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Occurrence and molecular characterization of hemoplasmas in domestic dogs and wild mammals in a Brazilian wetland

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ABSTRACT

Hemotropic mycoplasmas are known to cause anemia in several mammalian species. The present work aimed to investigate the occurrence of *Mycoplasma* spp. in wild mammals, domestic dogs and their respective ectoparasites, in southern Pantanal region, central-western Brazil. Between August 2013 and March 2015, 31 *Nasua nasua*, 78 *Cerdocyon thous*, seven *Leopardus pardalis*, 42 dogs, 110 wild rodents, and 30 marsupials were trapped and ectoparasites (ticks and fleas) found parasitizing the animals were collected. Mammals and ectoparasites DNA samples were submitted to conventional PCR assays for *Mycoplasma* spp. targeting 16S rRNA and *RnaseP* genes. Twenty-four *N. nasua*, three *C. thous,* two domestic dogs, one *L. pardalis* and one wild rodent were positive for 16S rRNA PCR protocols. Fourteen *N. nasua* samples were also positive in *RnaseP* PCR. No marsupial or arthropod showed positivity for *Mycoplasma* spp. The phylogenetic analyses based on 16S rRNA gene showed that all sequences obtained from dogs, two sequences obtained from *C. thous* and ten sequences obtained from *N. nasua* showed to be closely related to *Mycoplasma haemocanis/Mycoplasma haemofelis* species. Genotypes closely related to '*Candidatus* Mycoplasma haemominutum' and *Mycoplasma haemomuris* were detected in the *L. pardalis* and in the wild rodent, respectively. Probably a novel *Mycoplasma* genotype, closely related to a sequence obtained from a Brazilian capybara was detected in 14 *N. nasua*, based on a concatenated phylogenetic analysis of 16S rRNA and *RnaseP* genes. The present study revealed that wild animals in southern Pantanal region, Brazil, are exposed to different species of hemoplasmas.

1. Introduction

Hemotropic mycoplasmas (hemoplasmas) are epicellular erythrocytic bacteria lacking cell wall. In contrast to several mucosal mycoplasmas, hemoplasmas have never been grown successfully in culture so far. These pathogens are known to be the causative agents of infectious anemia in several mammalian species and may induce acute hemolysis in some cases (Tasker, 2010). The disease is characterized by anorexia, lethargy, dehydration, weight loss and in some cases, can lead to death (Willi et al., 2007). Furthermore, hemotropic mycoplasmas are considered emergent zoonotic agents, mainly in immunocompromised individuals or those highly exposed to arthropod vectors (dos Santos et al., 2008; Maggi et al., 2013a).

The transmission of hemoplasmas between domestic cats and dogs seems to occur mainly by bloodsucking arthropods, such as ticks and fleas, blood transfusion, contaminated fomites and transplacentally (Seneviratna et al., 1973; Messick, 2003; Lappin et al., 2006). In addition to this, infections through biting and fighting are considered another possible routes of hemoplasma transmission (Tasker, 2010).

Although hemoplasmas have been detected in domestic cats (Braga et al., 2012; Miceli et al., 2013; André et al., 2014; Santis et al., 2014) and dogs (Ramos et al., 2010; Alves et al., 2014; Valle et al., 2014; Soares et al., 2016) from several localities in Brazil, few reports have documented the occurrence of hemoplasma species in wild animals. For

