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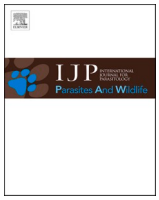

Morphological and molecular characterization of parabasilids isolated from ex situ nonhuman primates and their keepers at different institutions in Brazil

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ABSTRACT

Intestinal protozoa, which can be asymptomatic or cause diarrhea, dysentery and even death, are among the main agents that affect nonhuman primates (NHPs) kept under human care. Nevertheless, information on the molecular and morphometric profiles of parabasilids in the Neotropics is still scarce. In this context, the objective of this study was to isolate the Parabasalia protozoa detected in the feces of NHPs and their keepers in Pavlova and TYSGM9 media and to characterize the isolates by molecular biology and morphometry. Fecal samples from NHPs from five Brazilian institutions were analyzed. Direct examination was performed immediately after obtaining the samples. A total of 511 fecal samples from NHPs were collected, and 10.6% contained parabasilids. Regarding the handlers, of the 74 samples analyzed, three were positive. In vitro-generated parabasilid isolates were successfully obtained from all positive samples, as identified via microscopy. Isolates of the parasite were obtained both from New World NHPs, including the genera *Leontopithecus*, *Saguinus*, *Leontocebus*, *Aotus*, *Saimiri*, *Sapajus*, and *Alouatta,* and from the Old World primate *Pan troglodytes*. Forty-nine NHP isolates were molecularly identified: *Pentatrichomonas hominis* (16), *Trichomitus batrachorum* (14), *Tetratrichomonas brumpti* (13) and *Hypotrichomonas hampli* (6). The human isolates were identified as *Tetratrichomonas* sp. (2) and *T. batrachorum* (1). Visualization and morphometric analysis revealed trophozoites with piriform or rounded shapes that presented variable measurements. The isolates previously characterized as *P. hominis* had up to five free flagella, while *T. batrachorum* and *Tetratrichomonas* sp. had up to four free flagella, and *H. hampli* had a maximum of three free flagella. These morphometric characteristics corroborated the molecular identification. In general, a variety of parabasilids were observed to infect NHPs, and *T. batrachorum* was isolated from biological samples from both NHPs and their keepers, a finding that reinforces the susceptibility of these hosts to infections by parabasilids in Brazil.

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1. Introduction

Parabasilids are a very diverse group of unicellular, anaerobic, amitochondrial and flagellate organisms that can be symbiotic, free-living or pathogenic (Smejkalová et al., 2012; Čepička et al., 2016). For the most part, these microorganisms live symbiotically in the gastrointestinal tract of invertebrates, such as termites and cockroaches. However, pathogenic species that infect several animals, including humans and nonhuman primates (NHPs) have been described (Čepička et al., 2016).

Among the parabasilids most commonly detected in vertebrate hosts, such as humans and NHPs, the genus *Tetratrichomonas* stands out because it has the largest number of species among the group Parabasalia (Čepička et al., 2006). This group contains many taxa of veterinary importance for NHPs, including *Pentatrichomonas hominis* and *Tritrichomonas mobilensis,* which were already detected in *Saimiri* (Pindak et al., 1985, Culberson et al., 1986, Pindak and Pindak, 1998; Midlej et al., 2011), and *Dientamoeba fragilis,* whose natural hosts are gorillas, baboons and monkeys of the genus *Macaca* (Windsor and Johnson, 1999; Stark et al., 2008; Lankester et al., 2010; Barratt et al., 2011).

Among humans, one of the most studied species is *Trichomonas vaginalis*, which inhabits the genitourinary tract, especially in women (Baumeister and Hollinger, 1941; Meites et al., 2015). In addition, *Dientamoeba fragilis* and *P. hominis* have been observed in the intestinal region (Barratt et al., 2011; Li et al., 2016; Stark et al., 2016).

In general, intestinal parabasilids may be present in the intestinal lumen or crypts and are rarely responsible for triggering an inflammatory response or pathological mechanisms in NHPs (Blanchard and Baskin, 1988; Inoue et al., 2015). Nevertheless, reports of lesions and clinical manifestations induced by parabasilids have been increasingly reported worldwide in humans (Jongwutiwes et al., 2000; Mantini et al., 2009; Meloni et al., 2011; Vieira et al., 2012; David et al., 2015; Stark et al., 2016; Ali et al., 2017; Chieffi and Santos, 2018; Zhang et al., 2019) as well as in NHPs (Bunton et al., 1983; Pindak and Pindak, 1998; Kondova et al., 2005; Stark et al., 2008; Lankester et al., 2010; Westreich et al., 2019; Bailey and Hirt, 2023). In addition, there are reports on the possible ability of these parasitic agents to migrate to other organs of the host, adapt to new hosts and even carry out zoonotic transmission (Gookin et al., 2007; Kim et al., 2010; Dimasuay and Rivera, 2013).

In this context, increasing numbers of studies on the relationships between parabasilids and their hosts, as well as the virulence, tissue colonization, metabolism and zoonotic transmission of parabasilids, are being performed to elucidate various issues related to parasitism by the group Parabasalia (Gookin et al., 2007; Dimasuay and Rivera, 2013; Westrop et al., 2017; Handrich et al., 2019; Martínez-Herrero et al., 2019). It is important to note that Brazil is one of the countries with the highest biodiversity of NHPs in the world and moreover maintains NHPs from the Old World, including great apes, on various breeding farms both for biomedical research and for conservation, research, recreation and education (Barbosa et al., 2015, 2020; Dib et al., 2023). However, information on the parasitic agents that can infect these animals remains very limited, especially regarding the species of the group Parabasalia and their specificity for different primate taxa, including humans. Therefore, the objective of this study was to isolate and characterize parabasilids from ex situ NHPs and their handlers on different farms in Brazil via molecular and morphological methods.

2. Material and methods

2.1. Ethical aspects

The present study was approved by the Authorization and Information System on Biodiversity (SISBio) under license no. 74420-6 and authentication code 07442008202311120. Fecal samples were collected at five institutions (A–E). Institutions A, B and C were approved by the CEUA of Fiocruz, which decided to exempt the present study from the need to obtain a license through the Brazilian Directive for the Care and Use of Animals in Teaching or Scientific Research Activities - DBCA (Normative Resolution No 30., CONCEA, 2016), Item 6.1.10. Institution D was approved by CEUA/CENP/Evandro Chagas under registration no. 18/2020 and certificate no. 40/2021. Institution E was approved by the CEUA of the Regional Institution/University of Blumenau on 02/23/2022. In addition, this study was approved by the Human Ethics Committee of the Oswaldo Cruz Institute under protocol numbers 4,484, 952 and CAAE: 39957820.6.0000.5248.

