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Occurrence of *Hepatozoon* in Some Reptiles from Brazilian Biomes with Molecular and Morphological Characterization of *Hepatozoon caimani*

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Abstract: Amphibians and reptiles represent a considerable proportion of the vertebrate fauna in Brazil. Different blood parasitic infections have been reported in these groups, such as *Haemogregarina*, *Hepatozoon*, *Trypanosoma* and microfilariae. However, insufficient research on interactions between these parasites and their hosts has been carried out in some regions of the country. Samples were collected from populations of wild herpetofauna in different microhabitats throughout Brazil, totaling 111 samples of reptiles from the states of Mato Grosso and Pernambuco. We used an integrative approach, with classical microscopy, morphometry and molecular analysis, in order to identify hemoparasites present in the analyzed fauna. Genomic DNA was extracted for the PCR protocol based on the *18S* ribosomal RNA gene for *Hepatozoon* spp. A total of 53 positives were obtained with molecular screening (47.7%), all confirmed as *Hepatozoon* spp. using DNA sequencing. Among positive samples, 23 slides were examined, confirming the presence of *Hepatozoon* spp. in 91.3% of the smears. The phylogenetic analysis performed with sequences from 43 samples resulted in a tree containing several distinct clades. Sequences were generally grouped according to the taxonomic order of the host. Co-infections with microfilariae and *Trypanosoma* spp. were also found in microscopy analyses. This study describes the presence of *Hepatozoon caimani* in a new host species (*Paleosuchus palpebrosus*) that can be a paratenic host in the natural environment. The existence of parasitic co-infections in alligator species underscores the significance of recognizing the impact of infections by various parasitic taxa on the host populations.

Keywords: *Hepatozoon*; parasite diversity; phylogenetic diversity

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1. Introduction

Hepatozoon Miller 1908 is a protozoan genus of the Phylum Apicomplexa, which is inserted in the Suborder Adeleorina. It is the only genus in the Hepatozoidae family, and it encompasses the most common parasites in reptiles and invertebrate hosts, across a wide geographic distribution [1]. It has now been found infecting all living orders of reptiles,

and more than 200 species belonging to this parasite genus have been described in these vertebrate hosts worldwide [1–3].

Hepatozoon parasites are obligately heteroxenous, with gametocytes that can be found in the red blood cells or leukocytes of the vertebrate intermediate host [2,4] and several hematophagous arthropods serving as definitive hosts, including ticks, sandflies, culicine and anopheline mosquitoes, tsetse flies and others. After the blood meal, the gametocytes undergo syzygy and gametogenesis, and sporogonic development in the arthropod results in the formation of large polysporic oocysts (characteristic of the genus), usually with thick walls, which are found in the gut wall or in the arthropod hemocoel. Transmission occurs when the infected arthropod is ingested by the vertebrate host (which may also be predated by another vertebrate host) [2,4–6]. In vertebrate hosts, the infection is found in blood smears visualizing intracellular gametocytes with the nucleus of the host cell displaced to a lateral or polar position. With a single mature gametocyte, erythrocytes are rarely enlarged, but erythrocytes containing two or even three parasites undergo some deformation or enlargement [5].

Hepatozoonosis has been extensively studied in domesticated animals, especially in canids and felids, for which moderate signs and symptoms have been described. However, liver, lung, kidney and spleen tissues can be damaged during the parasite's asexual reproduction, and hemolytic anemia is also described in severe cases [7,8]. Symptoms or negative effects caused by an infection with *Hepatozoon* in wild vertebrate hosts remain largely unknown, either because the free-living individual host is not monitored after diagnostics or because the synergetic effect of co-infection with other parasitic and pathogenic organisms (e.g., bacterial, viruses, worms) constrains a precise effect of each pathogen.

Infections by different species of parasites of this genus have been frequently reported in hosts such as Anura, Squamata (mainly snakes) and Crocodylia [2,9–11]. In 1932, Hoare described the sporogony of *H. pettiti* in tsetse flies which fed on a Nile crocodile (*Crocodylus niloticus*). In Brazil, there are studies showing *Hepatozoon* infections in crocodilians in the Pantanal and Atlantic Forest regions, in frogs in the São Paulo State, in snakes and lizards associated with high tick infestation in the state of Bahia and in frogs in the state of Mato Grosso [1–3,5,6,12–17].

However, considering the vast territory of Brazil and the extreme diversity of ecoregions and their associated fauna, immense geographic and taxonomic gaps exist in the scientific knowledge about *Hepatozoon* parasites in the country, with relatively few regions and potential hosts sampled so far. Hence, the aim of this study was to assess the occurrence and genetic diversity of *Hepatozoon* in reptile communities recently sampled at different microhabitats, in four Brazilian biomes. In addition, we characterized the *Hepatozoon* species detected using a combination of morphological and molecular analyses.

2. Materials and Methods

2.1. Sampling

Hepatozoon infections were investigated in blood samples of 111 specimens of free-living reptiles collected in four biomes and transition areas in the Brazilian states of Mato Grosso (67 samples) and Pernambuco (44 samples) (Figure 1). Specimens belonged to nine species, grouped in six families and three orders: Alligatoridae (59.45%), Dipsadidae (1.80%), Kinosternidae (2.70%), Polychrotidae (0.90%), Teiidae (14.41%) and Tropiduridae (20.72%). These were collected in habitats within the biomes of Amazonia, Caatinga, Pantanal and Cerrado (Brazilian Savanna) (Figure 1).

