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Research paper

Molecular detection of *Hepatozoon* spp. in domestic dogs and wild mammals in southern Pantanal, Brazil with implications in the transmission route

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ABSTRACT

Hepatozoon parasites comprise intracellular apicomplexan parasites transmitted to vertebrate animals by ingestion of arthropods definitive hosts. The present work aimed to investigate the occurrence of *Hepatozoon* spp. in wild animals, domestic dogs and their respective ectoparasites, in southern Pantanal region, central-western Brazil, by molecular techniques. Between August 2013 and March 2015, 31 coatis (*Nasua nasua*), 78 crab-eating foxes (*Cerdocyon thous*), seven ocelots (*Leopardus pardalis*), 42 dogs (*Canis lupus familiaris*), 110 wild rodents (77 *Thichomys fosteri,* 25 *Oecomys mamorae,* and 8 *Clyomys laticeps*), 30 marsupials (14 *Thylamys macrurus,* 11 *Gracilinanus agilis,* 4 *Monodelphis domestica* and 1 *Didelphis albiventris*), and 1582 ticks and 80 fleas collected from the sampled animals were investigated. DNA samples were submitted to PCR assays for *Hepatozoon* spp. targeting 18S rRNA gene. Purified amplicons were directly sequenced and submitted to phylogenetic analysis. A high prevalence of *Hepatozoon* among carnivores (*C. thous* [91.02%], dogs [45.23%], *N. nasua* [41.9%] and *L. pardalis* [71.4%]) was found. However, ticks and fleas were negative to *Hepatozoon* PCR assays. By phylogenetic analysis based on 18S rRNA sequences, *Hepatozoon* sequences amplified from crab-eating foxes, dogs, coatis and ocelots clustered with sequences of *H. canis, H. americanum* and *H. felis.* The closely related positioning of *Hepatozoon* sequences amplified from wild rodents and *T. macrurus* marsupial to *Hepatozoon* from reptiles and amphibians suggest a possible transmission of those *Hepatozoon* species between hosts by ectoparasites or by predation. *Hepatozoon* haplotypes found circulating in wild rodents seem to present a higher degree of polymorphism when compared to those found in other groups of animals. Although rodents seem not to participate as source of *Hepatozoon* infection to wild carnivores and domestic dogs, they may play an important role in the transmission of *Hepatozoon* to reptiles and amphibians in Pantanal biome.

1. Introduction

Hepatozoon spp. are apicomplexan parasites that infect a wide variety of vertebrate hosts, which play a role as intermediate hosts and acquire infection through the ingestion of arthropod definitive host containing oocysts (Smith, 1996). Additionally, other routes of

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transmission, such as the predation of infected vertebrates containing *Hepatozoon* cysts in their tissues (Johnson et al., 2007) and transplacental transmission (Baneth et al., 2013), have been described.

Regarding the occurrence of *Hepatozoon* spp. in wild and captive animals in Brazil, the protozoa has been so far molecularly detected in crab-eating foxes (*Cerdocyon thous*) (André et al., 2010; Almeida et al., 2013); bush dogs (*Speothos venaticus*) (André et al., 2010); maned wolf (*Cerdocyon brachyurus*); ocelots (*Leopardus pardalis*) (Metzger et al., 2008; André et al., 2010); little-spotted-cats (*Leopardus tigrinus*) (André et al., 2010); yagourandi (*Puma yagouaroundi*), pumas (*Puma concolor*); rodents (*Akodon* sp., *Oligoryzomys nigripes*, *Oligoryzomys flavescens, Calomys callosus*) (Demoner et al., 2016; Wolf et al., 2016); rattlesnake (*Crotalus durissus terrificus*) (O'Dwyer et al., 2013); crocodiles (*Caiman yacare)* (Viana et al., 2010); lizards (*Hemidactylus mabouia, Phyllopezus periosus*, *Phyllopezus pollicaris*) (Harris et al., 2015); and anuran amphibians (*Leptodactylus chaquensis*, *Leptodactylus podicipinus*) (Leal et al., 2015).

Although *Hepatozoon* infections in wild animals are usually subclinical (Kocan et al., 2000; Metzger et al., 2008), some studies associated the *Hepatozoon* infection with the presence of clinical disease in coyotes (Kocan et al., 2000; Garrett et al., 2005), mortality in hyenas (East et al., 2008) and necrotizing inflammatory lesion in unnatural reptilian hosts (Wozniak et al., 1995). Moreover, *Hepatozoon* spp. may be a potential pathogen and an opportunistic parasite in immunocompromised animals or if occurring in concomitant infections (Davis et al., 1978; Baneth et al., 1998; Kubo et al., 2006). For that reason, there is a need to assess the distribution of *Hepatozoon* spp. in free-living animals, especially for endangered or elusive, free-living host species that are difficult to sample (Wobeser, 2007). Furthermore, large-scale parasite screening has the potential for determining the distribution of similar lineages in different hosts, providing information on parasite transmission dynamics, which represents an important issue for endangered species (Fayer et al., 2004).

