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ORIGINAL PAPER

A survey of hemoparasites and ectoparasites in *Nasua nasua* Linnaeus, 1766 with a redescription of *Hepatozoon procyonis* Richards, 1961 based on morphological and molecular data

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Abstract

Hemoparasites are vector-borne parasites that infect wild carnivores worldwide. Since data on hemoparasite infections in *Nasua nasua* from Brazil are lacking, the aim of this study was to investigate the occurrence of hemoparasites and ectoparasites in *N. nasua* from different areas of Brazil. Blood samples and ectoparasites from 83 *N. nasua* were collected in Botucatu, Palmital, and São Paulo municipalities. Samples were screened via microscopy and molecular methods to detect hemoparasites. Tissues from two *N. nasua* were obtained for histopathological and molecular analyses. All 83 samples were negative for piroplasms on morphological and molecular examination. Thin blood smears of nine animals were positive for *Hepatozoon* gamonts. The gamonts shared morphological characteristics of *Hepatozoon procyonis*. Meronts were detected in the liver and spleen tissue of one animal. Twenty-one blood samples and four tissue samples were PCR positive for *Hepatozoon* sp. The sequences obtained were 97% identical to those of *Hepatozoon felis*, *Hepatozoon ursi*, and *Hepatozoon* sp. Based on searches for similarity and morphology, we identified the sequences as belonging to *H. procyonis*. This study provides epidemiological data on hemoparasite infections and redescribe *H. procyonis* based on morphological, morphometrical, and molecular analyses.

Keywords Coatis · *Hepatozoon procyonis* · Histology · Molecular characterization · Tick-borne pathogens

Introduction

The ring-tailed coati (*Nasua nasua*) are medium-sized animals belonging to the order Carnivora, family Procyonidae. *Nasua nasua* are omnivores with generalist diet, composed mainly of insects, fruits, and small vertebrates (Alves-Costa et al. 2004). They are widely distributed in South America and adapt to different environments and food resources, making it possible for them to live in urban areas and to interchange between wild and domestic environments (Rodrigues et al. 2006). Vector-borne hemoparasites are important worldwide, as they can cause diseases in animals and humans. Many of these pathogens are maintained in wildlife reservoirs (Herrera et al. 2008; Shock et al. 2011; Yabsley and Shock 2013; Alvarado-Rybak et al. 2016). For example, the coatis are considered reservoirs for *Trypanosoma cruzi* and *Trypanosoma evansi* (phylum: Sarcomastigophora) and can become infected with several other hemoparasites, including members of phylum Apicomplexa, such as piroplasms and *Hepatozoon* spp. (Herrera et al. 2004, 2008; Rodrigues et al. 2007; Sousa et al. 2017a, b).

Piroplasms are intraerythrocytic tick-borne parasites that include species within the genera *Theileria*, *Babesia*, and *Cytauxzoon* (Barbosa et al. 2017). These protozoa have zoonotic and/or veterinary importance. *Cytauxzoon* sp. infection is mainly reported in felids (Alvarado-Rybak et al. 2016). Some species of

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Theileria are highly pathogenic to cattle and small ruminants, causing significant economic loss (Bishop et al. 2004). *Babesia* spp. are considered the causative agents of a zoonosis named babesiosis, and wildlife animals act as possible reservoirs for zoonotic *Babesia* (Yabsley and Shock 2013). Piroplasm infections have been reported in several carnivore species, including members of the Procyonidae family (Alvarado-Rybak et al. 2016). In the Brazilian Pantanal, *Theileria* sp. closely related to *Theileria equi* has been detected infecting *N. nasua* (Sousa et al. 2017b). However, an infection in *N. nasua* from other regions of Brazil has not been reported yet.

Hepatozoon species (Adeleorina: Hepatozoidae) are blood parasites that infect domestic and wild animals worldwide. They are mainly reported infecting carnivores and rodents, among the mammals (Modrý et al. 2017). Within the Procyonidae family, *Hepatozoon* sp. was first reported in the raccoon *Procyon lotor* from the USA and consequently named *Hepatozoon procyonis* (Richards 1961). *Hepatozoon procyonis* was also detected in *Procyon cancrivorus* (Schneider 1968; Rodrigues et al. 2007). In Brazil, *H. procyonis* was diagnosed in *N. nasua* for the first time by morphological analysis (Rodrigues et al. 2007). Sousa et al. (2017b) performed the first molecular detection of the *Hepatozoon* sp. in *N. nasua*. However, the studies that previously reported *Hepatozoon* sp. infection in coatis were based solely on either morphological (Rodrigues et al. 2007) or molecular examination (Sousa et al. 2017a). Studies involving both morphological and molecular analyses are needed to improve characterization of hemoparasite species in *N. nasua*, enabling a better understanding of their epidemiology.

Ticks and fleas are hematophagous ectoparasites that can cause irritation and spoliation. Moreover, they are of great medical and veterinary importance, because these ectoparasites play a role as vectors of pathogens for humans and animals (Muller et al. 2005). Therefore, the study of ectoparasites associated with wildlife is necessary to elucidate whether these arthropods may represent a risk to the health of humans and animals (Dantas-Torres et al. 2010). In Brazil, few recent studies have investigated the presence of ectoparasites infesting coatis (Dantas-Torres et al. 2010; Martins et al. 2017; Sousa et al. 2017a).

In this context, the aim of the present study was to investigate the occurrence of hemoparasites and ectoparasites in *N. nasua* from different regions in Brazil, using both morphological and molecular analyses. In addition, we redescribed *H. procyonis* based on its morphological and molecular characteristics.