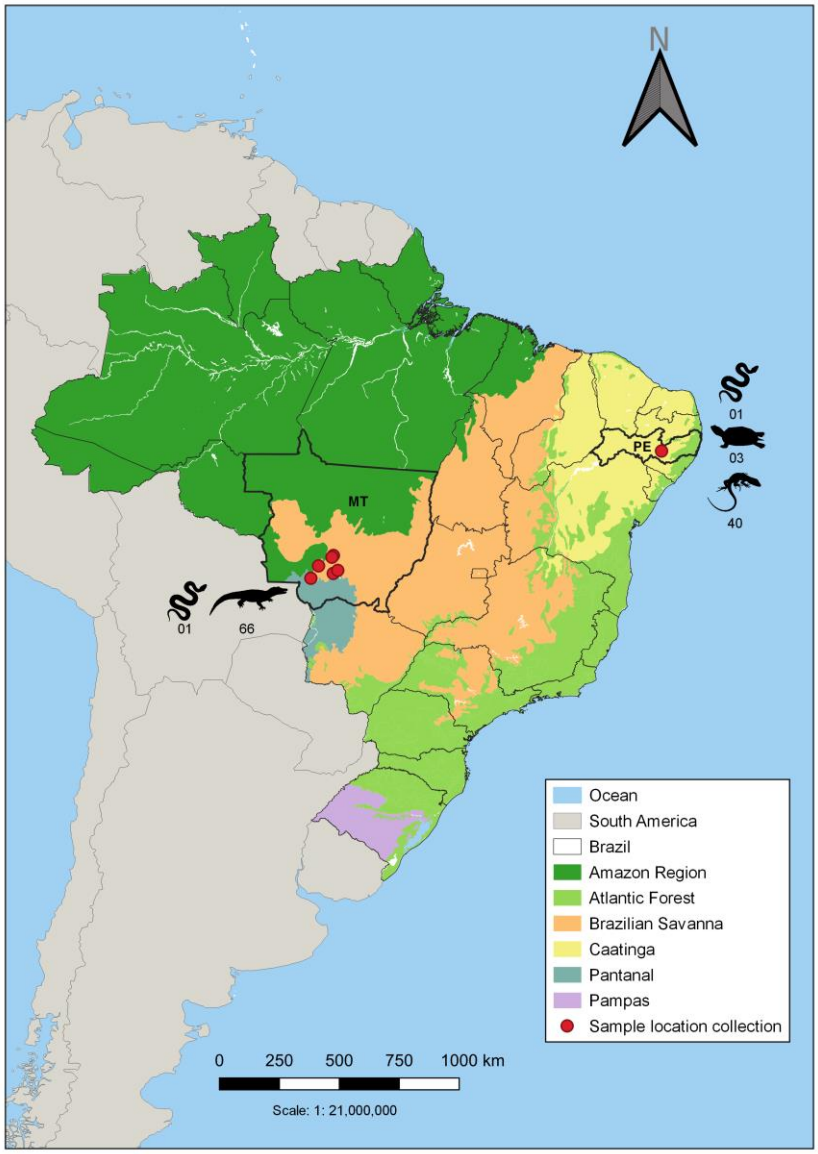

Figure 1. Spatial distribution of the collection sites sampled in this study.

From each individual, approximately 10 µL of blood were taken from the caudal vein, collected in heparinized microcapillary tubes and stored on ETOH 95%, immediately after the procedure. Thin blood smears were also prepared, fixed with 100% methanol and stained with a 10% Giemsa solution for 1 h.

All blood samples and reptiles were collected and handled under appropriate permits in Brazil. This project was approved by the Chico Mendes Institute for Biodiversity Conservation (license number 69767-2, 76058-1) and the Ethics in Use Committee of Animals of the Superintendência de Controle de Endemias (CEUA-SUCEN) number 0005/2020, approved on 28 April 2021.

2.2. Morphologic Detection of Parasites

Diagnosis of parasites was performed with light microscopy at 10×, 40× and 100× magnification using ZEISS® Axio Lab.A1 microscopes with integrated ZEISS® Axiocam 305 color camera (ZEISS®, Jena, Germany) and Leica light microscope® DM3000 LED (Leica Microsystems, Wetzlar, Germany). Slides identified as positive for blood parasites were examined in full to obtain photographs in the ZEISS® Axio Lab.A1 programs with integrated ZEISS® Axiocam 305 color camera (ZEISS®, Jena, Germany). Morphometric measurements of the parasites were taken in the ImageJ v 1.54f software [18]. Morphological

determination of the blood parasites and the morphometric measurements were taken following the original descriptions and the diagnostic keys for reptile hosts [1,19].

2.3. Molecular Detection of Parasites

Total genomic DNA was extracted from blood samples using the Wizard® Genomic DNA Purification Kit (PROMEGA®, Madison, WI, USA), following the manufacturer's instructions for whole blood matrixes. Lysates were transferred to columns and washed according to the manufacturer's instructions. Polymerase chain reactions (PCR) were conducted using a protocol targeting the *18S* ribosomal RNA gene (*SSU*, small subunit ribosomal RNA gene) of *Hepatozoon* species [20], with the primers HepF300/HepR900 and 50 ng of genomic DNA. In each PCR, positive controls were carried out in parallel containing *Hepatozoon* DNA, and ultrapure water was served as a negative control. PCR products were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit in ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

2.4. Phylogenetic Analysis

Phylogenetic relationship among reported parasites was inferred using partial *18S* ribosomal RNA gene sequences (~600 bp). GenBank accessions for sequences used in phylogenetic reconstruction are indicated in phylogenetic trees. Phylogenetic reconstructions were performed using Bayesian inference, as implemented in MrBayes v3.2.0 [21]. The evolutionary model GTR + G was used after a model selection analysis conducted in MEGAX [22]. Bayesian inference was executed with two Markov Chain Monte Carlo searches of 3 million generations, with each sampling 1 of 300 trees. After removing a burn-in of 25%, the remaining trees were used to calculate the 50% majority-rule consensus tree. The resulting phylogenetic tree was visualized using FigTree version 1.4.0 [23].

Estimates of evolutionary divergence over sequence pairs between groups and within groups were conducted using the Kimura 2-parameter model [24]. This analysis involved 54 nucleotide sequences. There were a total of 571 positions in the final dataset. Evolutionary analyses were conducted in MEGA X [22].

2.5. Statistical Analysis

The 95% confidence intervals (CI) of the mean were calculated using RStudio (v. 2023.09.1+494). The reported means in other studies that fall within that CI were considered not significantly different.

3. Results

Among all reptile blood samples evaluated, *Hepatozoon* infections were found only in samples belonging to the caiman's family (Alligatoridae), which comprised 59.4% of the specimens sampled (Table 1). The overall positivity of *Hepatozoon* in the Alligatoridae family was 47.7%, with higher positivity in *Caiman yacare* (93.6%) than in *Paleosuchus palpebrosus* (52.9%).

In some species, such as in Kinosternidae turtles, Dipsadidae snakes and Polychrotidae lizards, the number of samples analyzed was too low to accurately determine *Hepatozoon* occurrence. However, even in species with a reasonable number of specimens sampled, as among the ground dwelling lizards *Ameivula ocellifera* and *Tropidurus cocorobensis,* the positivity was 0% (Table 1).

Caiman yacare, the yacare caiman, also known commonly as the jacare caiman, Paraguayan caiman, piranha caiman, red caiman and southern spectacled caiman, represented 83% of all positive individuals; however, it represents 42.3% of the samples collected.