There is a lack of information on the vectorial competence of arthropods that may act as definitive hosts for *Hepatozoon* spp. in Brazil. Although *Rhipicephalus sanguineus* sensu lato (s. l.) is considered the main biological vector for *Hepatozoon canis* (Smith, 1996), preliminary studies indicated that this tick species has little or no importance in the transmission of *Hepatozoon* sp. in Brazil (Demoner et al., 2013). Additionally, *H. canis* oocysts have been found in the hemocoel of *Amblyomma ovale* (Forlano et al., 2005) and *Rhipicephalus* (*Boophilus*) *microplus* (Miranda et al., 2011). Furthermore, the role of other invertebrate hosts in the transmission cycles of *Hepatozoon* sp. has been investigated. For instance, Watkins et al. (2006) observed oocysts of *Hepatozoon* sp. in *Megabothris abantis* fleas collected from rodents, suggesting the participation of this ectoparasite in the biological cycle of *Hepatozoon* sp.

The predation of paratenic hosts seems to be another important infection route for *Hepatozoon* spp. (Johnson et al., 2009). Sequences of *Hepatozoon* spp. obtained from rodents and wild canids in South Africa showed to be phylogenetically related, suggesting a potential for transmission by predation of rodents by foxes (*Vulpes pallida*) (Maia et al., 2014). Additionally, *Hepatozoon* genotype sp. detected in snakes (*Python regius* and *Boa constrictor imperator*) were closely related to sequences of *Hepatozoon* spp. obtained from rodents in North America (Sloboda et al., 2008; Allen et al., 2011). In Brazil, the prevalence of *H. canis* among domestic dogs in rural areas has showed to be higher than in urban areas. Although dogs from rural areas often roam the forests and probably predate *Hepatozoon* sp.-infected animals (O'Dwyer, 2011), the analysis of sequences obtained from wild rodents from an endemic area for *H. canis* showed that wild rodent species in Brazil were infected with other *Hepatozoon* species, rejecting the hypothesis that rodents act as reservoirs for *H. canis* in the state of São Paulo, southeastern Brazil (Demoner et al., 2016). However, monozoic cysts were found in the sampled rodents' tissues, suggesting that rodents may act as paratenic hosts for *Hepatozoon* spp. in Brazil (Demoner et al., 2016).

The aim of the present study was to investigate the occurence of *Hepatozoon* spp. in wild mammals and domestic dogs and their respective ectoparasites in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

2. Material and methods

The fieldwork was conducted at the Nhumirim ranch (56°39′ W, 18°59′S), located in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul, central-western Brazil (Fig. 1). This region is characterized by a mosaic of semideciduous forest, arboreal savannas, seasonally flooded fields covered by grasslands with dispersed shrubs and several temporary and permanent ponds. The Pantanal is the largest Neotropical floodplain and it is well known for a rich biodiversity. Two well-defined seasons are recognized: a rainy summer (October to March) and a dry winter (April to September) (Alves et al., 2016).

Between August 2013 and March 2015, four field expeditions (August 2013, October 2013, August 2014 and March 2015) were performed. The free-ranging carnivores (*C. thous*, *N. nasua* and *L. pardalis*) were caught used a Zootech® (Curitiba, PR, Brazil) model wire box live trap (1 × 0.40 × 0.50 m), which was made with galvanized wire mesh and baited with a piece of bacon every afternoon. Traps were armed during 24 h and checked twice a day. The animals were immobilized with an intramuscular injection of a combination of zolazepan and tiletamine (Zoletil®) at dosages of 8 mg/kg for ocelots and 10 mg/kg crab-eating foxes and coatis. Blood samples were collected by puncture of the cephalic vein stored in Vacutainer® containing EDTA and stored at −20 °C until DNA extraction. Additionally, blood samples were collected from 42 domestic dogs, which were cohabiting the same studied area. In addition to this, blood smears were performed and fixed with methanol and stained with Giemsa® (Giemsa® stain, modified, Sigma-Aldrich, St. Louis, MO,USA).

Small mammals (rodents and marsupials) were captured using live traps (Sherman® –H. B. Sherman Traps, Tallahassee, FL, USA and Tomahawk® Tomahawk Live Traps, Tomahawk, WI, USA) baited with a mixture of banana, peanut butter, oat and sardines. Traps were set up for 7 consecutive nights along linear transects, placed on the ground at 10 m intervals and alternating between trap type in 2 field expeditions (August 2014 and March 2015). The total capture effort was 200 traps-nights, equally distributed between the 2 expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described by Bonvicino et al. (2005). The animals were firstly anesthetized with an intramuscular injection of ketamine (10–30 mg/kg) associated with acepromazine (5–10 mg/kg) for rodents (proportion 9:1), or xylazine (2 mg/kg) for marsupials (1:1). After anesthesia, the animals were euthanized with potassium chloride, which dosis ranged from 75 to 150 mg/kg (Leary et al., 2013). Spleen samples were collected and stored in absolute alcohol (Merck®, Kenilworth, Nova Jersey, USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian Government Environmental Agency (Brazilian Institute of Environment and Renewable Natural Resources) (IBAMA) (SISBIO licenses numbers 38145, 38787-2) and was also endorsed by the Ethics Committee of the FCAV/UNESP

