Original Article

Braz. J. Vet. Parasitol., Jaboticabal, v. 26, n. 3, p. 352-358, july-sept. 2017
ISSN 0103-846X (Print) / ISSN 1984-2961 (Electronic)
Doi: http://dx.doi.org/10.1590/S1984-29612017041

Hepatozoon caimani in *Caiman crocodilus yacare* (Crocodylia, Alligatoridae) from North Pantanal, Brazil

Hepatozoon caimani em *Caiman crocodilus yacare* (Crocodylia, Alligatoridae) do Norte do Pantanal, Brasil

Andréa Bouer[1]; Marcos Rogério André[1]; Luiz Ricardo Gonçalves[1]; Mayara de Cássia Luzzi[1]; Juliana Paula de Oliveira[1]; Adriana Carlos Rodrigues[1]; Alessandro de Melo Varani[1]; Vitor Fernandes Oliveira de Miranda[1]; Lívia Perles[1]; Karin Werther[1]; Rosangela Zacarias Machado[1]*

[1] Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista – UNESP, Jaboticabal, SP, Brasil

Received June 14, 2017
Accepted June 29, 2017

Abstract

Hepatozoon species are the most common intracellular hemoparasite found in reptiles. *Hepatozoon caimani*, whose vectors are *Culex* mosquitoes, has been detected in a high prevalence among caimans in Brazil by blood smears examinations. The present work aimed to detect and characterize the *Hepatozoon* spp. found in 33 caimans (24 free-ranging and 9 captive; 28 males and 5 females) (*Caiman crocodilus yacare)* sampled at Poconé, North Pantanal, state of Mato Grosso, Brazil, using blood smears examinations and molecular techniques. *Hepatozoon* spp.-gametocytes were found in 70.8% (17/24) and 88.8% (8/9) of blood smears from free-ranging and captive caimans, respectively. *Hepatozoon* spp. 18S rRNA DNA was found in 79.2% (19/24) and 88.8% (8/9) of free-ranging and captive caimans, respectively. Comparative analysis of parasitized and non-parasitized erythrocytes showed that all analyzed features were significantly different (P<0.05) for both linear and area dimensions. Phylogenetic analysis based on 18S rRNA sequences grouped the *Hepatozoon* spp. sequences detected in the present study together with *H. caimani*, recently detected in caimans in southern Pantanal.

Keywords: Caimans, *Hepatozoon caimani*, morphology, morphometry, molecular characterisation, Brazil.

Resumo

Espécies do gênero *Hepatozoon* são os hemoparasitas intracelulares mais comumente encontrados em répteis. *Hepatozoon caimani*, cujos vetores são mosquitos do gênero *Culex* sp., têm sido detectados em uma alta prevalência entre jacarés no Brasil, por meio da análise de esfregaços sanguíneos. O presente estudo objetivou detectar e caracterizar parasitas do gênero *Hepatozoon* spp. em 33 jacarés (24 de vida-livre e 9 de cativeiro; 28 machos e 5 fêmeas) (*Caiman crocodilus yacare)* amostrados em Poconé, região norte do Pantanal, estado do Mato Grosso, Brasil, por meio da análise de esfregaços sanguíneos e técnicas moleculares. Gametócitos de *Hepatozoon* spp. foram encontrados em 70,8% (17/24) e em 88,8% (8/9) dos esfregaços sanguíneos de jacarés de via-livre e cativeiro, respectivamente. 18S rRNA DNA de *Hepatozoon* spp. foi detectado em 79,2% (19/24) e 88,8% (8/9) das amostras de sangue de jacarés de vida-livre e cativeiro, respectivamente. A análise comparativa de eritrócitos parasitados e não parasitados mostrou diferença significativa (P<0,05) em todas as variáveis lineares e de área analisadas. A análise filogenética baseada em sequências de DNA do 18S rRNA agrupou as sequências de *Hepatozoon* spp. detectadas no presente estudo juntamente com aquelas de *H. caimani*, recentemente detectadas em jacarés do Pantanal do Mato Grosso do Sul.

Palavras-chave: Jacarés, *Hepatozoon caimani*, morfologia, morfometria, caracterização molecular, Brasil.

Introduction

Hepatozoon species are apicomplexan protozoans that infect a wide range of vertebrate and invertebrate hosts, and include the widest distributed and most common intracellular hemoparasites found in reptiles (TELFORD, 1984, 2009). Based on morphologic and morphometric characteristics, *Hepatozoon caimani* have been found described as the only *Hepatozoon* species parasitizing *Caiman latirostris* (SMITH, 1996), *Caiman crocodilus* (LAINSON, 1977; LAINSON et al., 2003) and *Caiman yacare* (LAINSON et al., 2003; VIANA & MARQUES, 2005; VIANA et al., 2010a) in South America.

Culex (Meloconion) mosquitoes are the *H. caimani* natural vectors, where sporogonic phase takes place (LAINSON et al., 2003; VIANA et al., 2010b). Despite of that, the possibility