Material and methods

Study area and sample collection

Between September 2013 and June 2017, blood samples and ectoparasites were collected from *N. nasua* in three municipalities of the state of São Paulo (Botucatu, Palmital, and São Paulo), Brazil (Fig. 1). In Botucatu (22° 53′ 25″ S, 48° 27′ 19″ W), 30 animals were sampled from two forest fragments within the facilities of São Paulo State University—UNESP. Forty *N. nasua* were captured in a park located in the central region of the Palmital municipality (22° 47′ 1.7″ S, 50° 12′ 24.8″ W). The animals sampled in Palmital were part of a population control program developed by veterinarians from the "Centre of Medicine and Research on Wild Animals (Centro de Medicina e Pesquisa em Animais Selvagens—CEMPAS)," in which the males and females were subjected to vasectomy and tubal ligation, respectively. Moreover, food (fruits and a mixture of cornmeal, bananas, and dog food) was offered daily to these animals. Thirteen samples were collected from *N. nasua* in the "Screening Centre of Wild Animals (Centro de Triagem de Animais Silvestres—CETAS)" of the "Tietê Ecological Park (Parque Ecológico do Tietê)," from the urban area of São Paulo municipality (23° 29′ 29.0″ S, 46° 31′ 15.3″ W). In Botucatu and São Paulo regions, the coatis were free to move between urban and

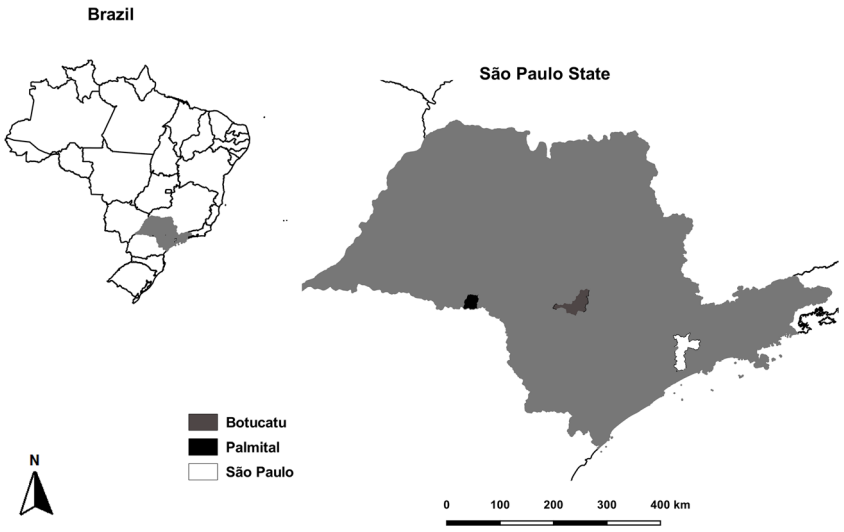

Fig. 1 Map of São Paulo State, Brazil, indicating the three municipalities where samples were collected

wild environments, whereas in the Palmital municipality, the animals remained restricted to the park (urban region) due to the absence of nearby floret fragments.

The individuals of *N. nasua* were anesthetized with a combination of ketamine and midazolam and had the sex and age recorded. Age was estimated based on tooth wear, body size, weight, and the animals were classified as either young or adult (Herrera et al. 2008). Blood samples were collected from the jugular vein and stored in EDTA tubes at − 20 °C until DNA isolation. For microscopic examination, thin blood smears were performed, fixed in absolute methanol, stained with 10% Giemsa, and screened for the presence of piroplasms and *Hepatozoon* spp. The parasitemia of the positive coatis was estimated by counting the number of gamonts in 500 leukocytes (Aktas et al. 2015).

Nasua nasua specimens were also inspected for the presence of ectoparasites. The tick and flea specimens found were stored in 70% ethanol. Morphological identification of arthropods was performed following taxonomic keys (Barros-Battesti et al. 2006; Martins et al. 2010; Linardi and Santos 2012). To detect the presence of *Hepatozoon* oocysts in the tick hemocoel, 35 specimens (nymphs and adults) were randomly selected and dissected as previously described by Demoner et al. (2013).

Histopathological analysis

All animals were apparently healthy except for two *N. nasua* that died in the CETAS of the "Parque Ecológico do Tietê." One animal was bitten by a dog and died by respiratory insufficiency, and the other was euthanized because it was severely injured with myiasis, muscular atrophy, and bone exposure of the lumbar spine. They were necropsied, and tissues samples (liver and spleen) were collected for histopathological and molecular analyses. For histopathology, tissues were formalin-fixed, paraffin-embedded, sliced at a thickness of 5 μm, and stained with hematoxylin and eosin (H&E).

Morphometric analysis

The parasites detected in the thin blood smears and in the histopathological analyses were measured via a computerized image analysis system, using the software QWin Lite 2.5 (Leica). All the detected *Hepatozoon* gamonts had their length, width, and cytoplasm projection measured. The number of gamonts measured by animal ranged from 1 to 23 gamonts. Nuclei, when visible, were also measured. We further measured the length and width of the *Hepatozoon* stages detected in the tissues.

DNA extraction, amplification, and sequencing

Genomic DNA of each sample was isolated from 200 μL of blood or 20–50 mg of tissues (liver and spleen), using the illustra™ blood genomicPrep Mini Spin Kit and the illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), respectively, following the manufacturer's instructions. DNA concentrations (ng/μL) and quality (A_{260nm}/A_{280nm}) were measured using spectrophotometry (NanoDrop® ND-2000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA).