2.2. Location and collection of fecal samples

The collection of fecal samples from ex situ NHPs and their handlers was performed at five institutions located in different regions of Brazil: Institution A in the state of São Paulo (SP), in the southeastern region of Brazil at coordinates 23°30′21 "S and 47°26′17"W; Institution B in Brasília in the Midwest Region, at coordinates 16°30"S and 46°30"W; Institution C in Rio de Janeiro, also in the Southeast Region, at coordinates 22°29′18"S and 42°54′48"W; Institution D in the state of Pará, northern Brazil, at coordinates 1°23′02"S and 48°22′ 51"W; and Institution E in the South Region of the State of Santa Catarina at coordinates 26°53′51"S and 49°13′36"W.

Fecal samples from NHPs of the families Callitrichidae (n = 225), Aotidae (n = 54), Cebidae (n = 96), Pitheciidae (n = 14), Atelidae (n = 95), Cercopithecidae (n = 16), Hominidae (n = 8), Lemuridae (n = 3) and their keepers (n = 74) were collected as described previously by Dib et al. (2023).

2.3. Coproparasitological analysis

Immediately after the collection of feces from NHPs and after receiving fecal samples from the keepers at the Institutions, the material was subjected to a qualitative direct examination for the detection of trophozoites of the group Parabasalia. This initial analysis was carried out at the primate institutions in order to detect most of the positive samples with viable trophozoites.

2.4. In vitro culture of protozoa

All fecal samples that showed motile flagellate trophozoites on direct examination were inoculated into of modified Pavlova xenic culture medium (Jones, 1946; Pavlova, 1938) and of TYSGM-9 medium (Diamond, 1982) in glass test tubes (15 × 1.8 cm) with screw caps. To make a 1000 ml of Pavlova medium was used 1.292 g of sodium hydrogen phosphate, 0.42 g of potassium dihydrogen phosphate, 7.27 g of sodium chloride, and 1,46 g of yeast extract. A total of 970 mL of TYSGM-9 medium was made by a nutrient broth composed of 2g of casein peptone, 1g of yeast extract, 7.5 g of sodium chloride, 2.8 g of anhydrous dipotassium hydrogen phosphate, 0.4 g of potassium dihydrogen phosphate and 2 g of porcine gastric mucin. Both xenic culture media were autoclaved.

A total of 200 µl of fresh stool solution and buffered saline solution used for direct examination was inoculated into each tube of a set of four tubes. Each tube had 8 mL of medium. Two of these tubes contained modified Pavlova medium, one supplemented with horse serum and the other supplemented with fetal bovine serum. The two remaining tubes contained the TYSGM-9 medium with the respective sera and 500 µl of Tween 80 solution 5%.

In addition, antibiotic solutions with 10,000 U/µl streptomycin and penicillin and a drop of rice starch suspension (dilution: 1 g of starch in 16 ml of the medium to be used) were added to the xenic medium. Once inoculated, each isolate was incubated in a bacteriological oven at 36 °C, and the sediment in the tube was observed every day at 24-h intervals for seven days. From the seventh day on, all samples that contained viable parasites, i.e., moving parasites, were considered to be successfully isolates. In vitro maintenance of the protozoa was performed at

48–72 h intervals of subculture, i.e., the isolate was inoculated into fresh medium.

2.5. Molecular characterization

The isolates were removed directly from the culture and subjected to DNA extraction using the High Pure PCR Template Preparation Kit by Roche® according to the manufacturer's instructions. First, an attempt was made to genetically analyze the cultures using the protocol described by Felleisen (1997). Subsequently, the forward primer was changed in order to obtain a greater number of amplifications from the parasites maintained in vitro. Finally, a molecular analysis was only performed by amplifying a fragment consisting of approximately 1600 base pairs of the 18S rRNA-ITS1-5.8S-ITS2 region (Ibañez-Escribano et al., 2013).

Subsequently, the PCR products were purified with a GE® kit following the manufacturer's recommendations. All samples were sequenced in the forward and reverse directions using a 3730 DNA Analyzer automated sequencer (Applied Biosystems) on the Fiocruz platform. The resulting nucleotide sequences were aligned and edited in SeqMan version 7.1 (DNASTAR LaserGene®). Next, the BLASTn analysis tool was used to compare the data obtained with reference sequences belonging to the same gene fragment stored in the GenBank database. The sequences were saved in Fasta mode and aligned with other homologous sequences obtained from GenBank using Mega X software.

Phylogenetic inferences were obtained from maximum likelihood analyses and confirmed with bootstrap access with 1000 replications, with the best evolutionary model selected based on the Akaike