***Corresponding author:** Rosangela Zacarias Machado. Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias – FCAV, Universidade Estadual Paulista – UNESP, Via de Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP 14884-900, Jaboticabal, SP, Brasil. e-mail: zacarias@fcav.unesp.br

of the infected-invertebrate hosts being eaten by caimans is minimal (LAINSON et al., 2003; PAPERNA & LAINSON, 2003). *Caiman yacare* become infected for the first time as juveniles, when its diet changes from ingestion of invertebrates to predation of anurans and fishes (VIANA et al., 2010a). In South America, predation of insectivorous vertebrates appears to be the main transmission route of *H. caimani* (VIANA et al., 2012). *Hepatozoon caimani*-cystozoites have been found in amphibian tissues of the following species: *Leptodactylus fuscus, Leptodactylus chaquensis, Leptodactylus podicipinis, Scinax nasicus* and *Rana catesbeiana* (LAINSON et al., 2003; VIANA et al., 2012), suggesting that anurans are paratenic hosts for *H. caimani,* although frogs are not part of caiman's diet. The characid fish *Metynnis* sp., when fed with *C. quinquefasciatus* previously engorged on naturally *H. caimani*-infected caiman showed cysts harbouring cystozoites identical to those of *H. caimani*, suggesting its role as paratenic hosts for this haemoprotozoa (PEREIRA et al., 2014). Although some attempts were unsuccessful in proving the experimental transmission of *Hepatozoon* to crocodilians (PESSÔA et al., 1972; LAINSON et al., 2003), structures similar to *Hepatozoon* oocystis have been recently described in these invertebrates collected from *Caiman yacare*'s oral cavity (SOARES et al., 2017a).

Although *H. caimani* has been detected in a high prevalence in *C. crocodilus* (76.7%) in the Amazon region (LAINSON, 1977) and in *C. yacare* in western (71.4%) and southeastern (76-79.5%) Pantanal (VIANA & MARQUES, 2005; VIANA et al., 2010b; SOARES et al., 2017a) in Brazil, by blood smear examinations, molecular characterization studies are scarce. In fact, only recently, a molecular confirmation has been performed in a population of *Caiman yacare* sampled in Miranda, state of Mato Grosso do Sul, central-western Brazil (SOARES et al., 2017a) and in *Cayman crocodilus* in Amazon (SOARES et al., 2017b). The present work aims to detect and characterize the *Hepatozoon* spp. found in wild and captive *Caiman yacare* sampled at Poconé, North Pantanal, Mato Grosso state (MT), Brazil, using blood smear examinations and molecular techniques.

Material and Methods

Blood samples of *C. yacare* were collected from wild and captivity animals from August to October 2010, for a total of 2 collections. In the wild, the samples were taken at the area called Corixo Verde (16°25′,08,3″S, 56°37′ 39,2″W) at Piuval Farm in Poconé-MT (Figure 1). Thirty-three caimans (24 free-ranging and 9 captive; 28 males and 5 females) were captured during the day and night by hand or by using nooses attached to long poles. Afterwards they were tied, weighed and also marked (just free-ranging caimans) in their tail ridges with a numbered

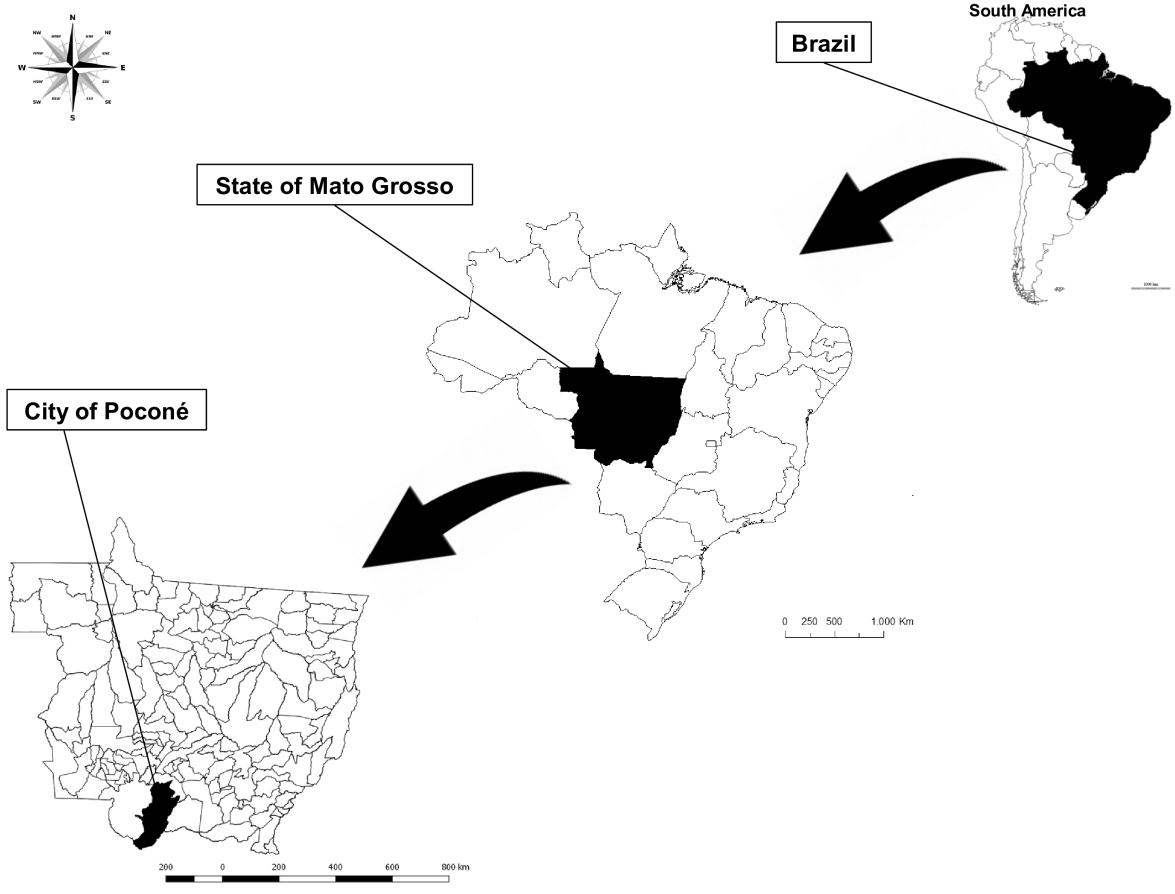

Figure 1. Capture site. Map of Mato Grosso state, central-western Brazil, showing the locality where caimans were sampled in the present study.

plastic ring and their snout-vent lengths were measured. Sex was determined by exposure of genitalia.