Piroplasm detection was performed by conventional PCR using the primers BAB2 143-167 (5′-CCG TGC TAA TTG TAG GGC TAA TAC A-3′) and BAB2 694-667 (5′-GCT TGA AAC ACT CTA RTT TTC TCA AAG-3′), targeting a ≈ 500-bp fragment of the 18S rRNA gene of *Babesia* spp., *Theileria* spp., and *Cytauxzoon* spp. (Almeida et al. 2012). PCR reactions were performed using the following amplification conditions: 95 °C for 5 min, 58 °C for 1 min, and 72 °C for 2 min with 35 repetitive cycles of 94 °C for 30 s, 58 °C for 20 s, and 72 °C for 30 s followed by a final extension at 72 °C for 7 min.

DNA samples were screened for the presence of *Hepatozoon* spp., using the HepF300 (5′-GTT TCT GAC CTA TCA GCT TTC GAC G-3′) and Hep900 (5′-CAA ATC TAA GAA TTT CAC CTC TGA C-3′) primer pair, which amplifies 600 bp of the 18S rRNA gene (Ujvari et al. 2004). The PCR conditions were 94 °C for 3 min, 35 cycles at 94 °C for 45 s, 56 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. All positive samples in the first PCR were also amplified by nested PCR with the primers HAM-1 (5′-GCC AGT AGT CAT ATG CTT GTC-3′) and HPF-2 (5′-GAC TTC TCC TTC GTC TAA G-3′) (Criado-Fornelio et al. 2006), followed by 4558 (5′-GCT AAT ACA TGA GCA AAA TCT CAA-3′) and 2733 (5′-CGG AAT TAA CCA GAC AAA T-3′) (Mathew et al. 2000), which targeted a larger fragment (1120 bp) of the 18S rRNA gene for sequencing and phylogenetic analysis. The PCR conditions for the primary PCR (primers HAM-1 and HPF-2) consisted of a pre-PCR step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, 56 °C for 1 min, an extension at 72 °C for 1 min and 30 s, and a final extension at 72 °C for 7 min. The PCR conditions of the secondary PCR (primers 4558 and 2733) consisted of a pre-PCR step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 2 min, an extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min.

To avoid misdiagnosis of *Hepatozoon* meronts with *Toxoplasma gondii* cysts, a PCR was performed on the *N. nasua* DNA tissues with the primers TOX4 (5′-CGC TGC AGG GAG GAA GAC GAA AGT TG-3′) and TOX5 (5′- CGC TGC AGA CAC AGT GCA TCT GGA TT-3′), which targeted a 529-bp fragment of the *T. gondii* repeated sequence (Homan et al. 2000).

All the PCRs were carried out in a total volume of 25 μL: 12.5 μL of GoTaq® Colorless Master Mix (Promega Corporation, WI, USA), 1.25 μL (10 pmol/μL) of each primer, 5 μL of DNA, and 5 μL of nuclease free water (Thermo Scientific). A negative (distilled sterile water) and a positive

control (*Hepatozoon canis* or *Babesia vogeli* DNA isolated from naturally infected dogs, or *T. gondii* DNA extracted from experimentally infected mice) were used in each reaction.

Amplified products were visualized by electrophoresis on 1% agarose gels stained with GelRed™ (Biotium, Hayward, USA). The amplicons were purified using illustra ExoProStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) in agreement with the manufacturer's instructions. PCR products were sequenced using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The obtained sequences were edited using the BioEdit software v7.2.5 (Hall 1999) and compared for similarity with sequences available in GenBank® by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignment, with 628 bp, for four sequences obtained in this study and 32 sequences retrieved from GenBank®, was performed using the MUSCLE algorithm and the Geneious v.7.1.3 software (Biomatters; http://www.geneious.com). The best evolutionary model for maximum likelihood analysis was identified using the jModelTest v.2.1.10 program (Darriba et al. 2012). The best fitting model based on the Akaike information criterion (AIC) was TPM2uf + G. The phylogenetic tree was estimated using maximum likelihood implemented in PhyML v.3.0 (Guindon et al. 2010). To estimate internal nodes of the tree, a bootstrap with 1000 replicates was used. An alignment, with 1085 bp from one *Hepatozoon* sp. sequence obtained in this study and five *Hepatozoon* sp. sequences isolated from carnivores available on GenBank®, was used to estimate the percentage of nucleotide divergence. This analysis was performed on the MEGA6 software, using the Kimura 2 Parameter substitution model (Tamura et al. 2013).

Statistical analysis

The prevalence and confidence intervals (95%) for *Hepatozoon* spp. were calculated using sampsize (Version 0.6), available at http://sampsize.sourceforge.net/iface/index.html (Glaziou 2005). Association between the number of positive animals in the PCR for *Hepatozoon* sp. with origin (Botucatu, Palmital, or São Paulo), age (young or adult), sex (female or male), tick (presence or absence), and fleas (presence or absence) was assessed using Pearson chi-squared test (all categories sample sizes > 5) or Fisher's exact test (any category sample size < 5). Association between the prevalence of infection with age, sex, and the presence/absence of ectoparasites was evaluated by region studied. Palmital region was excluded from the analysis, because all samples were negative for *Hepatozoon* spp. Data of parasitemia were checked for normality and homoscedasticity and Mann-Whitney's test was used to determine statistically significant differences between the parasitemia and age and sex. Analyses of the obtained data were carried out on the BioEstat 5.0 software (Ayres et al. 2007). p values ≤ 0.05 were considered to indicate statistically significant differences.