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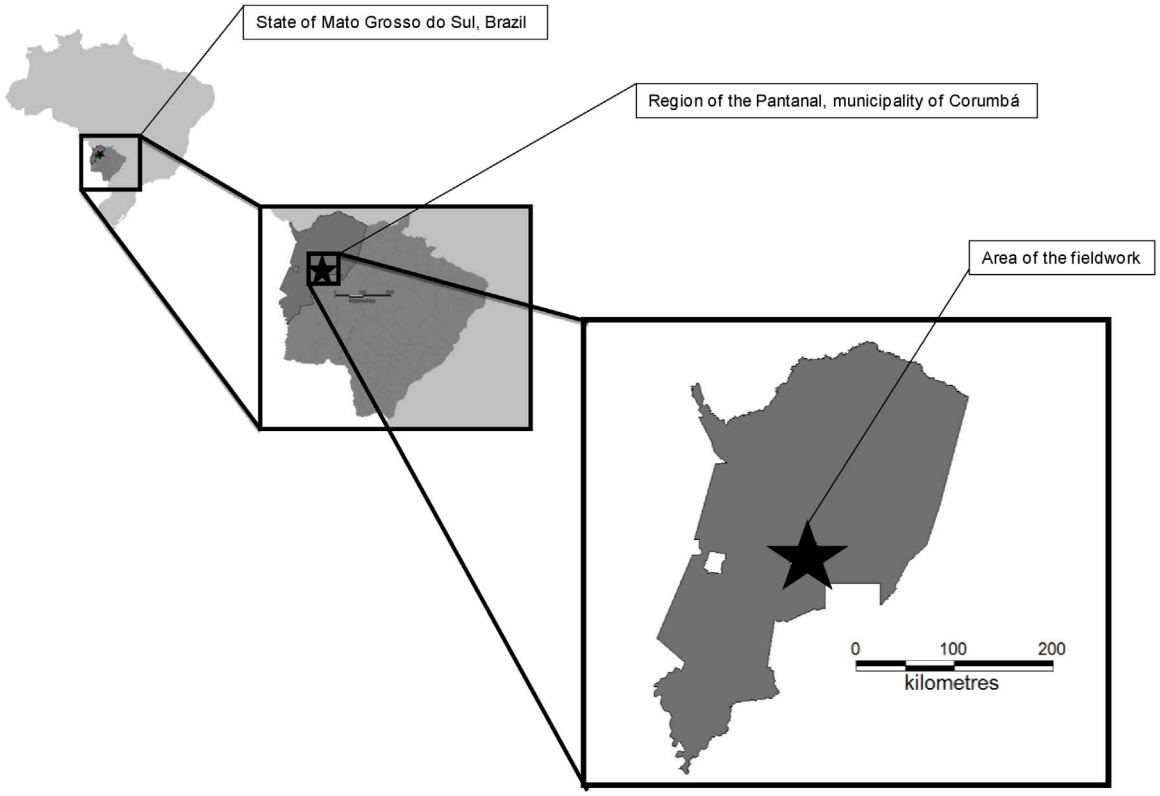

Fig. 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

instance, hemoplasmas have been detected in wild carnivores maintained in captivity in zoos (Willi et al., 2007; Guimarães et al., 2007; André et al., 2011), wild rodents (Vieira et al., 2009; Conrado et al., 2015; Gonçalves et al., 2015), monkeys (Bonato et al., 2015), and deer (Grazziotin et al., 2011) in Brazil.

Due to the lack of information about the epidemiology and routes of transmission of hemoplasmas among the wildlife from Brazil, the present study aimed to investigate the occurrence of hemotropic mycoplasmas 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 semi deciduous 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, being well known for its rich biodiversity. Two well-defined seasons are recognized in the studied region: a rainy summer (October to March) and a dry winter (April to September) (Sousa et al., 2017).

Between August 2013 and March 2015, a total of 256 mammals were captured in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul, including 158 carnivores, among 78 crab-eating foxes (*C. thous*), 31 coatis (*N. nasua*) and seven ocelots (*L. pardalis*); 140 small mammals, among 110 wild rodents (77 *Thrichomys fosteri*, 25 *Oecomys mamorae* and 8 *Clyomys laticeps*) and 30 wild marsupials (14 *Thylamys macrurus,* 11 *Gracilinanus agilis*, 4 *Monodelphis domestica* and 1 *Didelphis albiventris*). Forty-two blood samples from domestic dogs cohabiting the same studied area were also collected. All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license numbers38145, 38787-2) and endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus Jaboticabal) n° 006772/13 (Sousa et al., 2017).