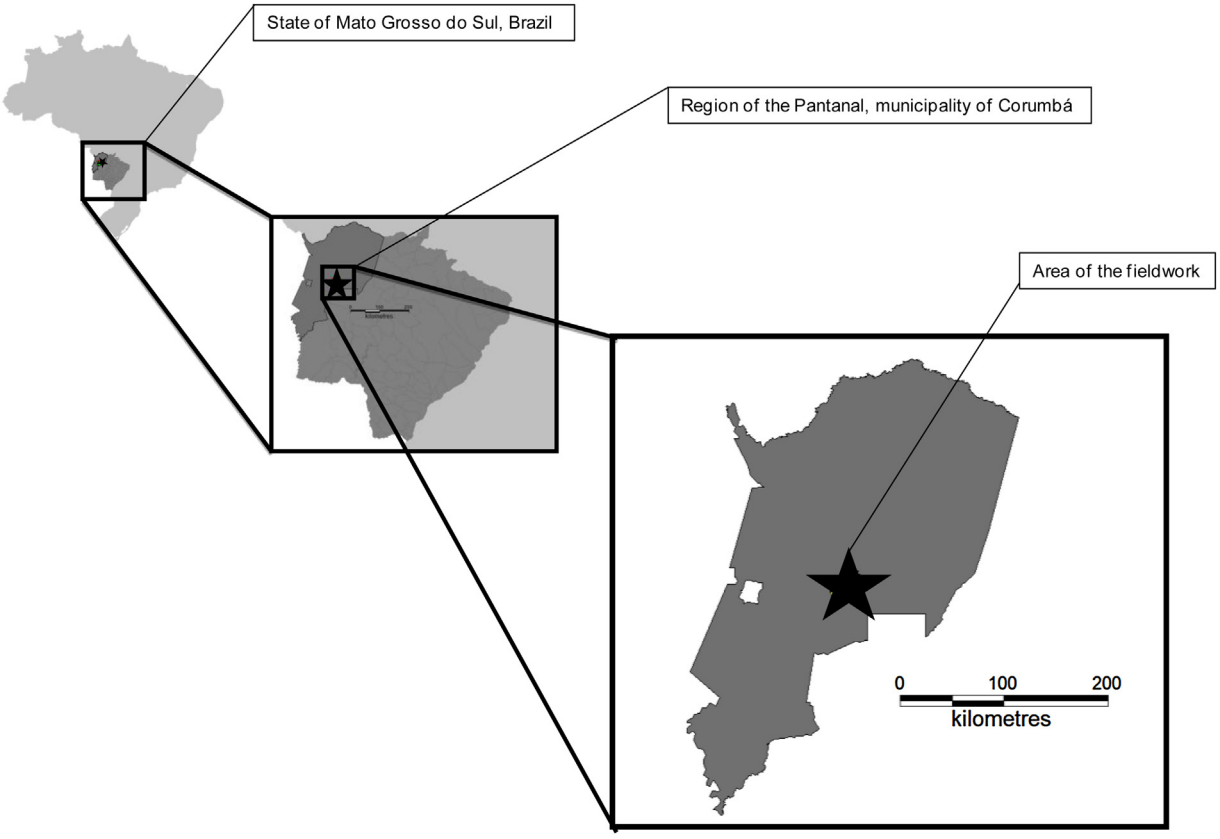

Fig. 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region where animals samples were collected in the present study.

University (CEUA –n° 006772/13), in accordance to Brazilian regulations.

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The specimens were stored in 100% alcohol (Merck®, Kenilworth, Nova Jersey, EUA) until identification using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera (Guimarães et al., 2001; Martins et al., 2016), and *Amblyomma* nymphs (Martins et al., 2010). *Amblyomma* larvae could not be identified to the species level because there is insufficient literature available until now. The identification of fleas was performed following the dichotomous keys elaborated by Linardi and Guimarães (2000).

DNA was extracted from 200 μL of each whole blood (wild carnivores and domestic dogs) and spleen (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. While ticks DNA extraction was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. The fleas DNA extraction also was processed in pools consisting of up to five individuals. Ticks and fleas were macerated and prepared for DNA extraction, using the same kit before mentioned. DNA concentration and quality was measured using absorbance ratio between 260/280 nm (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA).

In order to verify the existence of amplifiable DNA in the samples, internal control PCR assays targeting the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mammals gene (Birkenheuer et al., 2003), mitochondrial 16S rRNA ticks gene (Black and Piesman, 1994) and a fragment of the cytochrome c oxidase subunit I (cox1) coding for COX1 from fleas (Folmer et al., 1994) were performed. Two different PCR protocols were used aiming at amplifying different regions of 18SrRNA of *Hepatozoon*, based on Perkins and Keller (2001) and Ujvari et al. (2004) protocols (Table 1). Thus, the two *Hepatozoon* 18S rRNA sequences obtained by the two PCR protocols were concatenated in order to obtain a large 18S rRNA fragment (1300pb) to be used in phylogenetic analyses. DNA positive control was obtained from a naturally infected dog (Malheiros et al., 2016) and ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control.

The reaction products were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific®, Waltham, MA, USA). The sequencing of the two different regions of 18S rRNA *Hepatozoon* spp. gene fragments of was carried out using the BigDye® Terminator v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, EUA) (Sanger et al., 1977).

The sequences obtained from positive samples were first submitted to a screening test using Phred-Phrap software version 23 (Ewing and Green, 1998; Ewing et al., 1998) to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences from international database (GenBank) (Benson et al., 2002). All sequences that showed appropriate quality standards and identity with *Hepatozoon* spp. were deposited in the international database Genbank. Samples showing positive results for both PCR protocols had their sequences concatenated, using the Fragment Merger software version 1 (Bell and Kramvis, 2013). The sequences were aligned with sequences published in GenBank using MAFFT software, version 7 (Katoh and Standley, 2013).