Results

The 83 samples examined were negative for piroplasms both in the thin blood smear and the PCR. Nine animals (10.8%; CI 5–19.5; $n = 83$) were positive for *Hepatozoon* gamonts (Table 1). All positive samples found in the thin blood smears were from the Botucatu municipality. The gamonts were elongated, with an eccentric nucleus and a curved cytoplasmic projection located opposite to the nucleus (Fig. 2), which is characteristic of *H. procyonis* (Richards 1961; Rodrigues et al. 2007). The average *Hepatozoon* parasitemia of the nine animals was 0.40%, ranging from 0.2 to 1%. No statistical differences were observed in parasitemia between different ages ($p = 0.66$) or sexes ($p = 0.19$). However, these results should be considered with caution, given the small number of infected animals ($n = 9$). In the histopathological analysis, several stages of *Hepatozoon* meronts were detected in the liver and spleen of one out of the two sampled animals (Figs. 3 and 4). The blood from positive animals in the histopathology was negative for *Hepatozoon* spp. in the microscopic examination and positive in the molecular analysis. All samples presented as positive for infection in the thin blood smear and histopathology were also positive in the PCR.

Hepatozoon DNA was detected in 21 animals (25.3%; CI 16.4–36; $n = 83$), using PCR (Table 1). A higher prevalence of *Hepatozoon* infection was registered in *N. nasua* from Botucatu, with 17 positive animals (56.7%; CI 37.4–74.5; $n = 30$). In São Paulo, four animals (30.8%; CI 9–61.4; $n = 13$) were infected with *Hepatozoon* sp. None of the samples from the Palmital municipality were positive for *Hepatozoon*. The prevalence of *Hepatozoon* infection between Botucatu and São Paulo municipalities was not statistically different ($p = 0.18$). However, statistic differences were observed between Botucatu and Palmital ($p < 0.05$), and between São Paulo and Palmital ($p < 0.05$), as in Palmital, no infected animal was detected. Although the prevalence of *Hepatozoon* sp. was higher in female than in male, and in adults than in young coatis, differences in both cases were not statistically significant (in both cases, $p > 0.05$, Table 2).

Table 1 Prevalence of *Hepatozoon procyonis* infection and ectoparasites infestation from *Nasua nasua* in the municipalities of Botucatu, Palmital, and São Paulo, São Paulo State, Brazil, and comparison of *Hepatozoon procyonis* prevalence by PCR in relation to presence/absence of ectoparasites

				Origin			
				Botucatu	Palmital	São Paulo	Total
N° of animals				30	40	13	83
Pos. for *H. p.* (*n*/%)	Blood smear			9 (30.0)	0	0	9 (10.8)
	PCR			17 (56.7)	0	4 (30.8)	21 (25.3)
Pos. for ectoparasites (*n*/%)	Ticks	Pos. for ticks		26 (86.7)	3 (7.5)	2 (15.4)	31 (37.3)
		Pos. *H. p.* and ticks		16 (61.5)	0	2 (100)	18 (58.1)
		p value		0.29	*	0.08	
	Fleas	Pos. for fleas		6 (20.0)	0	1 (7.7)	7 (8.4)
		Pos. for *H. p.* and fleas		4 (66.7)	0	1 (100)	5 (71.4)
		p value		0.67	*	0.30	
N° of ticks collected		*A. dubitatum*	N	5	1	0	6
		A. ovale	F	27	1	0	28
			M	27	0	0	27
		A. sculptum	F			1	1
			N	39		2	41
		Amblyomma sp.	L	20		5	25
		R. sanguineus	F		1		1
N° of fleas collected		*R. lutzi lutzi*	F	8			8
			M	2			2
		C. felis	F			2	2

N° number, *Pos.* positive, *H. p. Hepatozoon procyonis*, *A. dubitatum Amblyomma dubitatum*, *A. ovale Amblyomma ovale*, *A. sculptum Amblyomma sculptum*, *R. sanguineus s. l. Rhipicephalus sanguineus* sensu lato, *R. lutzi lutzi Rhopalopsyllus lutzi lutzi*, *C. felis Ctenocephalides felis*, *N* nymph, *F* female, *M* male, *L* larvae

**p* value not measured, because in Palmital municipality, all samples were negative for *Hepatozoon procyonis*

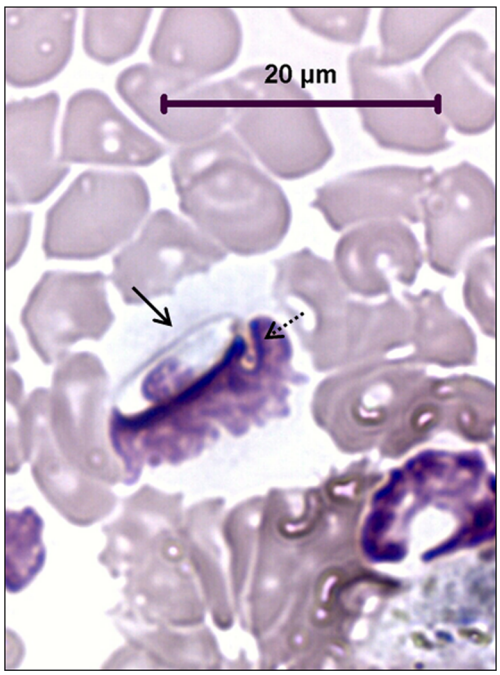

Fig. 2 Gamont of *Hepatozoon procyonis* in the total blood of a *Nasua nasua*. (⋯▶) Gamont. (⟶) cytoplasmic projection. Giemsa stain

The liver and spleen of the two necropsied coatis were also PCR-positive for *Hepatozoon* sp. and PCR-negative for piroplasm and *T. gondii*.