One thousand five hundred and eighty-two ticks parasitizing the sampled mammals were collected, including 1033 (65.2% [115 adults and 918 nymphs]) *Amblyomma sculptum* Berlese specimens, 241 (15.2% [78 adults and 163 nymphs]) *Amblyomma parvum* Aragão specimens, 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. Furthermore, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were also collected (Sousa et al., 2017).

DNA was extracted from 200 µL of each whole blood (158 wild carnivores and 42 domestic dogs) and 10 mg of spleen (140 small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. The amount of tick DNA extracted was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae. While DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. A total of 39 pooled fleas samples were submitted to DNA extraction. The fleas DNA extraction was also performed in pools consisting of up to five individuals. Ticks and fleas were macerated and submitted to DNA extraction, using the same kit before mentioned (Sousa et al., 2017).

In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH), ticks mitochondrial 16S rRNA and fleas cytochrome-c oxidase subunit I (Cox-1) genes were performed (Table 1). All 298 DNA animal samples amplified the predicted product for GAPDH gene. Out of 523 sampled ticks, 31

Table 1
Oligonucleotides sequences, target genes and thermal conditions used in conventional PCR assays targeting endogenous genes and 16S rRNA and *RNaseP* fragments of hemoplamas 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′)	Gene	Thermal conditions	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	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)
16S + 1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1 (CCGGTCTGAACTCAGATCAAGT)	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)
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	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)
HemMycop16S–41 s (GYATGCMTAAYACATGCAAGTCGARCG) HemMyco16S-938as (CTCCACCACTTGTTCAGGTCCCCGTC)	16S rRNA 1° set/ Hemoplasmas	94 °C for 2 min, 55 cycles of 94 °C for 15 s, 68 °C for 15 s and 72 °C for 18 s, and final extension of 72 °C for 1 min.	Maggi et al. (2013b)
HemMycop16S- 322 s (GCCCATATTCCTACGGGAAGCAGCAGT) HemMycop16S-1420as (GTTTGACGGGCGGTGTGTACAAGACC)	16S rRNA 2° set/ Hemoplasmas	94 °C for 2 min, 55 cycles of 94 °C for 15 s, 68 °C for 15 s and 72 °C for 18 s, and final extension of 72 °C for 1 min.	Maggi et al. (2013b)
HemoMyco RNaseP30 s (GATKGTGYGAGYATATAAAAAATAAARCTCRAC) HemoMyco RNaseP200as (GMGGRGTTTACCGCGTTTCAC)	*RNaseP*/ Hemoplasmas	94 °C for 2 min, 55 cycles of 94 °C for 15 s, 59 °C for 15 s and 72 °C for 18 s, and final extension of 72 °C for 1 min.	Maggi et al. (2013b)

(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 tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses (Sousa et al., 2017).

Previously described PCR protocols based on 16S rRNA gene were performed in order to amplify *Mycoplasma* spp. DNA, using two sets of oligonucleotides (Table 1). Five microliters of DNA were used as a template in 25 μL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0.8 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5U Taq DNA Polymerase (Life Technologies®) and 0.3 μM of each oligonucleotide. *Mycoplasma haemofelis* DNA obtained from a naturally infected cat (Miceli et al., 2013) and ultra-pure sterile water were used as positive and negative controls, respectively. 16S rRNA-*Mycoplasma*spp.-positive samples were additionally submitted to a previously described *RNaseP* gene-*Mycoplasma* spp. (165 bp) PCR assay (Table 1). The sequencing was performed using the BigDye® Terminator v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, USA) (Sanger et al., 1977).

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) in order to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of 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 previously deposited in an international database (GenBank) (Benson et al., 2002). All sequences that showed appropriate quality standards and identity with hemoplasmas were deposited in Genbank, except *RNaseP* sequences, whose small fragment size (165pb) precluded being deposited in the international database. Samples showing positive results for both 16S rRNA and *RNaseP* protocols had their sequences concatenated, using the fragment merger software, version 1 (Bell and Kramvis, 2013). The sequences were aligned using Clustal/W (Thompson et al., 1994) in Bioedit v. 7.0.5.3 (Hall, 1999) and MAFFT software, version 7 (Katoh and Standley, 2013).