Table 1. Reptile species sampled in this study; numbers in parentheses represent the samples positive for *Hepatozoon* sp. and *Hepatozoon caimani* parasites.

Order Family	Host Species	PCR Samples (Infected)	Microscopy Samples (Infected)	Biome * (Number of *Hepatozoon* Occurrences)
Crocodylia				
Alligatoridae	*Caiman crocodilus*	2	2	BS (0)
	Caiman yacare	47 (43)	21 (19)	AM (5), BS (20), PA (18)
	Paleosuchus palpebrosus	17 (9)	10 (3)	AM (1), BS (4), PA (4)
Chelonia				
Kinosternidae	*Kinosternon scorpioides*	3	3	CA (0)
Squamata				
Dipsadidae	*Erythrolamprus poecilogyrus*	1	0	BS (0)
	Oxyrhopus trigeminus	1	1	CA (0)
Polychrotidae	*Polychrus acutirostris*	1	1	CA (0)
Teiidae	*Ameivula ocellifera*	16	6	CA (0)
Tropiduridae	*Tropidurus cocorobensis*	23	9	CA (0)
Total		**111 (52)**	**53 (22)**	

* As the collected municipalities are in transition areas, we consider the main biome that occurs in the collection site. AM = Amazonia; CA = Caatinga; BS = Brazilian Savanna; PA = Pantanal.

In total, 53 thin blood smears were collected from eight reptile species (Table 1). *Hepatozoon* parasites were identified with microscopy in 22 smears, all belonging to *Caiman yacare* (n = 19) or *Paleosuchus palpebrosus* (n = 3). Figure 2A shows representative micrography of the *Hepatozoon* parasites present in *P. palpebrosus.* Additionally, we found co-infections of *Hepatozoon* with microfilariae (n = 10) (Figure 2B) and *Trypanosoma* spp. (n = 7) (Figure 2C) in *Caiman yacare* specimens.

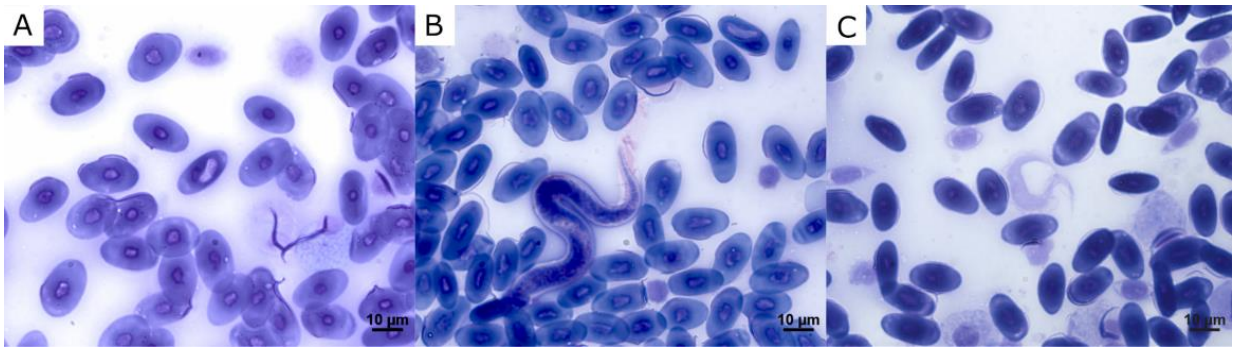

Figure 2. (**A**) Micrography of the *Hepatozoon* parasites present in *Paleosuchus palpebrosus.* (**B**) Microfilariae in *Caiman yacare*. (**C**) *Trypanosoma* sp. in *Caiman yacare*. Giemsa-stained thin blood films. Scale bar: 10 µm.

Hepatozoon caimani (identified by the free stages and intraerythrocytic gamonts and the presence of all the main features of this species) was confirmed with microscopy in sample ID 179 from *Caiman yacare* collected in Nossa Senhora do Livramento, Mato Grosso, Pantanal (Figure 3).

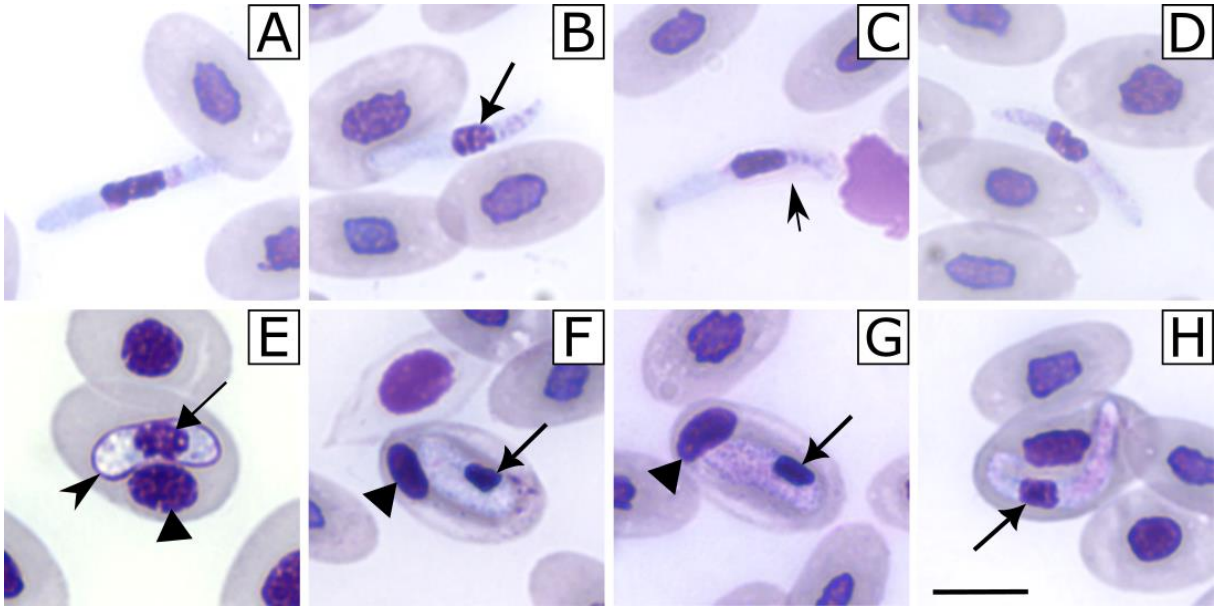

Figure 3. *Hepatozoon caimani* from *Caiman yacare* (ID 179). (**A**–**D**) Free stages. (**E**) Trophozoite. (**F**–**H**) Intraerythrocytic gamonts. Note the presence of an elongated body and a slightly thinner posterior end, where the apical ring is formed, which is a feature of the species in the free forms (**A**,**B**). Long curved arrows: parasite nuclei; long straight arrow: reticulated nucleus; simple arrowhead: conspicuous "capsule-like" structure; triangle arrowheads: host cell nuclei; short arrow: possible gamont emerging from a "capsule-like" structure. Giemsa-stained thin blood films. Scale bar: 10 µm.