Blood samples from tails from caimans were used for DNA extraction. DNA samples were extracted using QIAamp DNeasy Blood & Tissue Kit (QIAGEN®, Valencia, CA, USA), following manufacturer's instructions. Initially, the detection of the presence of *Hepatozoon* spp. DNA was made using 0.3 µM of primers targeting part of 18S rRNA gene, namely HEMO1 (5'-TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG- 3') and HEMO2 (5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC3-') (Síntese Biotecnologia®, Belo Horizonte, MG, BR), previously described by Perkins & Keller (2001). PCR amplifications were performed at 94 °C for 3 min followed by 35 repetitive cycles of 94 °C for 30 sec, 48 °C for 30 sec, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min (HARRIS et al., 2011). Each 5 µL sample of extracted DNA was used as a template in 25 µL reaction mixtures containing 1X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1.5 U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA). DNA samples were also used in another PCR targeting a region of 18S rRNA gene, using primers HepF300 (5'- GTT TCT GAC CTA TCA GCT TTC GAC -3') and HepR900 (5'-CAA ATC TAA GAA TTT CAC CTC TGA C -3'), described by Ujvari et al. (2004). The amplification conditions were performed with 25 µL PCR reactions, containing 5 µL DNA template, 1X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 0.2 mM of each dNTPs (Life Technologies®, Carlsbad, CA, USA), 1.5 mM $MgCl_2$, 0.6 µM of each primer (HepF300 and HepR900) (Síntese Biotecnologia®, Belo Horizonte, MG, BR), 1U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA). The cycling conditions were conducted following O'Dwyer et al. (2013) protocol, at 94 °C for 3 min followed by 35 repetitive cycles of 94 °C for 45 sec, 56 °C for 1 min, and 72 °C for 1 min, followed by a final extension at 72 °C for 7 min. *Hepatozoon* sp. DNA positive control was obtained from a naturally infected wild canid (ANDRÉ et al., 2010). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control. In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated rooms. The reaction products were purified using Silica Bead DNA Gel Extraction Kit (Thermo Fisher Scientific®, Waltham, MA, USA).

Purified amplified DNA fragments were submitted for sequence confirmation in an automatic sequencer (ABI Prism 310 DNA Analyser – Applied Biosystem®, Foster City, CA, EUA) (SANGER et al., 1977) in house and used for subsequent phylogenetic analysis. Phylogenetic reconstructions were based on DNA sequence alignment of positive samples. Samples showing positive results for both PCR protocols had their sequences concatenated (HepF300/HepR900 and HEMO1/HEMO2), using the Fragment Merger software version 1 (BELL & KRAMVIS, 2013). Comparisons with sequences deposited in GenBank (BENSON et al., 2002) were done using the nucleotides basic local alignment search tool (BLASTn) (ALTSCHUL et al., 1990). The sequences were aligned with sequences published in GenBank using MAFFT software, version 7 (KATOH & STANDLEY, 2013, 2016; YAMADA et al., 2016). *Adelina* sp. (Genbank access no. AF494059), *Eimeria* sp. (AF311644), *Haemogregarina* spp. (KX691418, HQ224959, KM887507, KM887508), *Isospora* sp. (U97523), *Sarcocystis* sp. (U97524) and *Theileria* sp. (FJ213586) were used as outgroups. Aligned sequences were edited by BioEdit Sequence Alignment Editor version 7.0.5.3 (HALL, 1999). Phylogenetic inference was based on Maximum Likelihood (ML) method. The Maximum-likelihood (ML) analysis was inferred with software IQ-TREE (NGUYEN et al., 2015) using W-IQ-TREE (TRIFINOPOULOS et al., 2016) (which includes an estimation of bootstrap node support), using 1000 bootstrapping replicates. The best model of evolution was selected using the software IQ-TREE (NGUYEN et al., 2015) by W-IQ-TREE (TRIFINOPOULOS et al., 2016), under the Akaike Information Criterion (AIC) (POSADA & BUCKLEY, 2004). The trees were examined in Treegraph 2.0.56-381 beta (STOVER & MULLER, 2010).

Additionally, an analysis of nucleotide polymorphisms of the 18S rRNA sequences obtained in the present study was performed. The sequences were aligned using MAFFT software, version 7 (KATOH & STANDLEY, 2013, 2016; YAMADA et al., 2016). The number of haplotypes, haplotype diversity (Hd) and nucleotide diversity (Pi) were determined using the program DnaSP 5, version 5.10.01 (LIBRADO & ROZAS, 2009).

Furthermore, the blood samples were used in blood smears for microscopic examination. Slides were air-dried, fixed with methanol and stained with Giemsa. Morphologic and morphometric features of gamonts, as well as the changes caused by the presence of parasites in erythrocytes were analyzed using the CellSens Imaging software (Olympus) in the Immunoparasitology Laboratory, Department of Veterinary Pathology, Universidade Estadual Paulista (UNESP Jaboticabal). Mean morphometric variables (nuclear and erythrocytes length, width, and area) of parasitized and non-parasitized cells were compared using non-paired t-test with Welch's Correction; F test was used for comparing variance values. The results were analyzed using GraphPrism 7.03.