Phylogenetic inferences were based on Bayesian and Maximum Likelihood (ML) methods. The Bayesian inference analysis 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). Phylogenetic inferences based on ML and Bayesian methods were performed using the evolutionary model GTR + G + I. *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) were used as outgroups in 16S rRNA analysis. *Clostridium innocuum* (GQ456215; U64878) was used as outgroup in concatenated analysis (16S rRNA + *RnaseP*).

Logistic regression models were employed to assess the effect of the putative predictor variables (i.e. gender and animal species) on the logit of the probability of positivity for hemoplasmas. All analyses were performed using R software 3.0.2 (R Core Team, 2013).

Additionaly, an analysis of nucleotide polymorphisms of 16S 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) and nucleotide diversity (Pi) were estimated to explore the levels of genetic differentiation among the species of hosts with higher number of positive samples for hemoplasmas, using the program DnaSP 5, version 5.10.01 (Librado and Rozas, 2009).

3. Results

Twenty-four (77.4%) *N. nasua,* three (3.8%) *C. thous,* two (4.7%) domestic dogs, one (14.2%) *L. pardalis* and one (0.9%) wild rodent (*O. mamorae*) were positive for both hemoplasmas-PCR protocols based on16S rRNA gene. Fourteen (4.6%) *N. nasua* were also positive in PCR for hemoplasmas based on *RnaseP* gene. None of sampled marsupials orarthropods (tick or flea) showed positivity for *Mycoplasma* spp. All 16S rRNA sequences obtained from positive animals were deposited in the international database Genbank under the following accession numbers: KY002649-KY002679. Logistic regression results evidenced no significant difference ($P > 0.05$) between sexes for positivity in PCR assays for *Mycoplasma* spp. However, compared to other animal species (ocelots, crab-eating foxes, domestic dogs and wild rodents), coatis showed a significantly (p-value < 0.01) higher chance to have *Mycoplasma* spp. DNA detected in whole blood samples. Odds ratios for *Mycoplasma* spp. positivity between coatis and each of other animal species were all above 20.57.

The BLAST analysis was performed with sequences obtained from

Table 2
Maximum identity of 16S rRNA and *RnaseP Mycoplasma* spp. sequences detected in wild and domestic mammals sampled in southern Pantanal, Brazil, by BLAST analysis.

Animal species	Number of sequences analized	Target Gene	Percentage of identity by BLAST®- analysis
O. mamorae	1	16S rRNA	Uncultured *Mycoplasma* sp. from a wild rodent, Brazil (KT215634) 100%
L. pardalis	1		*'Candidatus* Mycoplasma haemominutum' (EU839985) 99%
C. familaris	2		*Mycoplasma haemocanis* (KP715859) 100%
C. thous	3		*Mycoplasma haemocanis* (KP715859) 100%
N. nasua	24		*Mycoplasma* sp. (Raccon USA) (KF743735) 99%
N. nasua	12	*RNaseP*	*Mycoplasma haemofelis* (JN368074) 91%

an overlapping of approximately 600 bp of two hemoplasmas-16S rRNA fragments (800 bp and 1000 bp), and from 165 bp fragment of hemoplasmas-*RnaseP* gene. Twenty-four hemoplasmas-16S rRNA sequences obtained from *N. nasua* blood samples showed 99% identity with a sequence of *Mycoplasma* sp. detected in a raccon (*Procyon lotor*) sampled in the United States (KF743735). Three hemoplasmas-16S rRNA sequences obtained from *C. thous* blood samples and two hemoplasmas-16S rRNA sequences obtained from dogs' blood samples showed 100% identity with *M. haemocanis* (KP715859). Hemoplasmas-16S rRNA sequences obtained from one *L. pardalis* blood sample and one *O. mamorae* spleen sample showed 100% identity with '*Candidatus* Mycoplasma haemominutum' (EU839985) and with *Mycoplasma* sp. sequence obtained from a Brazilian wild rodent (KT215634), respectively, based on BLAST analysis. Additionally, all 14 hemoplasmas-*RNAseP* sequences obtained from *N. nasua* blood samples presented 91% of identity with *M. haemofelis* (JN368074) (Table 2).