In total, twenty-five 18S rRNA sequences were obtained from the blood, liver, and spleen samples of the 21 positive animals, and all isolated sequences were identical among them. A BLAST search showed that the *Hepatozoon* sp. sequences isolated in this study were distinct from the *Hepatozoon* sp. sequences available in GenBank®. The obtained sequences demonstrated a 97% similarity to those of *Hepatozoon felis* (KX017290, AY628681, and AY620232), *Hepatozoon* sp. (EF222257, KU198330, and FJ719813), and *Hepatozoon ursi* (EU041718), and a 96% similarity to those of *Hepatozoon canis* (KX712126, DQ439540, and AY150067). Based on the similarity search and morphological characteristics of the *Hepatozoon* gamonts, we identified the sequences as belonging to *H. procyonis*. All 25 *H. procyonis* sequences obtained in this study were deposited in GenBank® under the following accession numbers: MF685386–MF685410. The percentage of nucleotide divergence between *H. procyonis* from this study and *Hepatozoon* spp. from other carnivores ranged from 3.3 to 4.2% (Table 3).

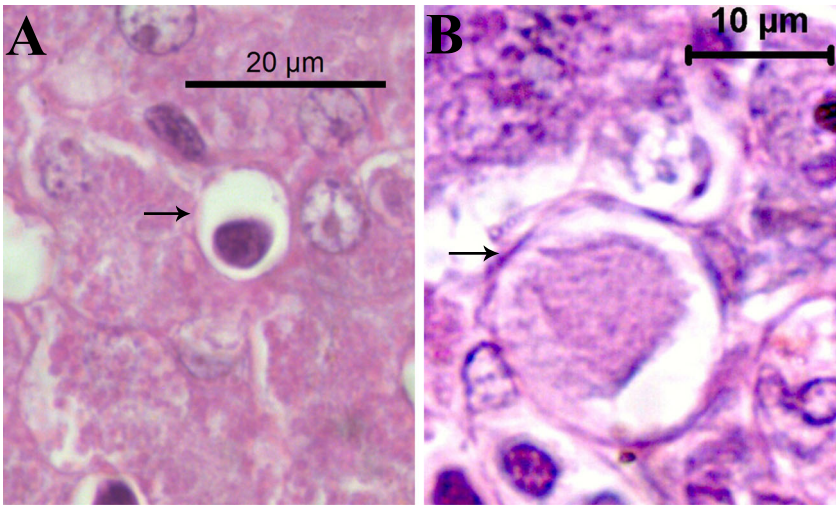

Fig. 3 Immature meronts of *Hepatozoon procyonis* in the liver and spleen tissues from *Nasua nasua*. **a** An early *H. procyonis* meront in the liver. **b** An immature *H. procyonis* meront in the spleen. Hematoxylin and eosin stain

The maximum likelihood tree (Fig. 5), inferred from a 640-bp fragment of the 18S rRNA gene from 32 *Hepatozoon* sequences, showed that *Hepatozoon* sp. sequences isolated in this study grouped within a large clade composed of *Hepatozoon* spp. sequences from carnivores and positioned in a distinct clade of *Hepatozoon* spp. sequences from amphibians, reptiles, rodents, and marsupials. Moreover, *H. procyonis* detected in this study clustered with *Hepatozoon* sp. sequences previously isolated from *N. nasua* in Brazil (KX779359 and KX779361) and formed a unique clade strongly supported with 100% bootstrap.

A total of 129 tick specimens were collected from 31 *N. nasua* (37.3%; CI 26.9–48.6; $n = 83$). Tick specimens were morphologically identified as *Amblyomma dubitatum*, *Amblyomma ovale*, *Amblyomma sculptum*, *Amblyomma* sp., and *Rhipicephalus sanguineus* sensu lato (Table 1). Of the 21 coatis that were positive for *H. procyonis*, 18 had ticks attached to their bodies (85.7%; CI 63.6–96.9). However, the presence of ticks was not significantly associated with the presence of *H. procyonis* ($p > 0.05$). *Amblyomma ovale* and *A. sculptum* were the most prevalent tick species infesting *N. nasua*. *Amblyomma ovale* was detected in 15 of the 31 animals infested with ticks (43.9%; CI 30.1–66.9; $n = 31$), and *A. sculptum* was present on 16 coatis (51.6%; CI 33–69.8; $n = 31$). To detect the presence of *Hepatozoon* oocysts, 35 tick specimens (30 *A. ovale* adults and five *A. sculptum* nymphs) were dissected. All the evaluated tick specimens were negative for *Hepatozoon* oocysts. Additionally, 12 specimens of fleas were also found infesting seven *N. nasua* (8.4%; CI 3.4–16.6; $n = 83$). Ten fleas were identified as *Rhopalopsyllus lutzi lutzi* and two as *Ctenocephalides felis* (Table 1). No significant association was observed between flea infestation and *H. procyonis* infection ($p > 0.05$).

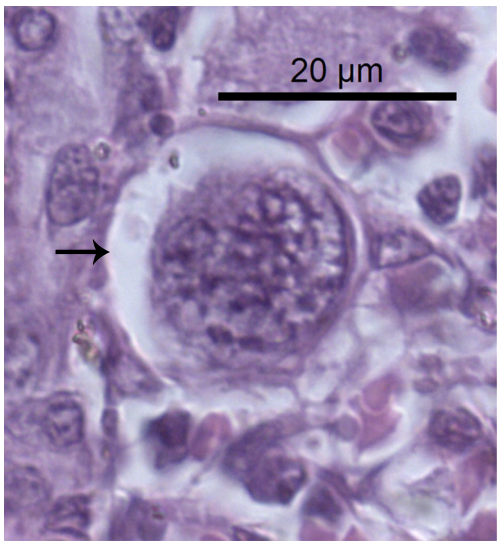

Fig. 4 Almost-mature meront of *Hepatozoon procyonis*, in the spleen of a *Nasua nasua*, with merozoites nuclei visible. Hematoxylin and eosin stain