Results

Hepatozoon sp.-gametocytes (Figure 2) were found in 70.8% (17/24) and 88.8% (8/9) of blood smears from free-ranging and captive caimans, respectively. DNA of *Hepatozoon* spp. was found in 83.3% (20/24) and 88.8% (8/9) of free-ranging and captive caimans, respectively. Thirteen (54.2%) free-ranging caimans showed *Hepatozoon* sp.-gametocytes in blood smear examinations and were also positive to *18S rRNA* PCR for *Hepatozoon* sp.; four (16.6%) showed positive results only in microscopic examinations. Seven (77.7%) captive caimans showed *Hepatozoon* sp.-gametocytes in blood smear examinations and were also positive to 18S rRNA PCR for *Hepatozoon* sp.; one (11.1%) showed positive results only in microscopic examinations.

The average morphometric measures of *H. caimani* gametocytes were: parasite whole cell (area = 53.2 μm^2 ± 14.6; lenght =12.9 µm ± 1.6; width = 4.81 µm ±1.1); nucleus (area = 13.1 μm^2 ± 4.71; lenght = 5.67 µm ± 1,5; width= 2.73 µm ± 0.88). Morphometric measures of non-parasitized and parasitized erythrocytes were

shown in Table 1. Mean morphometric variables (nuclear and erythrocytes length, width, and area) showed statistical differences between parasitized and non-parasitized cells (P<0.05), except for length parasite cell.

The two 18S rRNA *Hepatozoon* sequences obtained from *C. yacare* showed 98-99% identity (98-99% of coverage) with *Hepatozoon* spp. from lizards from North Africa, previously deposited in GenBank (HQ734787, HQ734789, HQ734807) by BLAST analysis. Additionally, the *Hepatozoon* sequences obtained showed 99% identity (97-98% of coverage) with *H. dormerguei* sequence obtained from chameleon and snake from Madagascar (KM234646 and KM234649) and 99% identity (54-57% of coverage) with *H. caimani* sequence amplified from *C. yacare* from Brazil (KU495924 and KU495925).

The phylogenetic tree of *Hepatozoon* spp. 18S rRNA sequences clustered in a monophyletic group. The phylogenetic tree was basically in two branches: one of them composed by *Hepatozoon* sequences amplified in the present study and sequences of *H. caimani*, recently detected in caimans in southern Pantanal, and *Hepatozoon* spp. from other reptile taxa (snakes, lizards and tortoise) and amphibians retrieved from Genbank. The other branch grouped *Hepatozoon* sequences amplified from mammals and ticks previously deposited in Genbank. *Adelina* sp., *Haemogregarina* spp., *Theileria* sp., *Isospora* sp., and *Sarcocystis* sp. were used as outgroups (Figure 3).

Nucleotide polymorphisms and DNA divergence between sequences obtained in the present study were also analyzed. The analysis of nucleotide polymorphisms of 18S rRNA sequences