All hemoplasmas-16SrRNA sequences obtained from dogs' blood samples (KY002678, KY002679), two hemoplasmas-16S rRNA sequences obtained from *C. thous* (KY002677, KY002675) and ten hemoplasmas-16S rRNA sequences obtained from *N. nasua* (KY002652, KY002653, KY002657, KY002661, KY002662, KY002665, KY002667, KY002672, KY002673, KY002674) were grouped in the same large branch comprising 16S rRNA sequences of *M. haemocanis* (HQ918287, AY150973) and *M. haemofelis* (EU930823, KJ135316) previously deposited in Genbank, with clade support of 98 and 100, based on ML and Bayesian analyses, respectively (Figs. 2 and 3). A hemoplasma-16S rRNA sequence obtained from one *C. thous* blood sample (KY002676) and 14 hemoplasmas-16S rRNA sequences obtained from *N. nasua* blood samples (KY002651, KY002654, KY002655, KY002656, KY002658, KY002659, KY002660, KY002663, KY002664, KY002666, KY002668, KY002669, KY002670, KY002671) were pooled in a separate clade from the others *Mycoplasma* species previously detected, with clade support of 87 and 100,based on ML and Bayesian analyses, respectively (Figs. 2 and 3), but closely related with a new hemoplasma genotype obtained from a Brazilian capybara (FJ667773) and in the same large branch of *M. coccoides* (AY171918), '*Candidatus* Mycoplasma turicensis' (KJ095696) and a new genotype detected in a synanthropic rodent from Brazil (KM203857), with clade support of 53 and 69, based on ML and Bayesian analyses, respectively (Figs. 2 and 3). The hemoplasma-16S rRNA sequence obtained from an *O. mamorae* wild rodent (KY002650) was positioned in the same clade of one hemoplasma-16S rRNA sequence obtained from *T. fosteri* wild rodent sampled in the same region of Pantanal, Brazil (KT215621), with clade support of 100 and 100, based on ML and Bayesian analyses, respectively (Figs. 2 and 3). All hemoplasmas-16S rRNA sequences obtained from *N. nasua, C. thous, O. mamorae* and dogs were grouped into the same large branch of "haemofelis group", based on ML and Bayesian analyses (Figs. 2 and 3). The hemoplasma-16S rRNA sequence obtained from one *L. pardalis* (KY002649) blood sample was placed in the same clade of '*Candidatus* Mycoplasma haemominutum' (KJ135327), with clade support of 93 and 100, based on ML and Bayesian analyses, respectively, and in the same large branch of "suis group" (Figs. 2 and 3).

Regarding the concatenated phylogenetic analysis of hemoplasmas based on *RnaseP* and 16S rRNA genes, six *Mycoplasma* spp. sequences obtained from *N. nasua* blood samples were pooled in a separate clade from the others *Mycoplasma* species previously detected, with clade support of 100 and 100, based on ML and Bayesian analyses, respectively (Figs. 4 and 5), but in the same large branch of *M. coccoides* (AY171918; EU078619) and '*Candidatus* Mycoplasma turicensis' (KJ095696; EF212002), with clade support of 91 and 100, based on ML and Bayesian analyses, respectively (Figs. 4 and 5). The remaining eight hemoplasmas *RnaseP* + 16S rRNA concatenated sequences obtained from *N. nasua* blood samples were grouped in the same large branch of *M. haemofelis* (KJ135316; EU078617) and *M. haemocanis* (AY150973; EU078618) with clade support of 100 and 100, based on ML and Bayesian analyses, respectively (Figs. 4 and 5).