Redescription of *Hepatozoon procyonis* Richards, 1961

Gamonts Gamonts were located in the cytoplasm of neutrophils and monocytes. They were elongated, surrounded by a capsule, and presented a mean size of $10.35 \pm 0.53 \times 3.21 \pm 0.29$ μm (9.06–11.34 × 2.53–4.01 μm; $n = 62$). The *H. procyonis* gamonts showed a curved cytoplasmic projection that was located opposite to the nucleus, which is characteristic of this species (Fig. 2). The cytoplasmic projection measured 4.33 ± 0.35 μm (3.66–5.15 μm; $n = 62$). It was not possible to visualize the nucleus in most of the gamonts. When visible, the compact nucleus was located eccentrically and showed a mean size of $4.07 \pm 0.44 \times 2.22 \pm 0.22$ μm (2.97–4.36 × 1.81–2.53 μm; $n = 13$).

Table 2 Comparison of *Hepatozoon procyonis* prevalence by PCR, in *Nasua nasua* from Botucatu, Palmital, and São Paulo, in relation to sex and age

Origin	N°	Gender					Age				
		Female		Male		*p* value	Young		Adult		*p* value
		N°	Positive (*n*/%)	N°	Positive (*n*/%)		N°	Positive (*n*/%)	N°	Positive (*n*/%)	
Botucatu	30	14	8 (57.1)	16	9 (56.2)	0.96	13	6 (46.1)	17	11 (64.7)	0.31
Palmital	40	24	0	16	0	*	15	0	25	0	*
São Paulo	13	6	3 (50.0)	7	1 (14.3)	0.26	8	1 (12.5)	5	3 (60.0)	0.21
Total	83	44	11 (25.0)	39	10 (25.6)		36	7 (19.4)	47	14 (29.8)	

N° number of animals sampled

**p* value not measured, because in Palmital municipality, all samples were negative for *Hepatozoon procyonis*

Meronts Several developmental stages of meronts were observed in the liver and spleen of *N. nasua* (Figs. 3 and 4). The *H. procyonis* meronts were round to oval and separated from the surrounding tissues by a thick membrane, which enveloped them. Immature meronts contained amorphous material (Fig. 3) and measured 15.39 ± 2.4 × 12.99 ± 2.29 μm (10.07–20.39 × 8.58–18.84 μm; $n = 53$). Almost-mature meronts presented visible nuclei without distinct merozoites (Fig. 4). The mean size was observed to be 21.63 ± 0.61 × 18.94 ± 2.31 μm (21.1–22.45 × 16.69–21.34 μm; $n = 4$). No mature meronts were observed.

Taxonomic summary

Type host: *Procyon lotor* Linnaeus, 1758 (Carnivora: Procyonidae) (Richards 1961; Clark et al. 1973).

Other hosts: *Procyon cancrivorus* Cuvier, 1798 (Carnivora: Procyonidae) (Schneider 1968; Rodrigues et al. 2007); *Nasua nasua* Linnaeus, 1766 (Carnivora: Procyonidae) (Rodrigues et al. 2007; present study).

Vector: Unknown.

Infection site: Gamonts were observed in peripheral and total blood (Richards 1961; Schneider 1968; Clark et al. 1973; Rodrigues et al. 2007; present study). Meront stages were detected in the heart (Richards 1961; Schneider 1968; Clark et al. 1973), skeletal muscle (Clark et al. 1973), and liver and spleen tissue (present study).

Type locality: Southwestern Georgia (Richards 1961).

Other localities: Panama (Schneider 1968); Texas, USA (Clark et al. 1973); Rio de Janeiro State, Brazil (Rodrigues et al. 2007); Botucatu (22° 53′ 25″ S, 48° 27′ 19″ W) and São Paulo (23° 29′ 29.0″ S, 46° 31′ 15.3″ W) municipalities, São Paulo State, Brazil (present study).

Prevalence (present study): Botucatu: 17 out of 30 (56.57%); São Paulo: four out of 13 (30.77%).

DNA sequences: Twenty-five 18S rRNA sequences (964–1064 bp) were deposited in GenBank® (accession numbers: MF685386–MF685410).

Material deposited: Blood smear and histology slides are deposited at the National Institute of Amazonian Research (INPA), Manaus, AM, Brazil (nº INPA13).

Discussion

Piroplasms have been reported to infect a wide range of wild carnivores worldwide, including members of the Procyonidae family such as the raccoon (*P. lotor*) in the USA and Japan and the white-nosed coatis (*Nasua narica*) in Costa Rica (Kawabuchi et al. 2005; Birkenheuer et al. 2006, 2008; Jinnai

Table 3 Distance matrix among *Hepatozoon* sp. sequences isolated from carnivorous. The upper triangle shows the percentage of nucleotide difference, while the lower triangle shows the number of nucleotide difference

Sequences	1 (AF176836)	2 (KU569168)	3 (EU041718)	4 (MF685403)*	5 (AY620232)	6 (KX757032)
1. *Hepatozoon americanum*	–	3.2%	3.4%	3.6%	3.3%	2.7%
2. *Hepatozoon canis*	32	–	4.4%	4.2%	3.6%	3.6%
3. *Hepatozoon ursi*	34	44	–	3.8%	3.4%	3.5%
4. *Hepatozoon procyonis**	36	42	38	–	3.3%	3.6%
5. *Hepatozoon felis*	33	37	35	34	–	2.2%
6. *Hepatozoon silvestris*	28	37	36	37	23	–