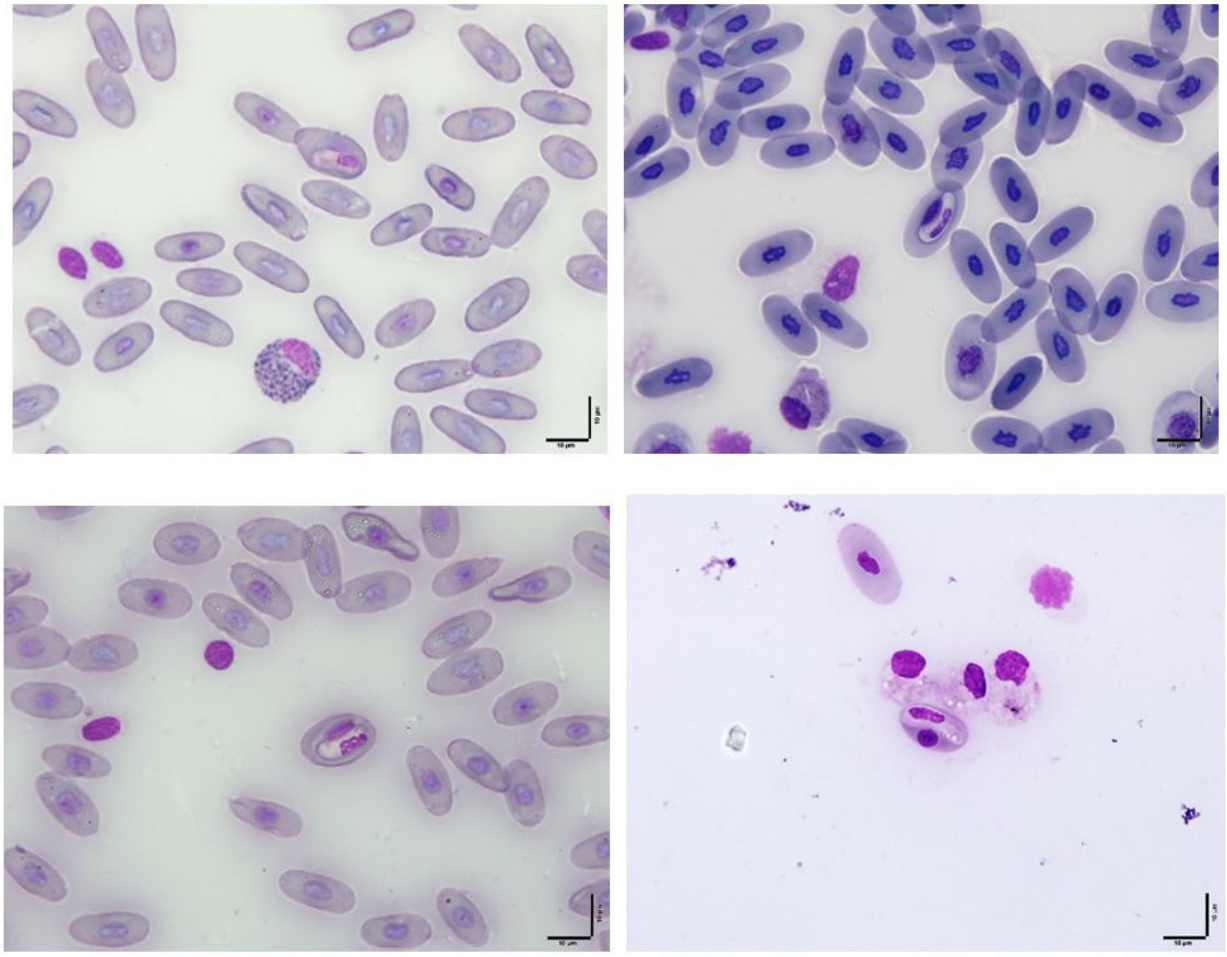

Figure 2. *Hepatozoon caimani* gamonts found in *Cayman yacare* sampled in Poconé, state of Mato Grosso, central-western Brazil. 1000x.

Table 1. Comparative analysis between non-parasitized and *H. caimani*-parasitized erythrocytes found in *Caiman crocodilus yacare* blood smears.

	Cell			Nucleus		
	Area (µm²)	**Lenght (µm)**	**Width (µm)**	**Area (µm²)**	**Lenght (µm)**	**Width (µm)**
N	130.9 (24.9)[a]	25.5 (10.4)[a]	8.66 (1.1)[a]	15.8 (4.4)[a]	5.8 (0.8)[a]	3.4 (0.6)[a]
PA	162.6 (32.6)[b]	19.3 (2.3)[a]	10.5 (1.6)[b]	17.5 (6.8)[b]	6.62 (1.3)[b]	3.3 (0.7)[b]

Same letters in columns = no statistic differences (P≥0,05); different letters in columns = statistic differences (P≤0,05); (Standard deviation); N = non-parasitized erythrocytes; PA = parasitized erythrocytes.

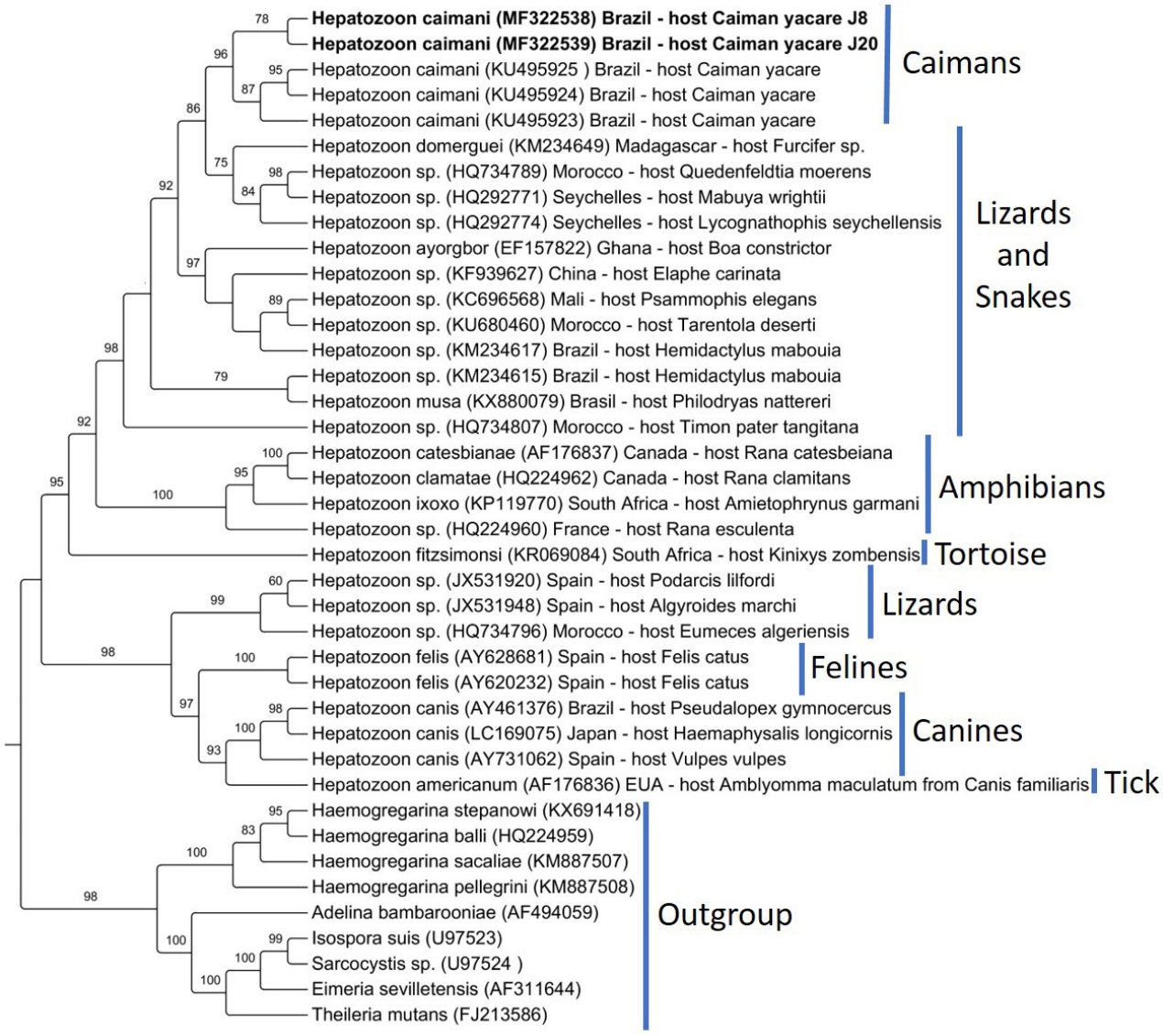

Figure 3. Phylogenetic tree based on an alignment of 1200bp fragment of *Hepatozoon* spp. 18SrRNA sequences from *Caiman yacare* blood samples, using Maximum Likelihood (ML) method and TVM+G+I evolutionary model. Bootstrap values for ML (≥50) are given above relevant nodes. The sequences indicated in bold represent those from this study and original sample codes were named.

showed two haplotypes (haplotype diversity (Hd): 1.000; variance of haplotype diversity: 0.25000; Standard Deviation (SD): =0.500) and nucleotide diversity (Pi) of 0.02113 (SD= 0.01057).