One sequence obtained from *C. thous* blood sample (KY002676) and 14 sequences obtained from *N. nasua* blood samples (KY002651, KY002654, KY002655, KY002656, KY002658, KY002659, KY002660, KY002663, KY002664, KY002666, KY002668, KY002669, KY002670, KY002671), were pooled in a separate clade from other *Mycoplasma* species previously detected, based on the 16S rRNA and *RnaseP* phylogenetic analysis (Figs. 2–5).

The 16S rRNA alignment (1247pb) of 24 sequences obtained from coatis blood samples showed the presence of four haplotypes, haplotype diversity (hd): 0.569; standard deviation (SD): = 0.074, nucleotide diversity (Pi): 0.05270 (SD = 0.00563) and average number of nucleotide differences (k):19.44565 (Table 3). Nucleotide polymorphisms were not found in 120 bp *RnaseP* gene fragment alignment of 14 sequences obtained from coatis blood samples. Fourteen sequences shared the same haplotype (#hap 1), while the sequences (10) closed related to *M. haemocanis/M. haemofelis* showed tree different haplotypes (#hap2, #hap3, #hap4) (Fig. 6).

4. Discussion

The present study showed the presence of *Mycoplasma* spp. DNA in blood or spleen samples of wild carnivores, domestic dogs and wild rodents in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil. To the best authors' knowledge, this was the first molecular detection of *Mycoplasma* spp. among wild coatis and crab-eating foxes from Brazil.

The molecular analyses of hemoplasmas-16S rRNA sequences revealed a low (2/42) occurrence of *M. haemocanis* among sampled dogs. The occurrence of *M. haemocanis* (4.7%) found in the present study was similar to that reported in dogs sampled in an urban area (4.2%) located in the same state of Mato Grosso do Sul (Soares et al., 2016). On the other hand, the found occurrence of *M. haemocanis* was higher than that found in dogs sampled in the states of Pernambuco (Ramos et al., 2010), northeastern Brazil (1/205), and São Paulo (Alves et al., 2014) (3/154), but lower than that found in dogs sampled in the state of Rio Grande do Sul (17/331) (Valle et al., 2014). Only two studies reported the molecular detection of '*Candidatus* M. haemato-parvum' in dogs from Brazil, in the states of São Paulo (Alves et al., 2014) and Rio Grande do Sul (Valle et al., 2014). The results of the present study reinforces that the mostly common species of *Mycoplasma* parasitizing dogs in Brazil is *M. haemocanis*.

A genotype closely related to *M. haemocanis/M. haemofelis* was detected in blood samples from two crab-eating foxes in the present study, based on the molecular analysis of 16SrRNA sequences.

Fig. 2. Phylogenetic tree constructed with 1535pb *Mycoplasma* spp.16S rRNA sequences, using Maximum Likelihood (ML) method and GTR + G + I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

Unfortunately, the two positive *C. thous* samples did not show positivity to *RnaseP* PCR protocol, precluding the differentiation of these *Mycoplasma* species. In previous studies, a genotype closely related to *M. haemocanis* was detected in raccoon dogs (*Nyctereutes procyonoides viverrinus*) from Japan (Harasawa et al., 2014) and arctic foxes (*Vulpes lagopus*) from Canada (Mascarelli et al., 2015). Besides, genotypes closely related to *M. haemocanis, M. haemofelis* and '*Candidatus* M. turicensis' were detected in Darwin's foxes (*Lycalopex fulvipes*) from Chile (Cabello et al., 2013). In Brazil, among wild canids maintained in captivity in zoos, two bush dogs (*Speothos venaticus*) and two European wolves (*Canis lupus*) were positive for *Mycoplasma* species closely related to '*Candidatus* M. haematoparvum and "*Candidatus* M. haemo-