Phylogenetic inference was based on Bayesian (BI) and Maximum Likelihood (ML) methods. The Bayesian inference (BI) analysis

Table 1
Oligonucleotides sequences, target genes and thermal conditions used in conventional PCR assays targeting endogenous genes and *Hepatozoon* 18S rRNA gene fragments in biological samples from wild mammals and domestic dogs, trapped and sampled, respectively, in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Oligonucleotides sequences (5′-3′)	Target gene	Thermal conditions	References
GAPDH-F (CCTTCATTGACCTCAACTACAT)	GAPDH/Mammals	95 °C for 5 min; 35 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s; and final extension of 72 °C for 5 min.	Birkenheuer et al. (2003)
GAPDH-R (CCAAAGTTGTCATGGATGACC)			
16S + 1 (CTGCTCAATGATTTTT-TAAATTGCTGTGG)	16SrRNA/Ticks	10 cycles of 92 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min, followed by 32 cycles of 92 °C for 1 min, 54 °C for 35 s and 72 °C for 1,35 min, and final extension of 72 °C for 7 min.	Black and Piesman (1994)
16S-1 (CCGGTCTGAACTCAGAT-CAAGT)			
HC02198 (TAAACTTCAGGGT-GACCAAAAAATCA)	COX1/ Fleas	95 °C for 1 min, 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 10 s, and final extension of 72 °C for 5 min.	Folmer et al. (1994)
LCO1490 (GGTCAACAAAT-CATAAAGATATTGG)			
HepF300 (GTTTCTGACC-TATCAGCTTTCGACG)	18SrRNA/*Hepatozoon* 18SrRNA/*Hepatozoon*	94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 7 min.	Ujvari et al. (2004)
HepR900 (CAAATCTAA-GAATTTCACCTCTGAC)			
HEMO1 (TATTGGTTTTAA-GAACTAATTTTATGATTG)		94 °C for 3 min, 35 cycles of 94 °C for 45 s, 48 °C for 60 s and 72 °C for 1 min, and final extension of 72 °C for 7 min.	Perkins and Keller (2001)
HEMO2 (CTTCTCCTTCCTT-TAAGTGATAAGGTTCAC)			

was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) simulations were run for 10^9 generations with a sampling frequency of every 100 generations and a burn-in of 25%. The Maximum-likelihood (ML) analysis was inferred with RAxML-HPC BlackBox 7.6.3 (Stamatakis et al., 2008) (which includes an estimation of bootstrap node support), using 1000 bootstrapping replicates. The best model of evolution was selected by the program jModelTest2 (version 2.1.6) on XSEDE (Darriba et al., 2012), under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). All phylogenetic analyses were performed using CIPRES Science Gateway (Miller et al., 2010). The trees were examined in Treegraph 2.0.56-381 beta (Stover and 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 Clustal/W (Thompson et al., 1994) in Bioedit v. 7.0.5.3 (Hall, 1999). The number of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi) and DNA divergence between populations (group of hosts different species) that were estimated to explore the levels of genetic differentiation among the populations were determined using the program DnaSP 5, version 5.10.01 (Librado and Rozas, 2009).

3. Results

A total of 256 animals were captured. One hundred fifty-eight carnivores: Seventy-eight crab-eating foxes, 31 coatis and seven ocelots. One hundred and forty small mammals: 110 wild rodents (77 *T. fosteri*, 25 *O. mamorae* and 8*C. laticeps*) and 30 wild marsupials (14 *T. macrurus,* 11 *G. agilis*, 4 *M. domestica* and 1 *D. albiventris*). We also sampled blood of 42 domestic dogs.

One thousand five hundred and eighty-two ticks found parasitizing the sampled mammals were collected, of which 1033 (65.2% [115 adults and 918 nymphs]) belonging to *Amblyomma sculptum* Berlese species, 241 (15.2% [78 adults and 163 nymphs]) belonging to *Amblyomma parvum* Aragão species, 32 (2%) *Amblyomma ovale* Koch adults, one (0.06%) *Amblyomma tigrinum* Koch adult, one (0.06%) *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one (0.06%) *Rhipicephalus sanguineus* s.l. (Latreille) adult, four (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae (Table 2). Besides, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were collected. Seventy-five fleas (93.7%) were collected from 16 (5.4%) *T. fosteri* rodents, four (5%) from two (0.6%) *M. domestica,* and one (1.25%) fleas from one (0.3%) specimen of *T. macrurus.*

All 298 DNA animal samples amplified the predicted product for GAPDH gene with an average concentration of 145.3 (SD = 95,3) ηG/μL, which indicated a successful DNA extraction. The number of tick DNA samples extracted was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs and 39 (7.4%) pooled larvae with an average concentration of 45.9 (SD = 84,3) ηG/μL. Out of 523 sampled ticks, 31 (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the mitochondrial 16S rRNA tick gene and were excluded from analysis. A total of 39 pooled fleas samples were extracted with an average concentration of 7 (SD = 8,43) ηG/μL and only one sample didn't amplified the predicted product for cox-1 from fleas and was also was excluded from further analysis.