The morphometric data of *Hepatozoon* sp. from this study (ID 179) and the comparison with the morphometric data from *Hepatozoon caimani* obtained from two other studies are shown in Table 2. CIs were calculated with the data reported in this study (Appendix A, Table A2) to determine whether there are significant differences with the morphometric measures reported in the previous studies for *H. caimani.* Although there are some significant differences in the measurements (Appendix A, Table A2), the morphological features demonstrate that this parasite is indeed *H. caimani*, as previously reported in the studies that were used in the comparison.

Table 2. Morphometric data of free stages and intraerythrocytic gamonts of *Hepatozoon* sp. from this study (ID 179) and *Hepatozoon caimani* obtained from two other studies.

	***Hepatozoon* (This Study)**		***H. caimani***	
			Soares et al. 2017 [25]	**Bouer et al. 2017 [3]**
Free stages (N = 50)				
		(Min–Max) values		(Min–Max) values
Area	55.319 ± 7.010 (μm^2)	(45.066–77.258)	50.689 ± 7.159 (μm^2)	(31.274–77.127)
Length	21.277 ± 1.429	(18.586–25.301)	23.891 ± 3.978	(14.674–34.183)
Width	2.974 ± 0.264	(2.298–3.556)	2.809 ± 0.650	(1.251–6.292)
Nucleus area	14.971 ± 2.832 (μm^2)	(12.079–24.005)	18.002 ± 3.917 (μm^2)	(10.254–31.229)
Nucleus length	6.040 ± 1.053	(4.739–9.215)	7.027 ± 1.627	(2.806–13.225)
Nucleus width	2.974 ± 0.264	(2.298–3.556)	2.752 ± 0.721	(1.073–6.455)

Table 2. *Cont.*

	***Hepatozoon* (This Study)**		***H. caimani***		
			Soares et al. 2017 [25]		**Bouer et al. 2017 [3]**
Intraerythrocytic gamonts (N = 50)					
		(Min–Max) values		(Min–Max) values	
Area	55.731 ± 10.123 (μm^2)	(33.456–78.023)	29.922 ± 4.588 (μm^2)	(22.585–52.082)	53.2 ± 14.6 (μm^2)
Length	12.971 ± 1.962	(10.197–21.143)	12.449 ± 1.797	(8.860–22.274)	12.9 ± 1.6
Width	5.152 ± 0.925	(3.355–9.953)	4.111 ± 1.001	(2.200–8.318)	4.81 ± 1.1
Nucleus area	13.951 ± 4.291 (μm^2)	(7.641–24.381)	16.150 ± 3.445 (μm^2)	(10.518–32.453)	13.1 ± 4.71 (μm^2)
Nucleus length	5.301 ± 1.222	(3.456–8.261)	5.895 ± 1.674	(2.410–12.873)	5.67 ± 1.5
Nucleus width	3.393 ± 0.797	(1.989–5.767)	3.080 ± 1.038	(1.403–6.025)	2.73 ± 0.88

Regarding the molecular analysis, a total of 52 PCR-positive individuals (43 samples from *Caiman yacare* and 9 samples from *Paleosuchus palpebrosus*) were found among 111 tested samples (Table 3). Of these, 43 sequences were used for further analysis. These sequences presented good criteria for use in the construction of the phylogenetic tree. The other obtained sequences did not show clear electropherogram (without double peaks), probably due to mixed infections, and were removed.

Table 3. Nucleic acid polymorphism in partial *18S* ribosomal RNA gene sequences (HepF300/HepR900) of *Hepatozoon* sp. isolates from Brazil (179-346) and references from GenBank (KJ413113, MF435046-MF435048, MF322538, MF322539, OM105596).

		Single Nucleotide Polymorphisms (SNPs)																					
Haplotype		9 8	2 7 9	3 1 5	3 1 9	3 4 0	3 5 3	3 5 4	3 5 7	3 6 4	3 6 5	3 7 6	3 8 0	3 8 1	3 8 4	3 8 5	3 8 8	4 3 3	4 3 8	5 1 4	5 1 8	5 2 4
1	179/286/288/292/299/325/329/334/338/340	T	G	A	G	T	C	C	T	T	A	T	A	A	T	A	A	G	T	G	G	A
2	181/346	.	.	.	A	.	.	.	.	.	T	C	.	.	A	G	.	.	C	.	.	G
3	285/290/300/302/303/304/306/323/309/314/339	C	A	.	A	C	.	.	G	.	C	.	.	G	.	.	T	.	C	A	A	.
4	289/294	C	A	.	A	C	.	.	G	.	C	.	.	.	.	.	T	.	C	A	A	.
5	293	C	A	.	A	C	.	.	G	.	T	.	.	G	.	.	T	.	C	.	A	.
6	296/337	.	.	.	A	C	**T**	.	.	C	T	.	.	.	A	G	.	.	C	.	.	G
7	297	.	.	.	A	C	.	.	.	C	.	.	.	.	.	.	.	.	C	.	.	G
8	310/312/330/331/341/342/343	C	A	.	A	C	.	.	G	.	C	.	.	G	.	.	T	.	C	.	A	.
9	322	.	.	.	A	C	.	.	.	C	.	.	.	.	A	.	.	.	C	.	.	G
10	326/344	C	A	**T**	A	C	.	**T**	G	.	C	.	.	G	.	.	T	.	C	.	A	.
11	332	.	.	.	A	C	.	.	.	C	.	.	T	.	A	G	.	.	C	.	.	G
12	333	.	.	.	A	C	.	.	.	C	.	.	.	.	A	.	.	.	C	A	A	G
13	335	C	A	.	A	C	.	.	G	.	C	.	.	.	.	.	T	.	C	.	A	.
14	345	C	A	.	A	C	.	.	G	.	T	.	.	.	.	.	T	.	C	.	A	.
1	KJ413113 *Hepatozoon* sp.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
3	MF435046/MF435047 *H. caimani*	C	A	.	A	C	.	.	G	.	C	.	.	G	.	.	T	.	C	A	A	.
11	MF322538/MF322539/MF435048 *H. caimani*	.	.	.	A	C	.	.	.	C	.	.	T	.	A	G	.	.	C	.	.	G
15	OM105596 *Hepatozoon* sp.	.	.	.	A	.	.	.	.	.	T	C	.	.	A	G	.	**A**	C	-	-	-

Sequences reported during this study obtained from animals collected in Brazilian Savanna (blue), Amazonia (green) and Pantanal (red) are shown (Appendix A, Table A1). Reference sequences are from Pantanal (KJ413113, MF322538 and MF322539), Amazonia (OM105596) and Brazilian Savanna (MF435046, MF435047 and MF435048). Singleton sites (where only one of the haplotypes has a distinct nucleotide, whereas all others are the same) are shown in bold.