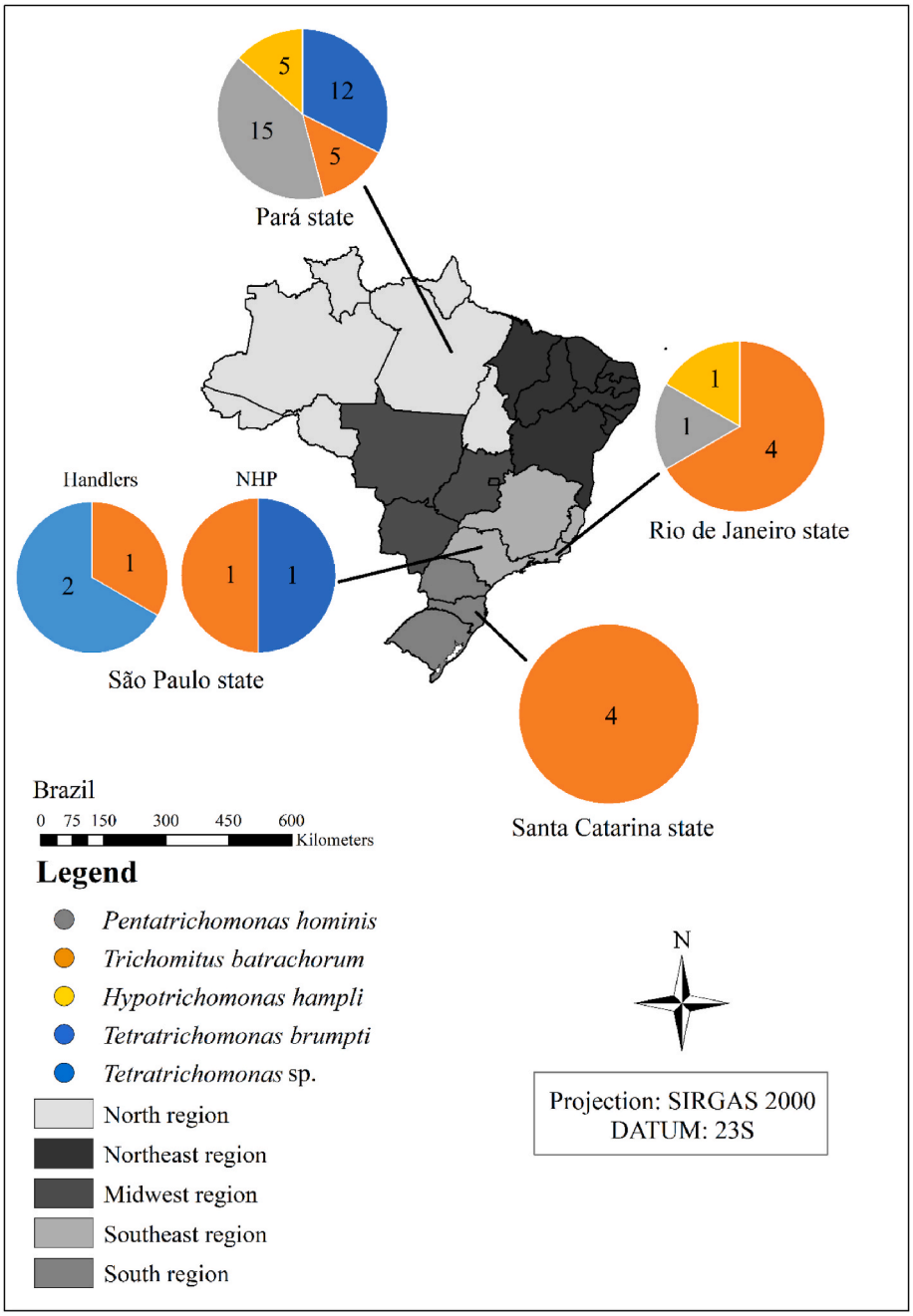

Fig. 1. Diversity of species of group Parabasalia, characterized by molecular techniques, in samples from NHPs and their keepers at institutions in the Brazilian states of Pará (northern region), Rio de Janeiro and São Paulo (southeast region) and Santa Catarina (southern region).

information criterion (AIC) using W-IQ-Tree software (http://iqtree.cibiv.univie.ac.at/). The phylogenetic tree was edited using MEGA-X software.

2.6. Morphological characterization

One milliliter of culture medium was removed, and the cells were washed twice with saline solution at 1500 RPM for 5 min to further clean the culture medium and remove excess bacteria and artifacts. After this procedure, a drop of the sediment was deposited on a slide, and the material was spread with the aid of a sterile tip to obtain a smear. The microscopy slides were then stained with a Rapid Panoptic Staining Kit (Laborclin®, Brazil). First, the slide bearing the smear was immersed in dye n°1, containing triarylmethane in methanol, for 10 s. Subsequently, the slide was immersed 4 times in stain n° 2, containing xanthenes in deionized water, and finally, the material was dipped once in stain n° 3, a solution of thiazines in deionized water.

Thirty parasitic cells of each isolate were measured under an Olympus BX 41 optical microscope at 1000× magnification and photomicrography with a BEL® EU12CONVS digital camera (Newcastle upon Tyne, UK). The parameters analyzed were trophozoite length, width and shape; nucleus length and width; number of visible flagella; flagellum length; undulating membrane length; and axostyle length.

2.7. Data analysis

All data are presented in tables and figures, including the absolute frequency of the Parabasalia species characterized based on molecular and morphological analysis. The minimum and maximum values and standard deviation of the morphological measurements of the parasitic forms are also presented.

3. Results

Direct examination of the 511 fecal samples of NHPs revealed that 54 samples from four of the five institutions that maintain primates ex situ in Brazil were positive for parabasilid trophozoites (Fig. 1). For all samples with positive results by the direct examination, isolates were successfully obtained in TYSGM-9 and modified Pavlova culture media with both serum sources (equine and fetal bovine). All parabasilid cultures remained viable for 3 years to undergo molecular techniques and morphological characterization.

Initially, the TFR1/TFR2 primers described by Felleisen (1997) were used, which amplify a fragment of the ITS-1 5.8S rRNA ITS-2 region. However, out of the 54 cultures analyzed, amplification and taxonomic classification of the parasitic species was possible in only 37 (68.5%) cases. Therefore, the analysis of the same gene region was performed using a different forward primer (TRICO-D), designed by Ibañez-Escribano et al. (2013). Finally, changing the primer enabled the molecular characterization of four species of the group Parabasalia in 49 (90.7%) of the 54 samples obtained from several NHP taxa and handlers in four Brazilian states: *Pentatrichomonas hominis* (29.6%), *Trichomitus batrachorum* (25.9%), *Tetratrichomonas brumpti* (24.1%) and *Hypotrichomonas hampli* (11.1%) (Table 1 and Fig. 1). Among the NHP species, *Saimiri collinsi* maintained under human care at Institution D, located in northern Brazil, presented the greatest number of isolates and the greatest diversity of parabasilids (Table 1).

In addition to feces from NHPs, fecal samples from animal handlers and food handlers at the institutions were analyzed. In the direct examination, fecal samples from humans were positive only for the group Entamoebidae, as previously reported in an article published by our research group (Dib et al., 2023). Attempts to isolate this group of parasites were also made; however, motile parabasilid trophozoites were unexpectedly found in the media of three human samples after approximately two weeks and were therefore included in the morphological and molecular analyses of this study. The three positive human samples were from Institution A of the state of São Paulo, southeastern Brazil, and were characterized as *Tetratrichomonas* sp. (2) and *T. batrachorum* (1). At the same institution, the same parabasilid genera were isolated from the fecal samples from the NHPs, including *Aotus trivirgatus* and *Pan troglodytes* (Fig. 1).

After analyzing the gene sequences of parabasilids isolated from the

Table 1
Molecular characterization of in vitro cultured trophozoites of the group Parabasalia obtained from fecal samples of Neotropical and Old World nonhuman primates kept ex situ at different institutions in Brazil.

