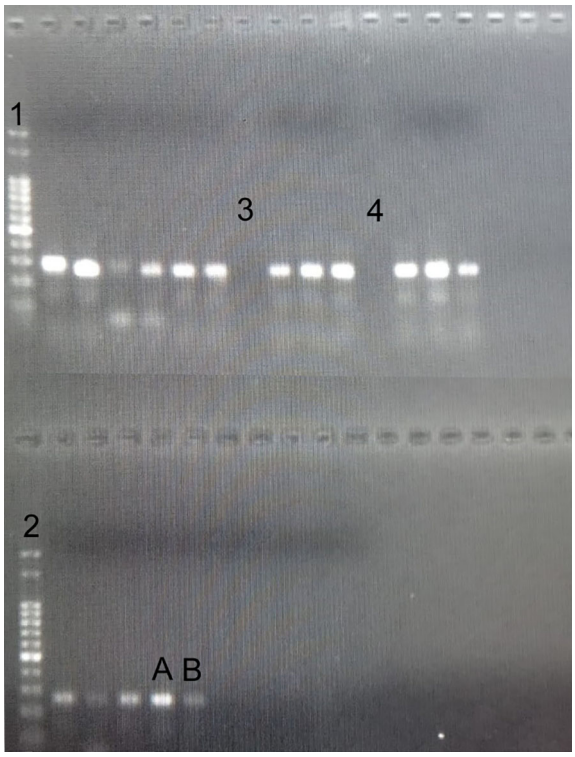

FIGURE 6
Electrophoresis gel with the results of Nested-PCR conducted to amplify the DNA of filarial parasites found in carcasses of *Saguinus bicolor*. Showing: numbers 1 and 2 corresponding to the DNA ladder; numbers 3 and 4 corresponding to negative control; letter A and B, to positive control.

The findings described in this study not only report the presence of filarial parasites in primates that live in great proximity to human beings, but also report a new host for *Mansonella* Sp. and *Dipetalonema gracile*, and the occurrence of filarial coinfections in *S. bicolor*. Furthermore, it points to a possibility of a filaria of the genus *Mansonella* in the Amazonian region still not registered in the GenBank database, that is causing infection to a highly threatened primate that lives in close proximity to the population in three Amazonian cities. These are all new steps towards unraveling answers about parasites and pathogens that can affect *S. bicolor*, a sparsely studied issue, and thus, help with conservation measures. In addition, these findings can help understand the outlook of filarial parasites in Brazilian Amazonian regions and their urban areas (6, 33).

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was reviewed and approved by Comissão de Ética no Uso de Animais of Universidade Federal do Amazonas (Authorization N. 017/2020) and Sistema de Autorização e Informação em Biodiversidade (SISBIO) by Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) (code: 0671530320200723). The licenses are provided in the Supplementary Material.

Author contributions

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fitd.2023.1080218/full#supplementary-material

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