No gamont suggestive of *Hepatozoon* was found in the blood smears from sampled animals. Out of 298 sampled animals, 61 (78.2%) crab-eating foxes, 16 (38%) domestic dogs, five (71.4%) ocelots, two (8%) *O. mamorae* and one (1.2%) *T. fosteri* were positive for 18SrRNA *Hepatozoon* spp.-PCR based on Perkins and Keller (2001) protocol. Thirty crab-eating foxes samples (38.4%), 13 (30.9%) domestic dogs, 13 (41.9%) coatis, 12 (15.5%) *T. fosteri,*

Table 2
Ticks species collected from wild mammals captured between August 2013 and March 2015 in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Animal species	TICKS[a]									
	N° of anim.	Infest (%)	*A. sculptum*	*A. parvum*	*A.tigrinum*	*A. ovale*	*A.auricularium*	*R.(B.) microplus*	*R.sanguineus* s. l.	*Amblyomma*spp.
Cerdocyon thous	78	35 (44.8)	34 M;. 55F;.643N	21 M;. 34F;. 3N	1F	4 M. 1F				204L
Nasua nasua	31	22 (70.9)	10 M; 13F; 275N	11 M;. 6F;.12N		20 M;7F	3N	1F		21L
Leopardus pardalis	7	2 (28.5)		3 M;. 3F						
Canis familiaris	42	1 (2.3)	1F						1M	
Thrichomys fosteri	77	23 (29.8)	2N	116N						36L
Oecomys mamorae	25	1 (4)		1N						
Clyomys laticeps	8	3 (37.5)		13N			1N			7L
Thylamys macrurus	14	1 (7.1)		18N						1L
Monodelphis domestica	4	0 (0)								
Gracilinanus agilis	11	0 (0)								
Didelphis. albiventris	1	0 (0)								
Total	298	88 (29.6)	1033	241	1	32	4	1	1	269

L – larvae, N – nymph, M – adult male, F – adult female, N° anim. – number of sampled animals, No infest. – number of infested animals according to host species.
[a] *A. sculptum – Amblyomma sculptum. A. parvum – Amblyomma parvum. A. tigrinum – Amblyomma tigrinum. A. ovale – Amblyomma ovale. A. auricularium – Amblyomma auricularium. R. (B.) microplus – Rhipicephalus(Boophilus) microplus. R. sanguineus* s. l. *– Rhipicephalus sanguineus* sensu lato.

nine (36%) *O. mamorae* and one (7.1%) *T. macrurus* showed positive results in 18SrRNA *Hepatozoon* spp.-PCR based on Ujvari et al. (2004) protocol. Twenty (25.6%) crab-eating foxes and ten (23.8%) domestic dogs samples showed positive results for both protocols, which allowed the concatenation of obtained sequences. All arthropod (fleas and ticks) DNA samples were negative for both PCR protocols. All sequences obtained from the positive animals were deposited in the international database Genbank under the following accession numbers: KT881500 –KT881535 and KX776286 – KX776408.

All the 18S rRNA *Hepatozoon* sequences obtained from domestic dogs (n = 29), showed 99–100% identity (100% of coverage) with *H. canis* previously deposited in GenBank (AY150067) by BLAST analysis. The 18S rRNA *Hepatozoon* sequences obtained from *C. thous* (n = 91) showed 98–99% identity (97–100% of coverage) with *Hepatozoon* spp. sequences obtained from lizards from Portugal (JX531925), *H. felis* isolate Cuiaba (KM435071) and other *H. felis* genotype from Japan (AB771501). The sequences amplified from *T. fosteri* spleen samples (n = 13) showed 97–99% identity (97–100% of coverage) with *Hepatozoon* sp. sequence obtained from *Crotalus durissus terrificus* from Brazil (KC342523) and *Hepatozoon fitzsimonsi* (KR069084). Additionally, *Hepatozoon* sequences obtained from *O. mamorae* (n = 11) showed 99% identity (100% of coverage) with *Hepatozoon* sp. sequence amplified from a Brazilian lizard (KM234617) and *Hepatozoon* spp. sequences amplified from rodents in Brazil (KU667309). The unique 18S rRNA *Hepatozoon* sequence obtained from *T. macrurus* also showed 99% identity (88% of coverage) with *Hepatozoon* spp. sequences amplified from rodents in Brazil (KU667309). The 18S rRNA *Hepatozoon* sequences obtained from *L. pardalis* sequences (n = 5) and from *N. nasua* (n = 12) showed 98–99% identity (99–100% of coverage) with *H. felis* isolate Cuiaba (KM435071) and *H. felis* isolate Japan (AB771501), respectively (Table 3).

The phylogenetic tree of *Hepatozoon* spp. 18S rRNA sequences clustered basically in two large branches: one of them composed by *Hepatozoon* sequences amplified from *C. thous, C. L. familiaris, N. nasua* and *L. pardalis* from the present study and sequences of *H. canis, H. americanum* and *H. felis* retrieved from Genbank. The other large branch grouped: i. *Hepatozoon* sequences amplified in wild rodents species (*T. fosteri* and *O. mamorae*) and *T. macrurus* marsupial species from the present study; ii. *Hepatozoon* sequences amplified from rodents sampled in from Brazil (Mato Grosso and São Paulo, states) and other countries (Chile, Spain, Thailand, Ghana); iii. *Hepatozoon* sequences amplified from reptiles (snakes, tortoise and lizards) and amphibians previously deposited in Genbank, supported by significant clade support. *Adelina* sp., *Theileria* sp., *Isospora* sp., and *Sarcocystis* sp. were used as outgroups (Fig. 2).