*Sequence isolated in this study

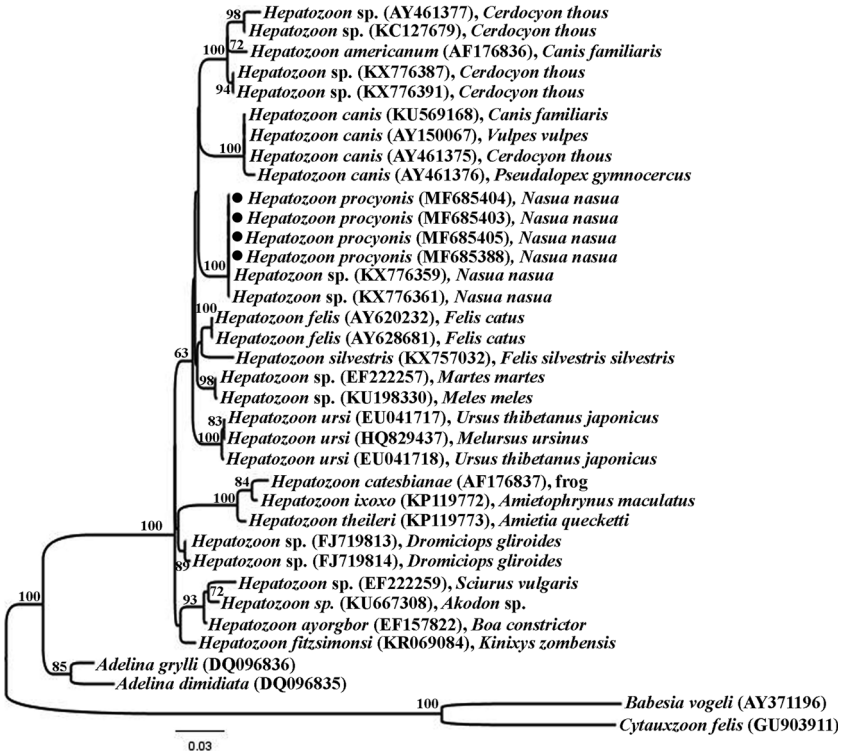

Fig. 5 Phylogenetic tree of maximum likelihood analysis based on the partial sequences (640 bp) of the 18S rRNA gene of *Hepatozoon* species from *Nasua nasua*, isolated in this study and sequences deposited in GenBank®. The branch length scale represents 0.03 substitutions per site. *Cytauxzoon felis*, *Babesia vogeli*, *Adelina dimidiata*, and *Adelina grylli* were used as outgroups. (●) sequences obtained in this study

et al. 2009; Clark et al. 2012; Mehrkens et al. 2013; Alvarado-Rybak et al. 2016). In Brazil, these protozoa have been detected via molecular methods in carnivores such as the pampas cat (*Oncifelis colocolo*), common genet (*Genetta genetta*), crab-eating fox (*Cerdocyon thous*), pampas fox (*Lycalopex gymnocercus*), jaguar (*Panthera onca*), little spotted cat (*Leopardus tigrinus*), margay (*Leopardus wiedii*), ocelot (*Leopardus pardalis*), Pallas's cat (*Otocolobus manul*), puma (*Puma concolor*), jaguarundi (*Puma yagouaroundi*), maned wolf (*Chrysocyon brachyurus*), and ring-tailed coati (*N. nasua*) (André et al. 2009, 2011; Soares et al. 2014, 2017; Silveira et al. 2016; Furtado et al. 2017; Sousa et al. 2017b). However, piroplasms were not detected in *N. nasua* from the Botucatu, Palmital, and São Paulo municipalities. The absence of piroplasm infections in coatis from these regions may be related to the geographical distribution of these protozoa or to the unavailability of competent vectors, specially given the fact that these parasites have been detected infecting ring-tailed coatis from Pantanal region of Brazil (Sousa et al. 2017b).

Hepatozoon procyonis was the only hemoparasite found infecting *N. nasua* from the regions of our study. This parasite was first reported infecting *P. lotor* specimens from Georgia, USA (Richards 1961). *Hepatozoon procyonis* was also detected in *P. cancrivorus* from Panama and Brazil (Schneider 1968; Rodrigues et al. 2007). By morphological analysis, Rodrigues et al. (2007) identified *H. procyonis* in *N. nasua* from Brazil and morphometrically characterized the gamonts of this species. Recently, *Hepatozoon* sp. was detected infecting *N. nasua* from the Brazilian Pantanal via molecular methods, but the *Hepatozoon* species that affected these animals was not identified (Sousa et al. 2017a). To our knowledge, this is the first report that characterizes *H. procyonis* from *N. nasua* morphologically, morphometrically, and molecularly. Based on these characteristics, we also redescribed this parasite.

We detected *Hepatozoon* gamonts in thin blood smears of the *N. nasua*. The gamonts were elongated with eccentric nuclei and presented a cytoplasmic projection. The morphological data obtained in this study agree with previously described gamont characteristics for *H. procyonis* (Richards 1961; Schneider 1968; Rodrigues et al. 2007). The study by Rodrigues et al. (2007) reported that the mean size of the *H. procyonis* gamonts from *N. nasua* was 10.45 × 4.03 μm. In our study, similar data was obtained, with gamonts measuring 10.35 × 3.21 μm on mean. The parasites observed in *P. lotor* had their nuclei located in the central region of the gamont (Richards 1961). However, the nuclei of the *H. procyonis* gamonts from *N. nasua* were reported to be eccentric and measured 4.20 × 2.03 μm on mean (Rodrigues et al. 2007). The gamonts identified in this study also exhibited eccentric nuclei with a mean size of 4.07 × 2.22 μm. However, the nuclei of most of the gamonts were not visible. The morphometric data obtained in this study with respect to cytoplasmic projection, a characteristic of *H. procyonis*, were similar to that described by Rodrigues et al. (2007). Cytoplasmic projection measured 4.33 μm in this study, whereas Rodrigues et al. (2007) reported measurements of 4.08 μm.