Twenty-one single nucleotide polymorphisms (SNPs) were obtained in fourteen different *Hepatozoon* haplotypes, with only four haplotypes having been described previously (Table 3). One haplotype (H1) was found in ten samples, including *Caiman yacare* and *Paleosuchus palpebrosus* hosts, belonging to three different populations from two biomes (Pantanal and Brazilian Savanna). Our GenBank survey indicated that it has been previously detected (GenBank KJ413113) but not yet linked to any *Hepatozoon* morphospecies. Sample ID 179, which was morphometrically diagnosed as *Hepatozoon caimani*, had the H1 haplotype. Other haplotypes also frequently found were H3 and H8, with 11 and 7 samples, respectively (Table 3). H3 was already described as belonging to *H. caimani* (MF435046 and MF435047) based on the samples collected in other areas of the Brazilian Savanna.

PCR data indicated that *Hepatozoon* positivity was highest in Amazonia transition areas (6, 100%), with the two haplotypes present among samples originating from this region. The highest diversity of *Hepatozoon* haplotypes (11 haplotypes) was identified in the samples collected in transition areas located in the Brazilian Savanna, and 7 haplotypes were unique to this region. One of these unique haplotypes identified in the Brazilian Savanna (H11) was previously reported for *H. caimani* from Pantanal (MF322538 and MF322539). In the Pantanal areas, we found 22 positives (91.7%) and recorded 6 different haplotypes (Table 3), with 3 haplotypes detected exclusively on these sites.

The final alignment used for the phylogenetic analysis combined the 14 detected *Hepatozoon* haplotypes from this study with previously recorded *Hepatozoon caimani* haplotypes. We also included sequences in the phylogenetic tree obtained from the GenBank database that were either closely matched to sequences from this study or represented *Hepatozoon* species described in reptiles, amphibians, fishes and small mammals. The Bayesian analysis (average standard deviation of split frequencies: 0.004186) resulted in a tree containing several distinct clades (Figure 4).

Hepatozoon sequences found in caiman samples were split in two clades. The first contained one haplotype (sequences 181 and 346) and the GenBank sequence OM105596 obtained from a fish specimen. The second was formed by three main subclades: A (332, MF322529, MF435048), B (MF435046, MF435047 and 25 sequences) and C (KJ413113 and 10 sequences, including sample ID 179 of *Hepatozoon caimani,* morphologically diagnosed in this study) (Figure 4).

Average K2P genetic distances among haplotypes obtained from crocodylian hosts were equal to or lower than 2% (Table 4). Estimates were relatively higher among sequences obtained from snake or lizard hosts (4%). Higher estimates of average genetic distances were observed among the comparisons of *Hepatozoon* sequences obtained from amphibian hosts in relation to reptile hosts (5–7%). Genetic distances among *Adelina* spp. and *Hepatozoon* spp. exceeded 10% in all comparisons.

Table 4. Average genetic distances (Kimura-2_parameter distances) over sequence pairs between groups (below the diagonal) and within groups (along the diagonal).

	Amphibia	**Crocodylia 1 ***	**Crocodylia 2A ***	**Crocodylia 2B ***	**Crocodylia 2C ***	**Squamata**	**Squamata (Ophidia) Brazilian**	**Testudines**	**Outgroup *Adelina* spp.**
Amphibia	0.02								
Crocodylia 1 *	0.06	0.00							
Crocodylia 2A *	0.06	0.01	0.00						
Crocodylia 2B *	0.07	0.02	0.02	0.00					
Crocodylia 2C *	0.06	0.01	0.01	0.02	0.00				
Squamata	0.06	0.03	0.04	0.04	0.04	0.04			
Squamata (Ophidia) Brazilian	0.05	0.03	0.03	0.04	0.03	0.04	0.00		
Testudines	0.05	0.03	0.03	0.04	0.03	0.04	0.02	0.00	
Outgroup *Adelina* spp.	0.15	0.12	0.13	0.13	0.12	0.13	0.11	0.11	0.04

* According to the phylogenetic tree (Figure 4). Crocodylia 1 (PP95): 181, 346, OM105596; Crocodylia 2A (PP97): 332, MF322539, MF435048; Crocodylia 2B (PP100): 335, 293, 289, 294, 285, 290, 300, 302, 303, 304, 306, 323, 309, 314, 339, MF435046, MF435047, 326, 344, 310, 312, 330, 331, 341, 342, 343, 345; Crocodylia 2C (PP100): 179, 286, 288, 292, 299, 325, 329, 334, 338, 340, KJ413113. PP = posterior probabilities.