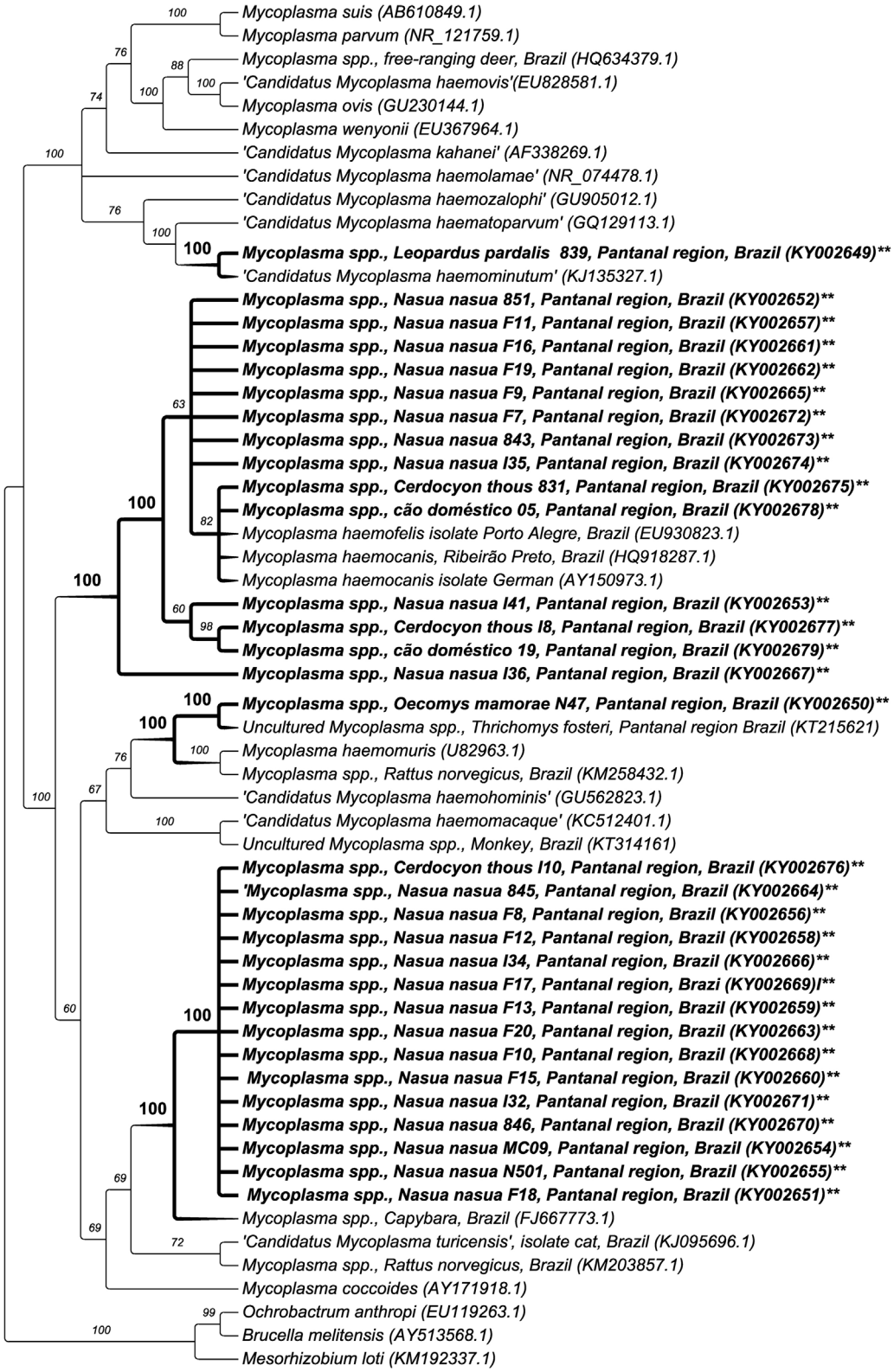

Fig. 3. Phylogenetic tree constructed with 1535pb *Mycoplasma* spp.16S rRNA sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

minutum", respectively (André et al., 2011).