Hepatozoon procyonis meronts have been found in the heart and skeletal muscle tissues of *P. lotor* and in the heart

tissue of *P. cancrivorus* (Richards 1961; Schneider 1968; Clark et al. 1973). We detected, for the first time, *H. procyonis* meronts in the liver and spleen tissues of *N. nasua*. The almost-mature meronts found in this study measured a mean of 21.63 × 18.94 μm and were smaller than the meronts detected in *P. lotor* and *P. cancrivorus* (Schneider 1968; Clark et al. 1973). *Hepatozoon procyonis* meronts observed in *P. lotor* measured 31.2 × 22.7 μm on mean (Clark et al. 1973), and those detected in *P. cancrivorus* presented a mean size of 40.3 × 27.1 μm (Schneider 1968). The differences observed in the size of the meronts may be due to the developmental stages of the measured meronts. Other explanations could be associated with the techniques of fixation and preparation of the samples, errors in morphometric analysis, or affiliation with other *Hepatozoon* species (Baneth and Shkap 2003; Kubo et al. 2010; Hodžić et al. 2017).

Phylogenetic analysis demonstrated that the *H. procyonis* sequence obtained in this study grouped in the same clade as the *Hepatozoon* sp. sequences isolated from *N. nasua* in the Brazilian Pantanal (Sousa et al. 2017a). Therefore, the *Hepatozoon* sp. detected in the Pantanal is probably *H. procyonis*, which suggests that this parasite appears to be common among coatis from several regions of Brazil.

Hepatozoon procyonis was detected in two (Botucatu and São Paulo) out of the three studied regions. No *H. procyonis* infection was observed in coatis from Palmital. This was probably because the animals lived restricted to the park and did not move between the urban and wild environments, consequently being less exposed to vectors. Moreover, the coatis from Palmital were fed daily and exposed to good nutritional conditions, suggesting robust immune systems for the animals. Our results are in agreement with Calegaro-Marques and Amato (2014) that stated the influence of the degree of urbanization on helminthic richness, abundance, and community structure of *Turdus rufiventris*. These authors reported that urbanization disrupts host-parasite interactions, and a higher environmental diversity allows the survival of intermediate hosts, vectors, and parasites (Calegaro-Marques and Amato 2014). Additionally, the prevalence of vectors like fleas and ticks infesting dogs is frequently higher in rural areas than in urban areas (Abarca et al. 2016).

Usually, the *Hepatozoon*-infected animals showed low parasitemia (Mundim et al. 2008). In this study, the parasitemia mean of *H. procyonis* was relatively low (0.4%), which indicates that the coatis evaluated were probably in the chronic stage of infection. In addition, no statistical difference was observed in the parasitemia with age or sex of animals. However, new studies using a higher number of animals are needed to assess this association.

The prevalence of hemoparasites was suggested to be affected by several factors, such as the host's sex and age (Aktas and Ozubek 2017). In our study, no significant difference was observed between a positive *H. procyonis* infection and the sex or age of the host. This information corroborates previous studies (Baneth et al. 2013; Cardoso et al. 2014; Aktas and Ozubek 2017) and suggests that coatis were infected when young. No association was observed between the *H. canis* infection in red foxes (*Vulpes vulpes*) and the sex or age of the animals, and the authors suggested that the foxes were infected by a vector or by transplacental transmission when young (Cardoso et al. 2014). However, the possibility of transplacental transmission for *H. procyonis* infection has not yet been studied.

In the present study, five tick species were detected on *N. nasua* (*A. dubitatum*, *A. ovale*, *A. sculptum*, *Amblyomma* sp., and *R. sanguineus* s. l.). We further identified, for the first time, nymphs of *A. dubitatum* infesting *N. nasua*. The other stages and species of ticks were previously detected on coatis in studies performed in Brazil (Dantas-Torres et al. 2010; Martins et al. 2017; Sousa et al. 2017a). However, the *H. procyonis* vector remains unknown. We failed to detect *Hepatozoon* oocysts in ticks. One explanation for not detecting the oocysts in the ticks might be the fact that the development of *Hepatozoon* oocysts takes about 30 days in *Rhipicephalus turanicus* and ~ 14 days in *A. ovale* (Giannelli et al. 2017). Probably, there was not enough time for the *Hepatozoon* to develop further and mature oocysts in the freshly taken ticks from the positive hosts. *Amblyomma ovale* was among the most frequent tick species collected from *N. nasua* in this study, and most of the *H. procyonis* positive animals were also found to be infested by *A. ovale*. Interestingly, *A. ovale* has been considered a potential vector for other *Hepatozoon* species (Forlano et al. 2005; Rubini et al. 2009; Demoner et al. 2013). These observations suggest that *A. ovale* could act as vector for *H. procyonis*, but further studies are required to confirm this hypothesis.

Conclusion

This study provides epidemiological data on hemoparasite infection in *N. nasua* from three distinct regions of Brazil. Furthermore, we characterized *H. procyonis* morphologically, morphometrically, and molecularly, and redescribed this species. Further studies are required to elucidate the life cycle of *H. procyonis* and its vectors.

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Compliance with ethical standards

All procedures used in this study were approved by the local ethics committee on animal use (protocol no.: 561-CEUA) and authorized by the

Brazilian Institute of Environment and Renewable Natural Resources (IBAMA; SISBIO authorization no.: 42263-2).

Conflict of interest The authors declare that they have no conflict of interest.

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