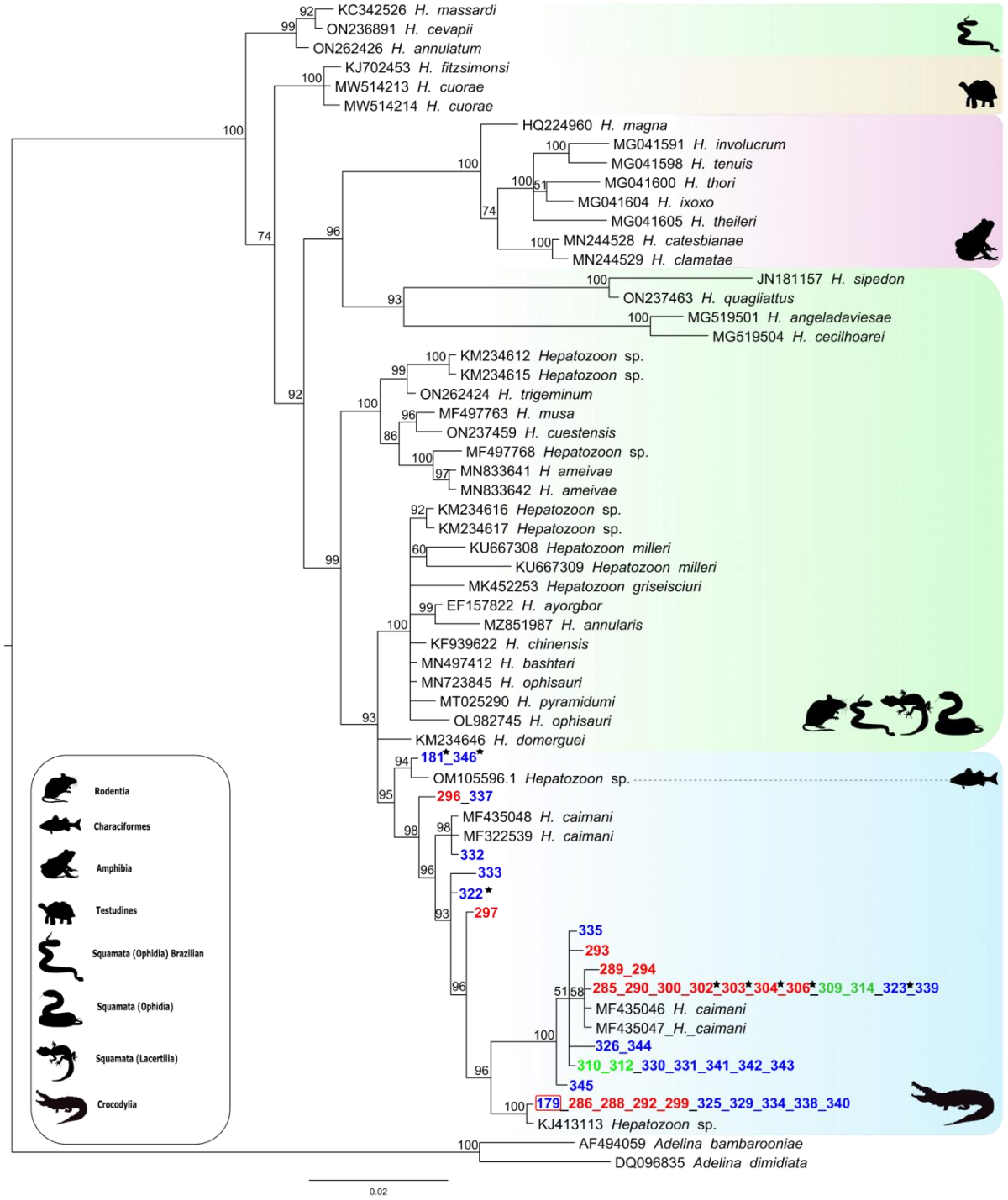

Figure 4. Bayesian phylogeny based on the partial *18S* ribosomal RNA sequences of *Hepatozoon* species (580 bp). *Adelina bambarooniae* and *Adelina dimidiata* were used as outgroup. Support values on nodes (in percentage) indicate posterior probabilities (PP). Green shading indicates sequences obtained from Squamata species; blue shading indicates sequences obtained from Crocodylia species (except for sequence OM105596 obtained in fish); pink shading indicates sequences obtained from Amphibia species; yellow shading indicates sequences obtained from Testudines species. Sequences obtained in this study are indicated in different font colors: Brazilian Savanna (blue), Amazonia (green) and Pantanal (red). *Hepatozoon* sequences obtained from *Paleosuchus palpebrosus* specimens are indicated with a star. The sequence of *Hepatozoon caimani* with morphometric data described in this study is highlighted in a red rectangle.

4. Discussion

In this study, we used an integrative approach using optic microscopy, morphometry, and molecular analyze in order to investigate the presence of *Hepatozoon* spp. in free-living reptiles sampled in four Brazilian biomes (Caatinga, Amazonia, Brazilian Savanna and Pantanal) and transition areas. *Hepatozoon* positivity was quite variable among different biomes: 0% in Caatinga, 64.9% in the Brazilian Savanna, 91.7% in Pantanal and 100% in Amazonia. Although it is not possible to collect samples from the same host species in all biomes to make a comparison among biomes, given the irregular distribution in the country of the host species studied, our results confirm the low occurrence of *Hepatozoon* in the Caatinga, which was previously reported by two other studies [26,27].

Using microscopy, only one positive sample (from Ceará state) has been found in fifteen *Tropidurus hispidus* (from Ceará and Pernambuco states) and no infected individual was found among fifteen *Ameivula ocellifera* (from Ceará state) [26]. In our study, with a molecular approach, no positives were found in the 39 individuals that were analyzed (23 *Tropidurus cocorobensis* and 16 *Ameivula ocellifera*). In addition, with molecular screening, 230 geckos from different states from all Brazilian regions, including the endemic species (*Hemidactylus agrius*, *H. brasilianus*, *Lygodactylus klugei*, *Phyllopezus pollicaris*, *P. periosus*) and an exotic species (*H. mabouia*) were analyzed [27]. An overall low prevalence of the *Hepatozoon* infection (3%) was found, with three host species infected (*H. mabouia*, *P. pollicaris* and *P. periosus*) being collected from Rio Grande do Norte, Ceará, Pernambuco and Paraíba [27]. Therefore, both previous studies have shown that *Hepatozoon* can infect reptiles in Caatinga, but with extremely low prevalence.

There is a significant difference between the morphometric values of the area in the intraerythrocytic stages reported in our study and the study by Bouer et al. [3] in comparison to the value reported in Soares et al. [25]. In the latter study, the reported value of 29.92 ± 4.588 does not correspond to the value that would be obtained if we multiply the length by width to determine the parasite area; this may be the result of a typing error. The values reported (Table 1) for the other morphometric measurements (length, width and parasite core measurements) in the three studies do not have a statistically significant difference. Unfortunately, in Soares et al. [25], the procedure for taking measurements or the number of parasites measured to calculate the mean values was not reported. This lack of information makes it difficult to determine if the values obtained are affected by any missing data; therefore, we cannot be certain if the results are unbiased.