Discussion

The occurrence of *Hepatozoon* sp. found in our study based on blood smears examinations (70.8%) among free-ranging *C. yacare* was lower than that found in *C. crocodilus* (76.7%) in the Amazon region (LAINSON, 1977) and in *C. yacare* in western (71.4%) and southeastern (76-79.5%) Pantanal (VIANA & MARQUES, 2005; VIANA et al., 2010a; SOARES et al., 2017a) in Brazil.

The detection sensitivity was slightly higher in PCR (83.3%) when compared to blood smear examinations (70.8%) of free-ranging caimans, corroborating previous studies involving molecular detection of *Hepatozoon* spp. in reptiles (UJVARI et al., 2004; HARRIS et al., 2011).

In the present study, morphometric alterations in parasitized cells by *Hepatozoon* spp. gamonts were markedly verified in all sampled yacares. The induction of changes in reptile parasitized cells by *Hepatozoon* spp. have already been reported in *Caiman crocodilus yacare* (VIANA & MARQUES, 2005; SOARES et al., 2017a) and in several species of snakes sampled in Brazil (MOÇO et al., 2002; O'DWYER et al., 2004; MOÇO et al., 2012). These changes in parasitize cells is probably mainly due to alterations in cellular erythrocyte cellular membrane's permeability (VIANA & MARQUES, 2005).

Herein, the average size of *Hepatozoon* sp. gametocytes observed (12.9 × 4.81 μM) was closely related to previously described *Hepatozoon caimani* gamonts in *Caiman crocodilus yacare* (LAINSON et al., 2003; VIANA & MARQUES, 2005; SOARES et al., 2017a) and

Caiman crocodilus crocodilus (LAINSON et al., 2003). Morphometric and morphologic features of sporogonic stages in the invertebrate vector, *Culex (Meloconion)*, and meronts in vertebrate hosts will allow a better characterization of *Hepatozoon* parasitizing caimans in Brazil. Recently, meronts structures were described in the wall of vessels from liver and kidney ducts of *H.* caimani-naturally infected *C. yacare* in southern Pantanal (SOARES et al., 2017a).

The variety of morphological and morphometric forms of gamonts verified in yacare' erythrocytes emphasizes the need for molecular confirmation of the involved *Hepatozoon* species. According to Moço et al. (2012), morphologically similar parasites showing different morphometric features may or may not represent different species. Furthermore, *Hepatozoon* spp. blood forms could show difference in their morphology depending on the host species involved (TELFORD, 2009). Besides, immature and mature gamonts, showing different morphological and morphometric features, could lead to a misdiagnosis of different species of *Hepatozoon* spp. (SMITH, 1996). Herein, we used a combination of morphometrical and phylogenetic assessment to confirm the occurrence of *H. caimani* in sampled caimans.

It seems that *Hepatozoon* species show limited host specificity, switching easily between different host species (MAIA et al., 2011). Regarding this high spectrum of host specificity found among *Hepatozoon* species, the host ecology appears to play a more important role in the complex relationship *Hepatozoon* – different host species than the phylogenetic relatedness among the hosts properly (SLOBODA et al., 2007). For instance, similar *Hepatozoon* spp. isolates appears to infect different genera of lizards, as previously reported (MAIA et al., 2011). On the other hand, some *Hepatozoon* species found in snakes have shown high specificity being restricted to determined hosts (TELFORD et al., 2001). Because of that, identification of new *Hepatozoon* species based solely on blood smears examinations should be avoided (MAIA et al., 2011). The identification of *Hepatozoon* species in different caiman species in Brazil should be done based on blood smears examinations and molecular phylogeny. Also, future studies should assess the phylogenetic relationships among *Hepatozoon* sp. isolates in different alligator species, *Culex* mosquitoes (vectors), anurans (paratenic hosts) and leeches in Brazil. Regarding the molecular characterization based on rDNA coding regions, such as that used in the present study (18S rRNA), although highly conserved and used frequently in molecular characterization of *Hepatozoon* spp. in several hosts (ANDRÉ et al., 2010; BORTOLI et al., 2011; MOÇO et al., 2012; O'DWYER et al., 2013; SOARES et al., 2017a; SOUSA et al., 2017), this target sequence may not be suitable for phylogenetic inferences of parasites which share some of intraspecific divergences (MOÇO et al., 2012), preventing attempts to differentiate those species. The search for new targets genes which allow a better phylogenetic differentiation of this group of parasites are much needed as an attempt to elucidate the diversity of *Hepatozoon* species that parasitize reptiles.

In summary, the present work showed through morphological, morphometric and molecular approaches, the occurrence of *H. caimani* in free-ranging and captive *Cayman yacare* in northern Pantanal.

Acknowledgements

The authors would like to thank Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for the financial support, Process nº 2015/14896-1.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. Basic local alignment search tool. *J Mol Biol* 1990; 215(3): 403-410. PMid:2231712. http://dx.doi.org/10.1016/S0022-2836(05)80360-2.