Nucleotide polymorphisms and DNA divergence between populations (groups of different host species) were also analyzed among the sequences obtained in the present study. The alignments were analyzed in separate, because Perkins and Keller (2001) and Ujvari et al. (2004) PCR protocols amplify different regions of 18SrRNA gene. Both DNA fragments obtained from two different regions from 18SrRNA gene showed to be quite conserved. The analysis of nucleotide polymorphisms of 18S rRNA sequences obtained from both protocols showed a small number of haplotypes (4 (Perkins and Keller (2001) PCR protocol) and 7 (Ujvari et al. (2004) protocol)) among the population of different hosts sampled. The alignment of sequences obtained from Ujvari et al. (2004) protocol showed seven haplotypes (haplotype diversity (hd): 0.771; Standard Deviation (SD): = 0.032) and nucleotide diversity (Pi) of 0.02084 (SD = 0.00142). Within the *T. fosteri* group, three different *Hepatozoon* haplotypes and a noteworthy Pi (0.00813) were observed. Except for the *Hepatozoon* sequences of rodents, divergence values between sampled host populations, based on the

Table 3
Maximum identity of 18S rRNA *Hepatozoon* spp. sequences detected in wild and domestic animals by BLAST analysis.

Animal species	Number of sequences analized	Protocol	% coverage by BLAST®	% identity by BLAST®
Canis familiaris	29	Perkins and Keller (2001) and Ujvari et al. (2004)	100%	99–100% *H. canis* (AY150067).
Cerdocyon thous	14	Perkins and Keller (2001)	97%	98–99% of identity with *Hepatozoon* spp. from lizards from Portugal (JX531925)
Cerdocyon thous	47	Perkins and Keller (2001)	99–100%	98–99% *H. felis* isolate Cuiaba (KM435071)
Cerdocyon thous	30	Ujvari et al. (2004)	100%	98% *H. felis* from Japan (AB771501)
Thrichomys fosteri	1	Perkins and Keller (2001)	100%	99% *Hepatozoon* spp. from *Crotalus durissus terrificus* from Brazil (KC342523)
Thrichomys fosteri	12	Ujvari et al. (2004)	97–99%	97% *H. fitzsimonsi* (KR069084).
Oecomys mamorae	2	Perkins and Keller (2001)	100%	99% *Hepatozoon* spp. from a lizard from Brazil (KM234617)
Oecomys mamorae	9	Ujvari et al. (2004)	100%	99% *Hepatozoon* spp. from a rodent in Brazil (KU667309)
Thylamys macrurus	1	Ujvari et al. (2004)	88%	99% *Hepatozoon* spp. from arodent in Brazil (KU667309)
Leopardus. pardalis	5	Perkins and Keller (2001)	98%	98–99% *H. felis* isolate Cuiaba (KM435071)
Nasua nasua	12	Ujvari et al. (2004)	99%	98–99% *H. felis* isolate Japan (AB771501)

alignment of sequences obtained from Perkins and Keller (2001) PCR protocol, was very low, probably because the alignment generated only four haplotypes (hd: 0.4392; SD: = 0.048) with low number of variable sites (3) to analyze. Although the number of haplotypes found had been small, some differences were observed analyzing the DNA divergence between populations (group of different host species). Analyzing the alignment of 18S rRNA *Hepatozoon* sequences from both protocols, the Pi value among the rodent populations of *T. fosteri* and *O. mamorae* was high compared to other host species. The alignment of 18S rRNA *Hepatozoon* sequences obtained from Ujvari et al. (2004) protocol showed a high level of divergence between the haplotypes found in domestic dogs and *T. fosteri* rodents groups (Pi = 0.04293). Additionally, the Pi value (0.0074) found between the 18S rRNA *Hepatozoon* sequences obtained from *N. nasua* and *C. thous* populations was lower in relation to other host species.

4. Discussion

The present study showed the presence of *Hepatozoon* spp. in blood or spleen samples of wild carnivorous, domestic dogs, rodents and marsupials in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil. Molecular analyses based on 18S rRNA gene revealed a high occurrence of *Hepatozoon* spp. among sampled animals.

Five out seven free-living ocelots captured showed to be positive for *Hepatozoon* spp. The occurrence of *Hepatozoon* was higher (83.3%) than that reported by Metzger et al. (2008) in wild *L. pardalis* (17.2%) sampled in the states of Maranhão and Ceará, northeastern Brazil. Based on the phylogenetic analysis, three 18S rRNA *Hepatozoon* sequences detected in sampled *L. pardalis* and sequences previously detected in wild felids from northeastern Brazil (Metzger et al., 2008) were closely related to *Hepatozoon* sp. genotype from Spanish cats. A genotype closely related to *Hepatozoon* sp. isolated from Spanish cats has also been found in a pampas gray fox (*Lycalopex gymnocercus*) that was co-infected with canine distemper virus in Argentina (Giannitti et al., 2012). The gray fox was euthanized after had showed severe incoordination; on necropsy, *Hepatozoon* cysts were observed in skeletal and myocardium muscles. Keeping in mind that this genotype closely related to *H. felis* is circulating in Brazil, more studies are much needed in order to monitor the impact of this parasite among the Brazilian wildlife.

The occurrence of *Hepatozoon* spp. among sampled crab-eating foxes found in the present study was very high (91%) when compared to previous studies, which had found percentages of positivity of 43.1% in wild *C. thous* sampled in the state of Espírito Santo, southeastern Brazil (Almeida et al., 2013), and 55% in wild *C. thous* sampled in the state of Rio Grande do Sul, southern Brazil (Criado-Fornelio et al., 2006). The phylogenetic analysis demonstrated that the *Hepatozoon* genotype infecting crab-eating foxes in the region of Pantanal was closely related to *Hepatozoon* spp. Isolate Curupira 2, an *H. americanum*-related organism, and *H. americanum*. The *H. americanum*-related haplotypes have been reported in crab-eating foxes from the Brazilian states of Rio Grande do Sul (Criado-Fornelio et al., 2006), São Paulo (André et al., 2010), and Espírito Santo (Almeida et al., 2013). Thus, this haplotype appears to be common among foxes in Brazil. Although *H. americanum* is