Host	Positive samples on microscopy and isolated in vitro	Molecular identification				US[a]
		Pentatrichomonas hominis	*Trichomitus batrachorum*	*Tetratrichomonas brumpti*	*Hypotrichomonas hampli*	
Callitrichidae						
Leontopithecus chrysomelas	2	–	2	–	–	–
Leontopithecus chrysopygus	1	1	–	–	–	–
Leonthopitecus hybrid	1	–	–	–	–	1
Saguinus ursulus	1	1	–	–	–	–
Leontocebus weddelli	6	–	–	1	5	–
Aotidae						
Aotus infulatus	1	–	–	1	–	–
Aotus trivirgatus	1	–	–	1	–	–
Cebidae						
Saimiri collinsi and *Saimiri boliviensis*	1	1	–	–	–	–
Saimiri collinsi	23	10	2	7	–	4
Sapajus xanthosternos	1	–	1	–	–	–
Sapajus apella and *Cebus albifrons*	1	1	–	–	–	–
Sapajus apella and *Cebus xanthosternos*	1	1	–	–	–	–
Sapajus apella	1	1	–	–	–	–
Atelidae						
Alouatta caraya	6	–	3	3	–	–
Alouatta guariba	6	–	5	–	1	–
Hominidae						
Pan troglodytes	1	–	1	–	–	–
Total	54	16	14	13	6	5

[a] - Unidentified samples.

feces of NHPs and humans, it was possible to observe a strong relationship with the reference nucleotide sequences of the group Parabasalia from the maximum likelihood test and Kimura evolutionary model 2 parameters (K2P) (Fig. 2). The sequences of *P. hominis* from *Sapajus*, *Cebus, Leonthopithecus, Saguinus* and *Saimiri* were showed 100% identity to those of *P. hominis* detected in domestic cats in the Czech Republic.

The nucleotide sequences identified as *T. brumpti* in *S. collinsi* and *A. caraya* were grouped within the same cluster of this species identified in feces of tortoises (*Chelonoidis carbonaria*) from the Czech Republic and anteaters (*Myrmecophaga tridactyla*) from Spain. However, the gene fragments obtained from the isolates from the zookeepers in São Paulo in the present study were identified only as *Tetratrichomonas* sp. and were not included within the same cluster of *T. brumpti* (Fig. 2).

The only parabasilid detected from both NHPs and a zookeeper was *T. batrachorum*. *T. batrachorum* was also the only species identified in apes kept at the four institutions included in this study and showed 100% phylogenetic identity with the nucleotide sequence obtained from iguanas (*Iguana iguana*) and 95.3% with the nucleotide sequence obtained from domestic pigs (*Sus scrofa*), both from a study conducted in the Philippines. In contrast, the least commonly identified parabasilid species in this study was *H. hampli,* which was isolated from the feces of *Leontocebus weddelli* kept in enclosures at Institution D in Pará and howler monkeys (*Alouatta guariba)* at Institution C in Rio de Janeiro (Table 1, Fig. 1). The phylogenetic analyses revealed a high degree of identity of *H. hampli* isolated from *L. weddelli* and *A. guariba* with the same parabasilid species detected in kangaroos (*Macropus rufus*) in the Czech Republic (Fig. 2). All sequences obtained were deposited in GenBank under accession numbers PP214325 to PP214376 (Supplementary data).

In addition to molecular techniques, morphometric analysis was performed on 30 trophozoites from each sample previously identified by molecular methods, totaling 1560 parasitic cells (1470 from NHP samples and 90 from zookeepers) (Table 2). Although four different species from the group Parabasalia were detected, the size, shape and number of structures analyzed in the trophozoites were very similar among these parasitic taxa. In general, *P. hominis* and *T. brumpti* had a minimum body length of 5 μm and a maximum of 20 μm, while the lengths of *T. batrachorum* and *H. hampli* ranged from 5 μm to 19 μm and from 6 μm to 19 μm, respectively. All species cells with nuclei that measured between 1 μm and 5 μm in both length and width. Regarding the shape of the cell, trophozoites with pyriform and rounded bodies were observed. These rounded bodies probably are endoflagellar forms or pseudocysts. The number of flagella visible upon staining ranged from 1 to 4 in *T. brumpti* and *T. batrachorum*, 1 to 5 in *P. hominis* and 1 to 3 in *H. hampli* (Table 2 and Fig. 3).

4. Discussion

In this study, we sought to analyze fecal samples of NHPs previously screened by microscopy, for which the direct examination was positive for viable trophozoites, to maximize the success of the isolation and subsequent characterization of the parasites. Despite the small dimensions of parabasilids, the irregular, contorted and directional movement of these forms was fundamental to their characterization in the fecal samples that were subjected to in vitro isolation. This characteristic movement of the group Parabasalia trophozoites was also identified as a key feature in microscopic visualization of NHP's feces in a study in Rio de Janeiro (Santos et al., 2017). However, it is likely that a larger number of isolates would have been obtained if all the fecal samples had been tested. Although testing all the samples would be ideal, in vitro culture is a very expensive and laborious method to use in epidemiological studies that involve the collection and prior processing of samples from animals kept in geographically distant institutions, which occurs in Brazil.

All samples that were positive in direct examination were successfully isolated in both modified Pavlova and TYSGM-9 media supplemented with fetal bovine or equine serum. The literature reporting successful in vitro isolation describe the use of widely diverse media, both monophasic and biphasic, including RK-13/GMP, TYM, CTLM, Medium 199, GMP, MMP-4, MMP-5, Boeck and Drbohlav Locke, Dobell and Leidlaw, TYSGM, RPMI, LIT, and CM0161 (Pindak and Pindak,

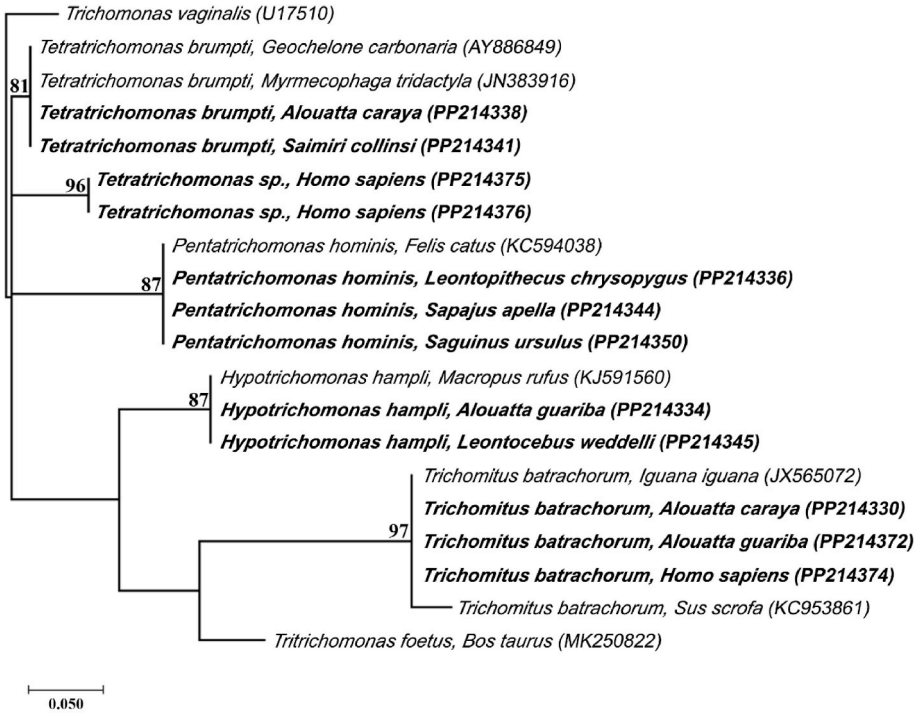

Fig. 2. Phylogenetic analysis of a 100 bp fragment of the ITS variable region by the maximum likelihood test and the Kimura 2-parameter (K2P) evolutionary model of parabasilids isolated from samples of ex situ nonhuman primates (NHPs) and their keepers at different institutions in Brazil. Tree not rooted.