Despite these differences, we are convinced that the morphological characteristics (see the Section 3) present in the parasites examined are consistent with *H. caimani* [3,5,25,28]. The difference in measurements may be because the process of the morphometric measurement is subjective to each researcher and also to the tools used for such a measurement (microscopy, camera, image analysis software). However, the morphological characteristics of the parasites present in our study and those used for the comparison concur with the previous descriptions made by other authors [5,28] as well as the original description [29]. The problem of substantial differences recorded within the parasite measurements by various authors has already been highlighted in other research related to parasites of the genus *Hepatozoon* [30]. Thus, it is necessary to consider the fact that there is no standardized methodology on how morphometric measurements should be performed on these parasites, resulting in conclusions that could end up in synonymies or misinterpretations of characteristics that may even be artifacts when preparing blood smears [30].

In relation to *Hepatozoon* occurrence in the other biomes, a lower positivity rate than that found in this study in *C. yacare* has been found in other Pantanal wetlands (71–79%) [8, 25,28] and in *C. crocodilus* in the Amazon region (76.7%) [31]. Therefore, we recorded the highest infection rates in Crocodylia species.

As mentioned in a previous work [25], the likely reason for the high prevalence of this parasite is attributed to the aggregate distribution behavior observed in alligator populations [32,33]. The host's population density facilitates a frequent contact between individuals, leading to a proportional linear increase in the transmission rate [34–36]. For

this reason, we believe that the tendency over time is to find increasingly higher positivity rates in these areas. Another explanation for the high prevalence of *Hepatozoon* in alligator populations may be the association between diet and transmission through paratenic vertebrate hosts [8], where the intermediate host (alligator) feeds on paratenic hosts (fishes, frogs, snakes and smaller alligators) containing parasite stages which do not develop [37].

Interestingly, *Hepatozoon* positivity per biome seems to be associated with each biome's forest cover. In biomes with the largest forest cover, such as the Amazon (78.74% in 2021 [38]), we observed the highest rates of positive tests for the presence of *Hepatozoon* (100%), while in the Caatinga, the biome with the lowest forest cover (8.23% in 2021 [38]), we did not observe any positive results. Biomes with a forest coverage of 14.46% (Pantanal) and 20.35% (Brazilian Savanna) showed positive rates of 91.7% and 64.9%, respectively. The increased number of positives found in the Pantanal may be a consequence of the higher presence of water bodies, which also favor (beyond the forest areas) the reproduction of culicine and anopheline mosquitoes, common vectors of *Hepatozoon* parasites [1].

The sensitivity of detection was higher in PCR (83%) than in blood smear examinations (71%) of free-ranging alligators, which agrees with the findings from previous studies involving the molecular detection of *Hepatozoon* spp. in reptiles [20,39]. It should be noted that we found the same positivity percentages in the Pantanal region for PCR and microscopy as those found by Bouer et al. [3].

It is possible that all the *Hepatozoon* sequences found in this study can belong to *Hepatozoon caimani* since they were grouped in the same clade along with different reference sequences reported for this species (MF435046, MF435047, MF435048 and MF322539). To determine if the all the haplotypes (including those most distant in the phylogenetic tree as 181, 346, 296 and 337) also belonged to *Hepatozoon caimani*, estimates of evolutionary divergence over sequence pairs between groups and within groups were conducted. The results showed distances among sequences from Crocodylia groups always being below 2%. Squamata (Ophidia and Lacertilia) group sequences presented a higher mean intraspecific distance, probably reflecting the great diversity of hosts grouped in this cluster. Moreover, all the sequences grouped in the Crocodylia clade presented >99% of identity with *Hepatozoon caimani* sequences from the GenBank database.

Six *Hepatozoon* species were reported as parasites of crocodylians: *H. crocodilinorum*, *H. hankini*, *H. sheppardi*, *H. petiti*, *H. serrei* and *H. caimani* [2], but only the last two species were reported parasitizing Brazilian crocodilians. *Hepatozoon serrei* was reported in *Paleosuchus trigonatus* [2], an Amazon alligator, found in small streams in the interior of the forest. *Hepatozoon caimani* was found described as the only *Hepatozoon* species parasitizing *Caiman latirostris* [40], *Caiman crocodilus* [5,31] and *Caiman yacare* [8,25,28,40] in South America. We are the first to report this parasite species infecting *Paleosuchus palpebrosus*, a host outside the genus *Caiman*. Thus, this lead us to confirm the limited host specificity of the *Hepatozoon* species [41]; however, the switching of *Hepatozoon caimani* between different host species does not seem to be that often. Future studies on the prevalence of this species in other alligator species are necessary.

This study represents the largest sampling of *Hepatozoon* sequences from crocodilians. Eleven new haplotypes of *Hepatozoon caimani* sequences were detected in this study, while four were already detected in alligator species from Brazil. Similar to the report on fish [42], the presence of this *Hepatozoon* species in *Paleosuchus palpebrosus* confirms the circulation of these parasites in vertebrates different from the *Caiman* species. The finding of gamonts on the blood smears of three *Paleosuchus palpebrosus* individuals confirms that these hosts are not paratenic hosts in the natural environment, although they can serve as prey for larger crocodiles [43,44]. This aspect plays a crucial role in the intricate life cycle of these parasites and warrants a further investigation.

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Institutional Review Board Statement: This project was approved by the Ethics in Use Committee of Animals—CEUA of the Institute of Tropical Medicine—USP (approval number 2019/000412A and date of approval 16 August 2019).

Data Availability Statement: The data presented in this study are available in Appendix A and in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) (accession numbers OR510629-OR510671).

Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. *Hepatozoon* parasite sequences identified in this study.