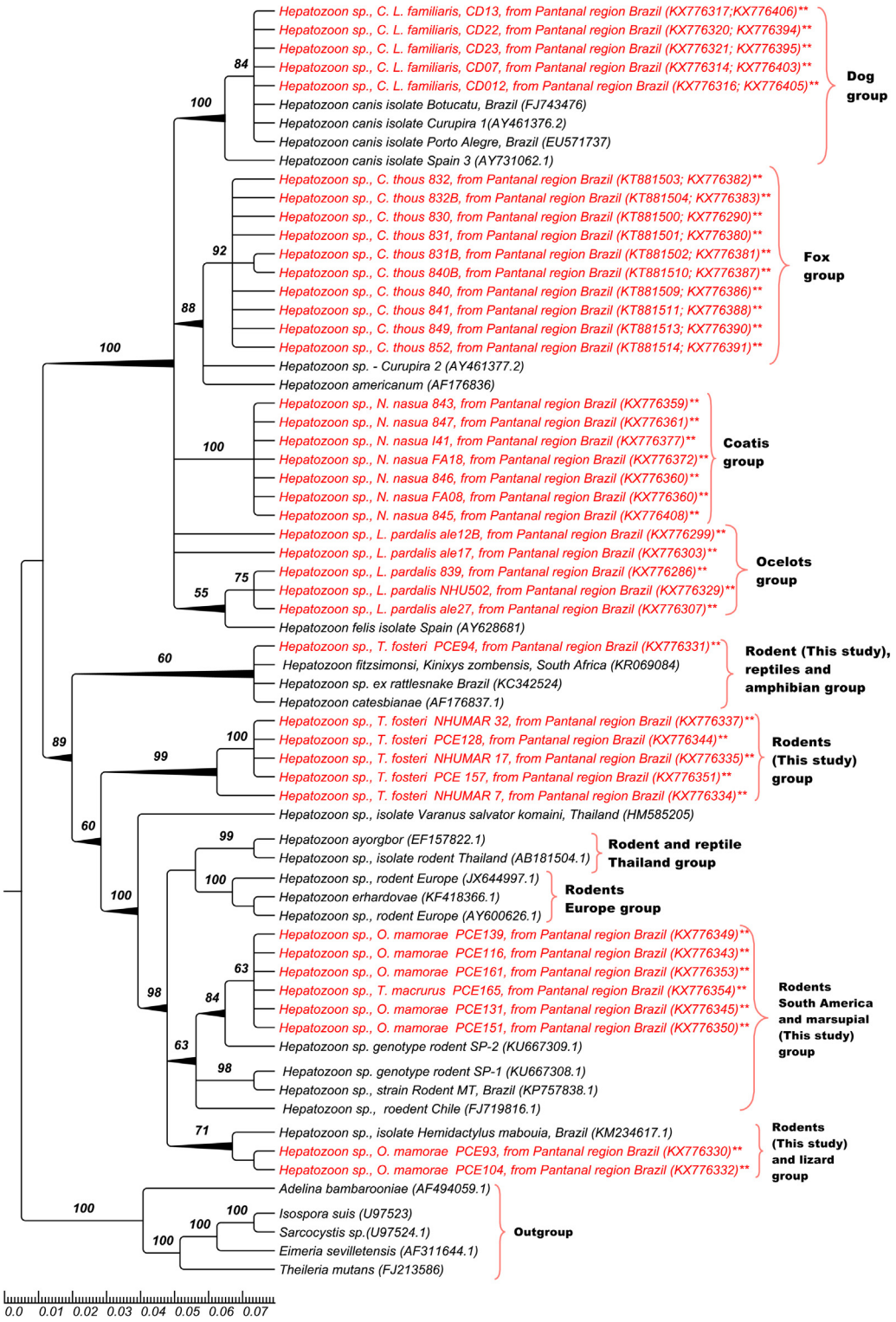

Fig. 2. Phylogenetic tree based on an alignment of 1900 bp fragment of *Hepatozoon* spp. 18SrRNA sequences, using Bayesian inference (BI) method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50.

considered a high pathogenic protozoa species for domestic dogs in the United States (Mathew et al., 1998), the *H. americanum*-related haplotype-infected crab-eating foxes in Brazil were apparently healthy (Criado-Fornelio et al., 2006; André et al., 2010; Almeida et al., 2013).

Hepatozoon canis infection was more prevalent (43.2%) among domestic dogs sampled in the present study than in dogs (3.63%) from urban areas from the same state of Mato Grosso do Sul, Brazil (Ramos et al., 2015). The higher prevalence of *H. canis* among dogs from rural areas when compared to dogs from urban areas has been already reported in several states from Brazil (Rubini et al., 2008; Gomes et al., 2010; Ramos et al., 2010; Miranda et al., 2014; Ramos et al., 2015). The phylogenetic positioning reinforced the hypothe-

sis that the domestic dogs from rural areas in Brazil are commonly infected by *H. canis* (Miranda et al., 2014).