Table 2
Morphological characterization of trophozoites of the group Parabasalia maintained in vitro obtained from samples of nonhuman primates (NHPs) and their keepers in different states of Brazil.

Host	Group Parabasalia	Body			Nucleus		Flagella		Undulating membrane's length	Axostyle's length
		Length	Width	Shape	Length	Width	Number	Length		
Leontopithecus chrysopygus (n = 30)	*Pentatrichomonas hominis*	7 - 13 ± 1.71	4 - 12 ± 1.95	P: 21 R: 9	1 - 4 ± 0.72	1 - 3 ± 0.61	1–4	5 - 19 ± 3.39	3 - 10 ± 1.93	1 - 6 ± 1.45
Saguinus ursulus (n = 30)		5 - 14 ± 2.23	2 - 11 ± 1.86	P: 24 R: 6	1 - 4 ± 0.71	1 - 4 ± 0.71	1–4	7 - 20 ± 3.11	3 - 10 ± 2.06	1 - 4 ± 0.91
Sapajus apella and *Cebus albifrons* (n = 30)		6 - 13 ± 1.83	5 - 9 ± 1.17	P: 15 R: 15	1 - 3 ± 0.58	1 - 3 ± 0.60	1–3	8 - 20 ± 3.29	4 - 12 ± 2.05	1 - 6 ± 1.36
Sapajus apella and *Cebus xanthosternos* (n = 30)		5 - 12 ± 1.54	4 - 10 ± 1.45	P: 24 R: 6	1 - 4 ± 0.71	1 - 4 ± 0.66	1–4	6 - 20 ± 3.92	4 - 11 ± 1.52	1 - 6 ± 1.74
Sapajus apella (n = 30)		7 - 16 ± 2.10	4 - 10 ± 1.70	P: 22 R: 8	1 - 3 ± 0.54	1 - 3 ± 0.54	1–4	8 - 20 ± 2.78	4 - 14 ± 2.33	1 - 6 ± 1.19
Saimiri collinsi and *Saimiri boliviensis* (n = 30)		6 - 16 ± 2.41	3 - 12 ± 2.26	P: 23 R: 7	1 - 3 ± 0.62	1 - 3 ± 0.51	1–4	7 - 28 ± 3.44	4 - 13 ± 2.00	1 - 5 ± 1.33
Saimiri collinsi (n = 300)		5 - 20 ± 2.35	3 - 15 ± 2.05	P: 207 R: 93	1 - 5 ± 0.80	1 - 5 ± 0.76	1–5	4 - 23 ± 3.48	3 - 13 ± 2.04	1 - 10 ± 1.68
Leontopithecus chrysomelas (n = 60)	*Trichomitus batrachorum*	6 - 16 ± 2.02	3 - 10 ± 1.94	P: 44 R: 16	2 - 5 ± 0.92	1 - 5 ± 0.84	1–4	4 - 20 ± 3.76	5 - 11 ± 1.39	1 - 6 ± 1.45
Saimiri collinsi (n = 60)		6 - 19 ± 2.69	3 - 12 ± 2.15	P: 35 R: 25	1 - 4 ± 0.61	1 - 4 ± 0.61	1–4	5 - 20 ± 3.67	5 - 10 ± 1.70	1 - 7 ± 1.43
Sapajus xanthosternos (n = 30)		7 - 13 ± 1.49	3 - 10 ± 1.87	P: 23 R: 7	2 - 4 ± 0.55	1 - 3 ± 0.51	1–4	6 - 20 ± 3.46	4 - 10 ± 1.72	1 - 6 ± 1.57
Alouatta guariba (n = 150)		5 - 15 ± 2.12	3 - 13 ± 1.90	P: 108 R: 42	1 - 5 ± 1.02	1 - 4 ± 0.84	1–4	4 - 21 ± 3.26	3 - 11 ± 1.84	1 - 7 ± 1.30
Alouatta caraya (n = 90)		5 - 15 ± 1.82	3 - 12 ± 2.01	P: 60 R: 30	1 - 4 ± 0.81	1 - 4 ± 0.79	1–4	7 - 20 ± 3.42	3 - 13 ± 2.14	1 - 10 ± 2.16
Pan troglodytes (n = 30)		8 - 12 ± 1.19	3 - 10 ± 1.88	P: 23 R: 7	2 - 4 ± 0.59	2 - 4 ± 0.62	1–4	5 - 20 ± 4.05	5 - 10 ± 1.67	1 - 8 ± 1.63
Homo sapiens (n = 30)		8 - 12 ± 1.26	4 - 10 ± 1.54	P: 25 R: 5	2 - 4 ± 0.64	2 - 3 ± 0.48	1–4	7 - 23 ± 3.84	5 - 12 ± 1.87	1 - 7 ± 1.48
Leontocebus weddelli (n = 30)	*Tetratrichomonas brumpti*	7 - 15 ± 2.42	4 - 13 ± 2.09	P: 21 R: 9	1 - 4 ± 0.95	1 - 4 ± 0.96	1–4	5 - 20 ± 3.65	3 - 11 ± 2.00	1 - 6 ± 1.57
Aotus trivirgatus (n = 30)		7 - 20 ± 3.02	3 - 14 ± 2.27	P: 21 R: 9	1 - 3 ± 0.61	1 - 3 ± 0.56	1–4	4 - 20 ± 3.83	3 - 9 ± 1.62	1 - 9 ± 1.96
Aotus infulatus (n = 30)		5 - 16 ± 2.48	3 - 12 ± 2.13	P: 15 R: 15	1 - 4 ± 0.71	1 - 4 ± 0.66	1–4	4 - 20 ± 3.59	4 - 10 ± 1.85	1 - 6 ± 1.42
Saimiri collinsi (n = 210)		5 - 19 ± 2.48	3 - 12 ± 2.01	P: 147 R: 63	1 - 5 ± 0.90	1 - 5 ± 0.80	1–4	4 - 23 ± 3.51	3 - 15 ± 2.06	1 - 10 ± 1.06
Alouatta caraya (n = 90)		5 - 20 ± 2.37	2 - 14 ± 1.96	P: 65 R: 25	1 - 4 ± 0.72	1 - 4 ± 0.70	1–4	4 - 20 ± 2.80	3 - 11 ± 1.95	1 - 9 ± 1.71
Homo sapiens (n = 60)	*Tetratrichomonas* sp.	5 - 13 ± 1.84	3 - 12 ± 2.32	P: 39 R: 21	1 - 5 ± 0.71	1 - 4 ± 0.64	1–4	4 - 20 ± 3.14	4 - 11 ± 1.82	1 - 6 ± 1.30
Leontocebus weddelli (n = 150)	*Hypotrichomonas hampli*	6 - 19 ± 2.32	3 - 12 ± 1.79	P: 101 R: 49	1 - 5 ± 0.69	1 - 5 ± 0.73	1–3	4 - 25 ± 4.12	3 - 10 ± 1.72	1 - 10 ± 1.62
Alouatta guariba (n = 30)		7 - 14 ± 1.84	4 - 9 ± 1.40	P: 24 R: 6	2 - 5 ± 0.94	2 - 5 ± 0.75	1–3	7 - 14 ± 1.66	5 - 9 ± 1.11	1 - 3 ± 0.70