Isolate	Host Species	Parasite Species	#GenBank®	Locality	Biome
179	*Caiman yacare*	*Hepatozoon caimani*	OR510629	Nossa Senhora do Livramento—MT	Pantanal
181	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510639	Nobre—MT	Brazilian Savanna
285	*Caiman yacare*	*Hepatozoon* sp.	OR510661	Cáceres—MT	Pantanal
286	*Caiman yacare*	*Hepatozoon caimani*	OR510630	Cáceres—MT	Pantanal
288	*Caiman yacare*	*Hepatozoon caimani*	OR510631	Cáceres—MT	Pantanal
289	*Caiman yacare*	*Hepatozoon* sp.	OR510650	Cáceres—MT	Pantanal
290	*Caiman yacare*	*Hepatozoon* sp.	OR510662	Cáceres—MT	Pantanal
292	*Caiman yacare*	*Hepatozoon caimani*	OR510632	Cáceres—MT	Pantanal
293	*Caiman yacare*	*Hepatozoon* sp.	OR510649	Cáceres—MT	Pantanal
294	*Caiman yacare*	*Hepatozoon* sp.	OR510649	Cáceres—MT	Pantanal
296	*Caiman yacare*	*Hepatozoon* sp.	OR510642	Cáceres—MT	Pantanal
297	*Caiman yacare*	*Hepatozoon* sp.	OR510646	Cáceres—MT	Pantanal
299	*Caiman yacare*	*Hepatozoon caimani*	OR510633	Cáceres—MT	Pantanal
300	*Caiman yacare*	*Hepatozoon* sp.	OR510663	Cáceres—MT	Pantanal
302	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510664	Cáceres—MT	Pantanal
303	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510665	Cáceres—MT	Pantanal
304	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510666	Cáceres—MT	Pantanal
306	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510667	Cáceres—MT	Pantanal
309	*Caiman yacare*	*Hepatozoon* sp.	OR510668	Barra do Bugres—MT	Amazonia
310	*Caiman yacare*	*Hepatozoon* sp.	OR510654	Barra do Bugres—MT	Amazonia
312	*Caiman yacare*	*Hepatozoon* sp.	OR510655	Barra do Bugres—MT	Amazonia
314	*Caiman yacare*	*Hepatozoon* sp.	OR510669	Barra do Bugres—MT	Amazonia
322	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510641	Porto Estrela—MT	Brazilian Savanna
323	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510670	Cuiabá—MT	Brazilian Savanna
325	*Caiman yacare*	*Hepatozoon caimani*	OR510634	Porto Estrela—MT	Brazilian Savanna
326	*Caiman yacare*	*Hepatozoon* sp.	OR510652	Porto Estrela—MT	Brazilian Savanna

Table A1. *Cont.*

Isolate	Host Species	Parasite Species	#GenBank®	Locality	Biome
329	*Caiman yacare*	*Hepatozoon caimani*	OR510635	Porto Estrela—MT	Brazilian Savanna
330	*Caiman yacare*	*Hepatozoon* sp.	OR510656	Porto Estrela—MT	Brazilian Savanna
331	*Caiman yacare*	*Hepatozoon* sp.	OR510657	Porto Estrela—MT	Brazilian Savanna
332	*Caiman yacare*	*Hepatozoon* sp.	OR510644	Porto Estrela—MT	Brazilian Savanna
333	*Caiman yacare*	*Hepatozoon* sp.	OR510645	Porto Estrela—MT	Brazilian Savanna
334	*Caiman yacare*	*Hepatozoon caimani*	OR510636	Porto Estrela—MT	Brazilian Savanna
335	*Caiman yacare*	*Hepatozoon* sp.	OR510647	Porto Estrela—MT	Brazilian Savanna
337	*Caiman yacare*	*Hepatozoon* sp.	OR510643	Porto Estrela—MT	Brazilian Savanna
338	*Caiman yacare*	*Hepatozoon caimani*	OR510637	Porto Estrela—MT	Brazilian Savanna
339	*Caiman yacare*	*Hepatozoon* sp.	OR510671	Porto Estrela—MT	Brazilian Savanna
340	*Caiman yacare*	*Hepatozoon caimani*	OR510638	Porto Estrela—MT	Brazilian Savanna
341	*Caiman yacare*	*Hepatozoon* sp.	OR510658	Porto Estrela—MT	Brazilian Savanna
342	*Caiman yacare*	*Hepatozoon* sp.	OR510659	Porto Estrela—MT	Brazilian Savanna
343	*Caiman yacare*	*Hepatozoon* sp.	OR510660	Porto Estrela—MT	Brazilian Savanna
344	*Caiman yacare*	*Hepatozoon* sp.	OR510653	Porto Estrela—MT	Brazilian Savanna
345	*Caiman yacare*	*Hepatozoon* sp.	OR510648	Porto Estrela—MT	Brazilian Savanna
346	*Paleosuchus palpebrosus*	*Hepatozoon* sp.	OR510639	Nobre—MT	Brazilian Savanna

Table A2. Statistical analysis between *Hepatozoon caimani* reported in our study and *Hepatozoon caimani* reported by other authors in Brazil.

	Hepatozoon **(This Study)**			***Hepatozoon caimani*** **Soares et al. 2017**		***Hepatozoon caimani*** **Bouer et al. 2017 [3]**
Free stages (N = 50)						
	SD	(Min–Max) values	Confidence intervals (95%)	SD	(Min–Max) values	SD
Area	55.319 ± 7.010	(45.066–77.258)	55.319 ± 1.586	50.689 ± 7.159	(31.274–77.127)	-
Length	21.277 ± 1.429	(18.586–25.301)	21.277 ± 0.323	23.891 ± 3.978	(14.674–34.183)	-
Width	2.974 ± 0.264	(2.298–3.556)	2.974 ± 0.0597	2.809 ± 0.650	(1.251–6.292)	-
Nucleus area	14.971 ± 2.832	(12.079–24.005)	14.971 ± 0.641	18.002 ± 3.917	(10.254–31.229)	-
Nucleus length	6.040 ± 1.053	(4.739–9.215)	6.040 ± 0.238	7.027 ± 1.627	(2.806–13.225)	-
Nucleus width	2.974 ± 0.264	(2.298–3.556)	2.974 ± 0.0597	2.752 ± 0.721	(1.073–6.455)	-
Intraerythrocytic stages (N = 50)						
		(Min–Max) values			(Min–Max) values	
Area	55.73 ± 10.123	(33.456–78.023)	55.731 ± 2.806	29.92 ± 4.588	(22.585–52.082)	53.20 ± 14.6
Length	12.97 ± 1.962	(10.197–21.143)	12.971 ± 0.544	12.45 ± 1.797	(8.860–22.274)	12.90 ± 1.6
Width	5.15 ± 0.925	(3.355–9.953)	5.152 ± 0.256	4.11 ± 1.001	(2.200–8.318)	4.81 ± 1.1
Nucleus area	13.95 ± 4.291	(7.641–24.381)	13.951 ± 1.189	16.15 ± 3.445	(10.518–32.453)	13.10 ± 4.71
Nucleus length	5.30 ± 1.222	(3.456–8.261)	5.301 ± 0.339	5.90 ± 1.674	(2.410–12.873)	5.67 ± 1.5
Nucleus width	3.39 ± 0.797	(1.989–5.767)	3.393 ± 0.221	3.08 ± 1.038	(1.403–6.025)	2.73 ± 0.88

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