To the best of authors' knowledge, the present study reported the first molecular detection of *Hepatozoon* spp. among coatis. Although Rodrigues et al. (2007) had previously detected *Hepatozoon* in two coatis from the state of Minas Gerais, southeastern Brazil, the diagnosis relied only on morphological and morphometric features of gametocytes in blood-stained smears. The *Hepatozoon* sequences detected in coatis were grouped within the large branch composed by the closely related *H. canis, H. americanum* and *H. felis* sequences. Similarly, *Hepatozoon* detected in raccoons (*Procyon lotor),* another member of Procyonidae family from the United States, showed to be closely related to *H. canis* (Allen et al., 2011). Considering that coatis can represent preys for wild felids (Novack et al., 2005), future studies should be performed in order to investigate the role of coatis as source of *Hepatozoon* infection for wild carnivores in Pantanal biome.

Although *Hepatozoon* spp. oocysts have been found in *A. ovale* (Rubini et al., 2009) and *R.* (*B.*) *microplus* (Miranda et al., 2011) ticks hemocoel, only *A. ovale* has been showed to be a competent vector for *Hepatozoon* in Brazil (Rubini et al., 2009; Demoner et al., 2013). In the present study, no tick, even *A. ovale,* was positive for *Hepatozoon* spp. Similar results have been found in northern Pantanal (Melo et al., 2016), where only one (1/930) *A. sculptum* was positive for *Hepatozoon* spp. Similarly, *H. canis* oocysts were found in only one (1/31) *R.* (*B.*) *microplus* ticks' hemocoel in an endemic area for canine hepatozoonosis in southeastern Brazil (Demoner et al., 2016). In fact, both *A. sculptum* and *R. microplus* species seem to show little or no importance in the hepatozoonosis epidemiology (Demoner et al., 2013; Demoner et al., 2016). Although the role of fleas as invertebrate hosts for *Hepatozoon* species infecting rodents in the United States have been proposed by Watkins et al. (2006), the *P.* (*P.*) *b. bohlsi* fleas collected from rodents in the present study did not show positivity for *Hepatozoon* in the molecular assays. Also, the role of other transmission routes, such as transplacental and predation, and the participation of different arthropods species as source of *Hepatozoon* infection should be better investigated in endemic areas.

The prevalence of *Hepatozoon* among wild rodents (*T. fosteri* and *O. mamorae*) was 21.8% (n = 24/110), lower than that reported (55.2%) in rodents (*O. nigripes, O. flavescens, Akodon sp., Necromys lasiurus* and *Sooretamys angouya*) sampled in an endemic area for canine hepatozoonosis in the state of São Paulo state, southeastern Brazil (Demoner et al., 2016). However, the found prevalence was higher than that reported (7.1%) in *C. callosus* rodents from northern Pantanal, state of Mato Grosso (Wolf et al., 2016). Although *Hepatozoon* DNA has been detected in wild rodents from Brazil (Demoner et al., 2016; Wolf et al., 2016), this is the first molecular detection of this parasite in *T. fosteri* and *O. mamorae* rodents and in *T. macrurus* marsupial in Brazil.

Although the transmission of *H. americanum* has been experimentally confirmed by the ingestion of cysts containing – rodent tissues by dogs (Johnson et al., 2009), previous studies showed evidence that the *Hepatozoon* species found in free-living rodents in Brazil differ from those detected in domestic and wild canids (Maia et al., 2014; Demoner et al., 2016). Herein, the phylogenetic positioning and DNA divergence analysis reinforce the hypothesis that the transmission of *Hepatozoon* from preys (rodents) to canids is a rare event and may not contribute to the spread of the parasite among canids in Brazil (Maia et al., 2014; Demoner et al., 2016).

Although the transmission of *Hepatozoon* spp. by carnivorism has been well documented in systems involving snakes as intermediate hosts, and frogs or lizards as paratenic hosts (Smith, 1996; Smith et al., 1999), Sloboda et al. (2008) showed that snakes could get experimentally infected by feeding tissues from *Hepatozoon*-infected rodents. Furthermore, Allen et al. (2011) and Demoner et al. (2016) detected *Hepatozoon*-rodent sequences closely related to *Hepatozoon*-reptile sequences. In addition to this, monozoic cysts containing cystozoites have been recently found in the lung of a free-living wild rodent sampled in the state of São Paulo State, Brazil (Demoner et al., 2016). Herein, the phylogenetic positioning of *Hepatozoon* detected in wild rodents suggested a possible transmission of *Hepatozoon* species between rodents and reptiles and amphibians by predation. Further studies focusing on the detection of monozoic cysts in rodents' tissues should be performed in order to support this hypothesis.

Although the *Hepatozoon* fragments obtained from two 18SrRNA different regions were quite conserved, nucleotide polymorphisms and DNA divergence between populations (groups of different host species) were observed in the present study. The present study provided some novel data concerning the *Hepatozoon* spp. diversity in Brazil. Within the *T. fosteri* group, three different *Hepatozoon* haplotypes and a noteworthy Pi value (0.00813) were observed. In addition to this, the Pi value found among the rodent populations of *T. fosteri* and *O. mamorae* was higher than that found among other host species, suggesting some degree of *Hepatozoon* genetic diversity among the population of wild rodents from Brazil. This fact may be due to the diversity of hosts (rodents, reptiles and amphibians) sharing the same closely related *Hepatozoon* species, revealed by the phylogenetic analysis.

In conclusion, the present study showed a high occurence of *Hepatozoon* spp. among wild animals in southern Pantanal region, Brazil. Carnivores, rodents and domestic dogs seemed not to share the same *Hepatozoon* species in the studied region. Rodents may play a role in the routes of *Hepatozoon* transmission to reptiles and amphibians. An evidence of some degree of *Hepatozoon* genetic diversity among the population of wild rodents from Brazil was reported for the first time.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetpar.2017.02.023.

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