n: number of measured trophozoites; P: piriform shape; R: rounded shape.

1998; Jongwutiwes et al., 2000; Čepička et al., 2006; Smejkalová et al., 2012; Ibañez-Escribano et al., 2013; Inoue et al., 2015; Santos et al., 2017; Petrželková et al., 2020). The successful isolation of parabasilids in these different media, including those used in this study, highlights the possibility that these parasites have low nutrient specificity. For our isolates, we chose to use culture media that have already been standardized by our research group and by other authors for the isolation and maintenance of protozoa that inhabit the large intestine, such as *Entamoeba histolytica*, *Entamoeba coli*, *Dientamoeba fragilis* and *Balantioides coli* (Carneri 1972; Diamond, 1982; Lima and Hirschfeld, 1996; Silva, 1997; Barbosa et al., 2017, 2020).

Regarding the sudden growth in human sample cultures, we hypothesize that the parasitic load of the sample was so low that it may not have been collected in the aliquot that we analyzed from the direct examination or that the very low quantity of cells present may have gone unnoticed when reading the slides. Even so, after inoculating the samples, it appears that the cells obtained sufficient nutrients that favored their multiplication, given that the human cultures remained alive for 3 years with a high number of trophozoites at each maintenance.

Although the efficiency of the modified Pavlova and TYSGM-9 media was not compared, all parabasilid isolates were successfully stabilized in all four xenic culture systems used. Notably, however, the modified Pavlova medium was found to be the most suitable for the long-term maintenance of parasitic cells due to its simplicity of preparation, which was associated with a better cost benefit ratio. These characteristics have been previously highlighted (Barbosa et al., 2018).

The polymerase chain reaction (PCR) results showed that amplified DNA products were obtained from more than 90% of the isolates. However, five isolates did not show the expected bands in electrophoresis, despite the vigorous growth of parasitic cells in the culture tubes. This negative PCR result might be related to the lack of specificity of the primer used for these parabasilid isolates, to insufficient amounts and optimization of the reagents used in the reaction, or to excess DNA from the parasite itself and even from bacteria, since the cultures analyzed were xenic. Notably, the pair of primers used in this study was recommended to the isolation and characterization of parabasilids from

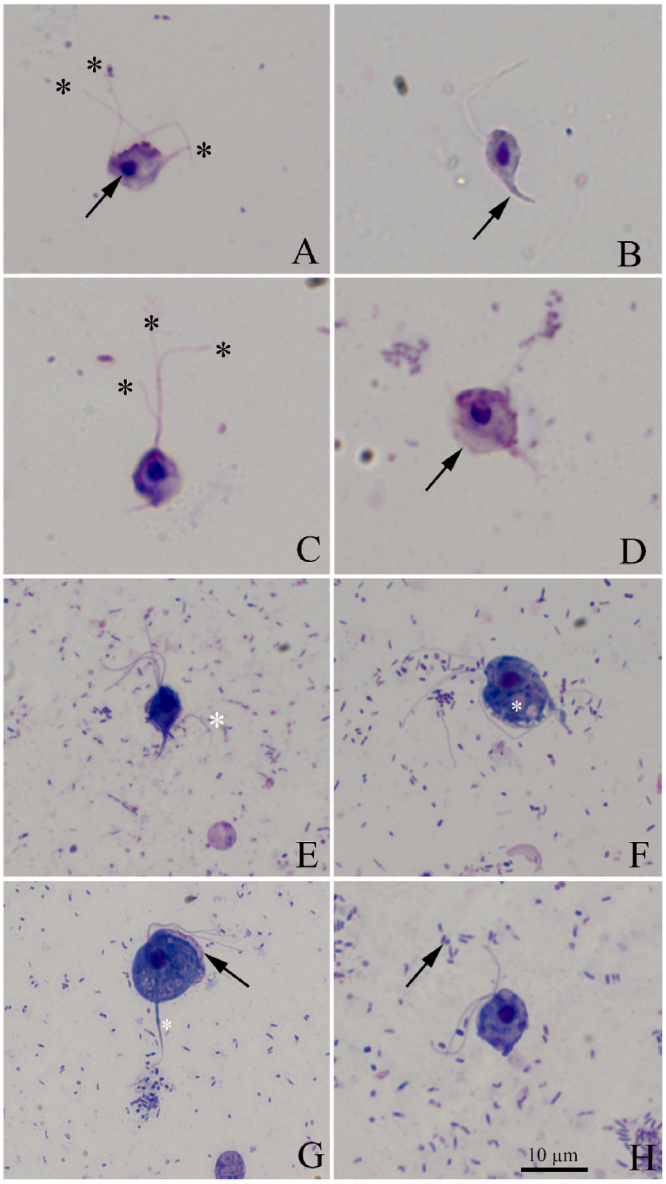

Fig. 3. *T. brumpti* trophozoite isolates obtained from *A. trivirgatus* stool samples were subjected to rapid Panoptic staining kit (A–D). **A.** Trophozoite with three flagella visible in the anterior region (*) and a rounded nucleus (black arrow). **B.** Trophozoite with prominent axostyle in the posterior region (black arrow). **C.** Trophozoite with three flagella visible in the anterior region (black *). **D.** Trophozoite with a rounded body and visible undulating membrane (black arrow). **E.** Human isolate of *Tetratrichomonas* sp. **F.** *T. batrachorum* isolated from *L. chrysomelas* with many vacuoles inside the cell (white *). **G.** *P. hominis* isolated from *S. collinsi* with clearly visible axostyle (white *) and undulating membrane. ***H.*** *H. hampli* trophozoite with three flagella detected in howler monkey (*A. guariba)*, surrounded by bacteria (black arrow). Maginification: 1000x.

biological samples of *Myrmecophaga tridactyla* (Ibañez-Escribano et al., 2013). Similar to the aforementioned authors, in the present study we chose to change only the forward primer previously described (Felleisen, 1997) since, after five PCR attempts, this change generate a larger number of amplifications and better quality electropherograms.