André MR, Adania CH, Teixeira RHF, Vargas GH, Falcade M, Sousa L, et al. Molecular detection of *Hepatozoon* spp. in Brazilian and exotic wild carnivores. *Vet Parasitol* 2010; 173(1-2): 134-138. PMid:20630658. http://dx.doi.org/10.1016/j.vetpar.2010.06.014.

Bell TG, Kramvis A. Fragment merger: an online tool to merge overlapping long sequence fragments. *Viruses* 2013; 5(3): 824-833. PMid:23482300. http://dx.doi.org/10.3390/v5030824.

Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Rapp BA, Wheeler DL. GenBank. *Nucleic Acids Res* 2002; 30(1): 17-20. PMid:11752243. http://dx.doi.org/10.1093/nar/30.1.17.

Bortoli CP, André MR, Braga MSCO, Machado RZ. Molecular characterization of *Hepatozoon* sp. in cats from São Luís Island, Maranhão, Northeastern Brazil. *Parasitol Res* 2011; 109(4): 1189-1192. PMid:21607692. http://dx.doi.org/10.1007/s00436-011-2376-6.

Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 1999; 41: 95-98.

Harris DJ, Maia JP, Perera A. Molecular characterization of *Hepatozoon* species in reptiles from the Seychelles. *J Parasitol* 2011; 97(1): 106-110. PMid:21348615. http://dx.doi.org/10.1645/GE-2470.1.

Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. *Mol Biol Evol* 2013; 30(4): 772-780. PMid:23329690. http://dx.doi.org/10.1093/molbev/mst010.

Katoh K, Standley DM. A simple method to control over-alignment in the MAFFT multiple sequence alignment program. *Bioinformatics* 2016; 32(13): 1933-1942. PMid:27153688. http://dx.doi.org/10.1093/bioinformatics/btw108.

Lainson R. *Trypanosoma cecili* n. sp., a parasite of the South American cayman *Caiman crocodilus crocodilus* (Linnaeus, 1758) (Crocodilia: Alligatoridae). In: Canning EU, editor. *Protozoology*. Berkhampstead: Clunbury Cottrell Press; 1977. vol. III. p. 87-93.

Lainson R, Paperna I, Naiff RD. Development of *Hepatozoon caimani* (Carini, 1909) Pessôa, De Biasi & De Souza, 1972 in the Caiman *Caiman c. crocodilus*, the frog *Rana catesbeiana* and the mosquito *Culex fatigans*. *Mem Inst Oswaldo Cruz* 2003; 98(1): 103-113. PMid:12700868. http://dx.doi.org/10.1590/S0074-02762003000100014.

Librado P, Rozas J. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 2009; 25(11): 1451-1452. PMid:19346325. http://dx.doi.org/10.1093/bioinformatics/btp187.

Maia JP, Harris DJ, Perera A. Molecular survey of *Hepatozoon* species in lizards from North Africa. *J Parasitol* 2011; 97(3): 513-517. PMid:21506764. http://dx.doi.org/10.1645/GE-2666.1.

Moço TC, O'Dwyer LH, Vilela FC, Barrella TH, Silva RJ. Morphologic and morphometric analysis of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) of snakes. *Mem Inst Oswaldo Cruz* 2002; 97(8): 1169-1176. PMid:12563486. http://dx.doi.org/10.1590/S0074-02762002000800019.

Moço TC, Silva RJ, Madeira NG, Paduan KS, Rubini AS, Leal DDM, et al. Morphological, morphometric, and molecular characterization of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) from naturally infected *Caudisona durissa terrifica* (Serpentes, Viperidae). *Parasitol Res* 2012; 110(4): 1393-1401. PMid:21922238. http://dx.doi.org/10.1007/s00436-011-2639-2.

Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol* 2015; 32(1): 268-274. PMid:25371430. http://dx.doi.org/10.1093/molbev/msu300.

O'Dwyer LH, Moço TC, Paduan KS, Spenassatto C, Silva RJ, Ribolla PE. Description of three new species of *Hepatozoon* (Apicomplexa, Hepatozoidae) from Rattlesnakes (*Crotalus durissus terrificus*) based on molecular, morphometric and morphologic characters. *Exp Parasitol* 2013; 135(2): 200-207. PMid:23867148. http://dx.doi.org/10.1016/j.exppara.2013.06.019.

O'Dwyer LH, Moço TC, Silva RJ. Description of the gamonts of a small species of *Hepatozoon* sp. (Apicomplexa, Hepatozoidae) found in *Crotalus durissus terrificus* (Serpentes, Viperidae). *Parasitol Res* 2004; 92(2): 110-112. PMid:14628216. http://dx.doi.org/10.1007/s00436-003-1005-4.

Paperna I, Lainson R. Ultrastructural studies on the sporogony of *Hepatozoon* spp. in *Culex quinquefasciatus* Say, 1823 fed on infected *Caiman crocodilus* and *Boa constrictor* from northern Brazil. *Parasitology* 2003; 127(Pt 2): 147-154. PMid:12954016. http://dx.doi.org/10.1017/S0031182003003482.

Pereira GR, Soares P, Gomes MQ, Viana LA, Manso PP, Machado MP, et al. Are fish paratenic natural hosts of the caiman haemoparasite *Hepatozoon caimani? Parasitol Res* 2014; 113(1): 39-45. PMid:24142284. http://dx.doi.org/10.1007/s00436-013-3623-9.

Perkins SL, Keller AK. Phylogeny of nuclear small subunit rRNA genes of hemogregarines amplified with specific primers. *J Parasitol* 2001; 87(4): 870-876. PMid:11534653. http://dx.doi.org/10.1645/0022-3395(2001)087[0870:PONSSR]2.0.CO;2.