From the analysis of the nucleotide sequences, four distinct genera of parabasilids were identified. Among the taxa characterized, the species *P. hominis* stands out. It was identified only in NHPs, especially *Saimiri collinsi*, from the Institution in Pará state. In the present study, *P. hominis* was also identified in Old World NHPs *Colobus angolensis* and the prosimian *Varecia variegata,* and also in New World NHP *Callithrix jacchus* (Smejkalová et al., 2012; Inoue et al., 2015). In the stained preparations, isolates with a maximum of five free anterior flagella were visualized; however, the literature emphasizes that *P. hominis* can have up to six free flagella (Benchimol, 2010; Čepička et al., 2016). In general, a great variety of trophozoites sizes were observed in different culture media. This size range was also reported for *P. hominis* detected in *Callithrix jacchus* from Japan (Inoue et al., 2015).

P. hominis infects a wide variety of mammals, including several species of NHPs and humans, but has been considered mostly asymptomatic in these hosts (Inoue et al., 2015; Abdo et al., 2022). However, *P. hominis* has already been diagnosed morphologically by staining and by molecular tools in two patients with gastrointestinal disorders with symptoms of diarrhea and abdominal pain, in France (Meloni et al., 2011). In addition, this species has been molecularly characterized in stool samples and respiratory secretions from a Thai woman who died (Jongwutiwes et al., 2000). Importantly, a case–control study conducted in China revealed a significantly greater prevalence of *P. hominis*, identified by molecular biology, in stool samples from patients with gastrointestinal cancer than in those from controls (Zhang et al., 2019). These results emphasize the need for further metagenomic studies to determine whether *P. hominis* is actually part of the microbiota, since it has been widely detected in different NHP taxa, as identified in this study, and it is generally associated with asymptomatic infection.

The second most frequently species was *T. brumpti*. These isolates were detected mainly in NHPs maintained in an institution located in northern Brazil and were characterized as *T. brumpti,* since the nucleotide sequences aligned 100% with those identified in the diarrheal feces of anteaters kept in the Madrid Zoo (Ibañez-Escribano et al., 2013), as well as tortoise isolates analyzed in the Czech Republic (Čepička et al., 2006). In recent years, *Tetratrichomonas* has been widely detected in the feces of Old World NHPs (Smejkalová et al., 2012; Petrželková et al., 2020; Bailey and Hirt, 2023) and has also been identified in Neotropical NHP isolates kept at a Wild Animals Center and the Zoo of Volta Redonda in Rio de Janeiro, Brazil (Santos et al., 2017).

Although the NHP isolates clustered perfectly with nucleotide sequences previously characterized as *T. brumpti*, the fragments of two isolates from the keepers did not cluster in this species and, therefore, were characterized only as *Tetratrichomonas* sp. Several phylogenetic studies have shown that the genus *Tetratrichomonas* is paraphyletic and is subdivided into several lineages (Čepička et al., 2006; Mantini et al., 2009; Smejkalová et al., 2012; Petrželková et al., 2020). The high diversity of this genus may explain the formation of an additional branch in the phylogenetic tree of this study, which contained the sequences of *Tetratrichomonas* sp. obtained from human feces isolates. The morphology of the isolates was another parameter that corroborated the classification of the genus *Tetratrichomonas* evidenced in these isolates, as they exhibited a pyriform to round shape and up to four free flagella. These morphological characteristics were similar to those previously described for *Tetratrichomonas* sp. isolates from NHPs and anteater (Ibañez-Escribano et al., 2013; Santos et al., 2017).

It should be noted that the scarcity of studies of the group Parabasalia, associated with the difficult visualization of these parasites under microscopy, may have contributed to the underdiagnosis of these agents and consequently obscured the relevance of this group within human and veterinary parasitology. However, several symptomatic clinical cases in humans have been reported, and the parasitic agent *Tetratrichomonas* sp. is potentially associated with these cases (Kutisova et al., 2005; Mantini et al., 2009). This issue highlights the need for further clinical studies involving NHPs, which have been identified as hosts of these agents, to verify the pathogenic potential of parabasilids in this group. It is also worth noting that this study analyzed the NHPs from 5 institutions out of hundreds of sites in Brazil that maintain ex situ NHPs, such as zoos, wild animal triage centers and wildlife preserves, which reinforces the need for additional parasitological surveys, such as this one, in the country.

In addition to these parabasilids, *T. batrachorum* was also characterized in at least one isolate from NHPs and, interestingly, also in an isolate from a zookeeper from an institution located in São Paulo. This was the only species identified in isolates from both New World and Old World NHPs, the latter represented only by *Pan troglodytes*. It is important to highlight that the positive handler worked exclusively with PNH and did not manipulate fecal material from other animals. Furthermore, in an epidemiological questionnaire published in our previous study (Dib et al., 2023), this zookeeper reported diarrheal episodes about 6 months before collection in this study. This is a relevant fact that should be analyzed carefully and in more depth since it may or may not be related to *T. batrachorum* infection. Regarding NHPs, no animal positive for *T. batrachorum* presented extraintestinal disorders during the study or in the last 6 months. Even so, based on the results obtained, it is extremely important that management issues be reviewed in order to avoid future contamination in the enclosures.

As in the present study, this parabasilid was also identified in the feces of Old World NHPs by a research group from the United Kingdom (Bailey and Hirt, 2023). Other comparisons with the literature were limited because none of the articles analyzed reported the identification of *Trichomitus* in human feces. These results highlighted the low specificity of *T. batrachorum* for the NHP taxon, since it was isolated from the feces of Callitrichidae, Cebidae, Atelidae and Hominidae, which have very distinct biometric patterns. In addition, *T. batrachorum* exhibited high adaptability to different environments, since this protozoan was identified in several Brazilian regions.

A wide range of sizes and a variaty of shapes were observed in all parabasilid species. Nevertheless, in this wide range of measurements, it was possible to observe sizes compatible with the dimensions of *T. batrachorum* detected in the cane toad (*Rhinella marina*) from the Philippines (Agripo et al., 2020). In addition, the isolates showed up to four flagella when analyzed by microscopy. In the present study, these structures were unequal in size, as has also been reported by other authors (Čepička et al., 2016; Agripo et al., 2020).

In *in-vitro* conditions, the parasitic cells are in different stages of the cell cycle, which can directly influence the phenotype of trophozoites. All cultures remained stable for years with an abundance of parasitic cells during the check for the production of subcultures every 48–72 h. During each maintenance of the parasites, piriform-shaped trophozoites were mostly observed, along with more rounded forms, which may indicate the predominance of cells in the G2 phase of the cell cycle. The predominance of teardrop cells in vitro was also observed in in vitro studies of *T. vaginalis* and *T. foetus* in Brazil (Ribeiro et al., 2000). The observation of this latter form may indicate the formation of endoflagellar cells or even pseudocysts that are formed under stress conditions. However, this result can also indicate an evolutionary strategy already described for *T. foetus*, in which an increase in DNA is observed before the end of cytokinesis under stress conditions, with a later rapid multiplication when nutritional conditions become more favorable again (Iriarte et al., 2023).

Notably, the taxonomic and morphological classification of *T. batrachorum* are still very complex topics and are the subject of several studies (Honigberg, 1953; Čepička et al., 2016; Agripo et al., 2020). Currently, *T. batrachorum* has three synonyms, *Trichomonas batrachorum, Tritrichomonas batrachorum* (Perty) and *Trichomonas natricis* (Coutelen, Biguet and Cochet) (Agripo et al., 2020), and is part of a complex that groups together several species of trichomonads with great morphological similarities described in amphibians (Honigberg, 1953). Even so, this parabasilid taxon has large gaps in terms of its infectivity and its morphological and molecular characterization. Therefore, the morphological analysis performed in this study in association with the molecular characterization provided important additional information on the biometry and parasitism of this parabasilid in NHPs and in humans.

The parabasilid taxon least detected in the present study was *H. hampli*. The identification of this species was possible only because the sequences were grouped with *H. hampli* previously characterized in red kangaroo feces from the Czech Republic Zoo (Céza et al., 2015). In this study, *H. hampli* was detected only in isolates from *L. weddelli* from Pará (Institution D) and *A. guariba* from Rio de Janeiro (Institution C). Morphologically, in the stained preparations of these isolates, up to three anterior flagella were visualized, which reinforced the classification in the genus *Hypotrichomonas,* since this number of anterior flagella is expected for this taxon (Céza et al., 2015). However, the dimensions of the cell body were larger than those described by the aforementioned authors. This size change is related to the amount of nutrients available in the xenic culture medium used, especially supplementation with rice starch, which influences the pleomorphic characteristics of the parasite.

In contrast to previously mentioned genera that have been gaining increasing prominence in scientific studies, the genus *Hypotrichomonas*, which is one of the least known taxa among parabasilids, as well as *Trichomitus*, belongs to the second smallest order within the group Parabasalia, Hypotrichomonadida (Céza et al., 2015). Currently, there are eleven species of the genus *Hypotrichomonas*, the majority of which inhabit the gastrointestinal tracts of reptiles (Céza et al., 2015). Notably, *H. acosta* has already been characterized (Smejkalová et al., 2012). However, in the present study, another species of this genus was characterized, representing the first identification of *H. hampli* in NHPs.

In general, the greatest richness of parabasilid taxa was observed in the populations with the highest number of NHPs, i.e., in the institution located in the North Region of Brazil, followed by those located in the Southeast and South Regions. The greater number of individuals within the enclosures of the Institution of Pará, Northern Brazil, may have favored the transmission of these protozoa. It is known that physical proximity between animals, including the habit of social grooming by NHPs, facilitates the transmission of protozoa, especially those that do not form cysts and not survive in the environment for too long. Interestingly, human fecal samples positive for parabasilid taxa were obtained from keepers working at an institution located in an urbanized environment. Although the zoonotic transmission route of parabasilids has not yet been fully elucidated in the literature, as noted by Petrželková et al. (2020), the proximity of NHPs to urban centers, such as the Intitution A located in the state of São Paulo, may favor transmission between these hosts.

5. Conclusions

Using different biological, morphological and molecular laboratory tools, it was possible to identify four distinct taxa of parabasilids in the feces of NHPs kept in institutions in different regions of Brazil. In addition, the genus *Tetratrichomonas* and the species *T. batrachorum* were characterized for the first time in Brazil in the feces of both NHPs and their keepers, highlighting the susceptibility of these hosts to infections by the group Parabasalia. The detection of *T. batrachorum* in both NHPs and a sample from a handler draws attention to a possible zoonotic transmission. However, further studies with a larger sample size and robust phylogenetic analyses are essential to confirm this isolated finding highlighted in this study.

CRediT authorship contribution statement

Lais Verdan Dib: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Alynne da Silva Barbosa:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lais Lisboa Correa:** Validation, Software, Methodology, Formal analysis, Data curation. **Breno da Silva Torres:** Methodology, Data curation, Conceptualization. **Alcides Pissinatti:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Project

administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Silvia Bahadian Moreira:** Writing – review & editing, Supervision, Methodology, Formal analysis, Conceptualization. **Rodrigo Hidalgo Friciello Teixeira:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **André Luíz Mota da Costa:** Writing – review & editing, Methodology, Investigation. **José Augusto Pereira Carneiro Muniz:** Writing – review & editing, Methodology, Conceptualization. **Amauri Michel Junglos:** Writing – review & editing, Methodology, Investigation. **Zelinda Maria Braga Hirano:** Writing – review & editing, Supervision, Methodology, Investigation, Conceptualization. **Maria Regina Reis Amendoeira:** Writing – review & editing, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

We, Lais Verdan Dib, Alynne da Silva Barbosa, Lais Lisboa Correa, Breno da Silva Torres, Alcides Pissinatti, Silvia Bahadian Moreira, Rodrigo Hidalgo Friciello Teixeira, André Luíz Mota da Costa, José Augusto Pereira Carneiro Muniz, Amauri Michel Junglos, Zelinda Maria Braga Hirano, and Maria Regina Reis Amendoeira, authors from the manuscript intitled "Morphological and molecular characterization of parabasilids isolated from ex situ nonhuman primates and their keepers at different institutions in Brazil" report no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2024.100946.

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