Pessôa SB, Biasi P, Souza D. Esporulação do *Hepatozoon caimani* (Carini,1909), parasita do jacaré-de-papo-amarelo: *Caiman latirostris* Daud, no *Culex dolosus* (L. Arribálzaga). *Mem Inst Oswaldo Cruz* 1972; 70(3): 379-383. http://dx.doi.org/10.1590/S0074-02761972000300008.

Posada D, Buckley TR. Model selection and model averaging in phylogenetics: advantages of akaike information criterion and bayesian approaches over likelihood ratio tests. *Syst Biol* 2004; 53(5): 793-808. PMid:15545256. http://dx.doi.org/10.1080/10635150490522304.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* 1977; 74(12): 5463-5467. PMid:271968. http://dx.doi.org/10.1073/pnas.74.12.5463.

Sloboda M, Kamler M, Bulantová J, Votýpka J, Modrý D. A new species of *Hepatozoon* (Apicomplexa: Adeleorina) from *Python regius* (Serpentes: Pythonidae) and its experimental transmission by a mosquito vector. *J Parasitol* 2007; 93(5): 1189-1198. PMid:18163356. http://dx.doi.org/10.1645/GE-1200R.1.

Smith TG. The genus *Hepatozoon* (Apicomplexa: Adeleina). *J Parasitol* 1996; 82(4): 565-585. PMid:8691364. http://dx.doi.org/10.2307/3283781.

Soares HS, Marcili A, Barbieri ARM, Minervino AHH, Moreira TR, Gennari SM, et al. Novel piroplasmid and *Hepatozoon* organisms infecting the wildlife of two regions of the Brazilian Amazon. *Int J Parasitol Parasites Wildl* 2017b; 6(2): 115-121. PMid:28603688. http://dx.doi.org/10.1016/j.ijppaw.2017.05.002.

Soares P, Borghesan TC, Tavares LER, Ferreira VL, Teixeira MMG, Paiva F. *Hepatozoon caimani* Carini, 1909 (Adeleina: Hepatozoidae) in wild population of *Caiman yacare* Daudin, 1801 (Crocodylia: Alligatoridae), Pantanal, Brazil. *Parasitol Res* 2017a; 116(7): 1907-1916. PMid:28512673. http://dx.doi.org/10.1007/s00436-017-5467-1.

Sousa KC, Fernandes MP, Herrera HM, Benevenute JL, Santos FM, Rocha FL, et al. Molecular detection of *Hepatozoon* spp. in domestic dogs and wild mammals in southern Pantanal, Brazil with implications in the transmission route. *Vet Parasitol* 2017; 237: 37-46. PMid:28291601. http://dx.doi.org/10.1016/j.vetpar.2017.02.023.

Stöver BC, Muller KF. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. *BMC Bioinformatics* 2010; 11(1): PMC2806359. PMid:20051126. http://dx.doi.org/10.1186/1471-2105-11-7.

Telford SR. Haemoparasites of reptiles. In: Hoff GL, Frye FL, Jacobson ER, editors. *Diseases of amphibians and reptiles.* New York: Plenum Publishing Corporation; 1984. p. 385-517.

Telford SR. *Hemoparasites of the Reptilia: color atlas and text*. Boca Raton: CRC Press; 2009.

Telford SR Jr, Wozniak EJ, Butler JF. Haemogregarine specificity in two communities of Florida snakes, with descriptions of six new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) and possible species of *Haemogregarina* (Apicomplexa: Haemogregarinidae). *J Parasitol* 2001; 87(4): 890-905. PMid:11534655. http://dx.doi.org/10.1645/0022-3395(2001)087[0890:HSITCO]2.0.CO;2.

Trifinopoulos J, Nguyen LT, Von Haeseler A, Minh BQ. W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res* 2016; 44(W1): W232-W235. PMid:27084950. http://dx.doi.org/10.1093/nar/gkw256.

Ujvari B, Madsen T, Olsson M. High prevalence of *Hepatozoon* spp. (Apicomplexa, Hepatozoidae) infection in water pythons (*Liasis fuscus*) from tropical Australia. *J Parasitol* 2004; 90(3): 670-672. PMid:15270125. http://dx.doi.org/10.1645/GE-204R.

Viana LA, Marques EJ. Haemogregarine parasites (Apicomplexa: Hepatozoidae) in *Caiman crocodilus yacare* (Crocodilia: Alligatoridae) from Pantanal, Corumbá, MS, Brazil. *Rev Bras Parasitol Vet* 2005; 14(4): 173-175. PMid:16445875.

Viana LA, Paiva F, Coutinho ME, Lourenço-de-Oliveira R. *Hepatozoon caimani* (Apicomplexa: Hepatozoidae) in wild caiman, *Caiman yacare*, from the Pantanal Region, Brazil. *J Parasitol* 2010a; 96(1): 83-88. PMid:19685936. http://dx.doi.org/10.1645/GE-2150.1.

Viana LA, Soares P, Paiva F, Lourenço-De-Oliveira R. Caiman-biting mosquitoes and the natural vectors of *Hepatozoon caimani* in Brazil. *J Med Entomol* 2010b; 47(4): 670-676. PMid:20695284. http://dx.doi.org/10.1093/jmedent/47.4.670.

Viana LA, Soares P, Silva JE, Paiva F, Coutinho ME. Anurans as paratenic hosts in the transmission of *Hepatozoon caimani* to caimans *Caiman yacare* and *Caiman latirostris. Parasitol Res* 2012; 110(2): 883-886. PMid:21808978. http://dx.doi.org/10.1007/s00436-011-2570-6.

Yamada KD, Tomii K, Katoh K. Application of the MAFFT sequence alignment program to large data - reexamination of the usefulness of chained guide trees. *Bioinformatics* 2016; 32(21): 3246-3251. PMid:27378296. http://dx.doi.org/10.1093/bioinformatics/btw412.