Mycoplasma haemofelis and '*Candidatus* Mycoplasma haemominutum' were detected in the saliva and salivary glands of hemoplasma infected cats, suggesting that these bacteria could be transmitted by social interactions (Dean et al., 2008). Considering that, it could be hypothesized that the gregarious behavior of coatis (Blanco and Hirsch, 2006) may have contributed for a higher incidence of *Mycoplasma* spp. infection among this carnivore species compared to the other animal species sampled in the present study.

Among wild coatis, ten hemoplasmas sequences showed to be closely related to *M. haemocanis/M. haemofelis* based on 16S rRNA and *RnaseP* phylogenetic analyses. Additionally, a new '*Candidatus*

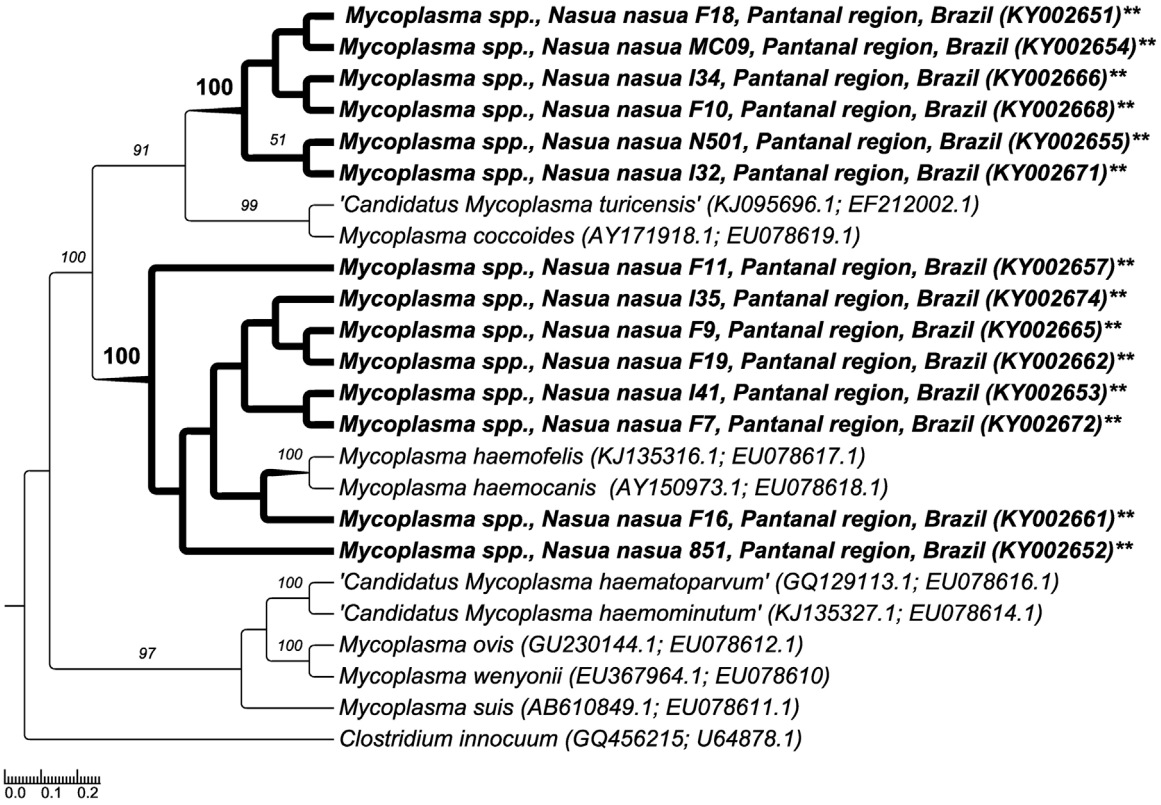

Fig. 4. Phylogenetic tree constructed with 1750pb *Mycoplasma* spp.16S rRNA + *RnaseP* sequences, using Maximum Likelihood (ML) method and GTR + G + I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50, using *Clostridium innocuum* (GQ456215; U64878) as outgroup.

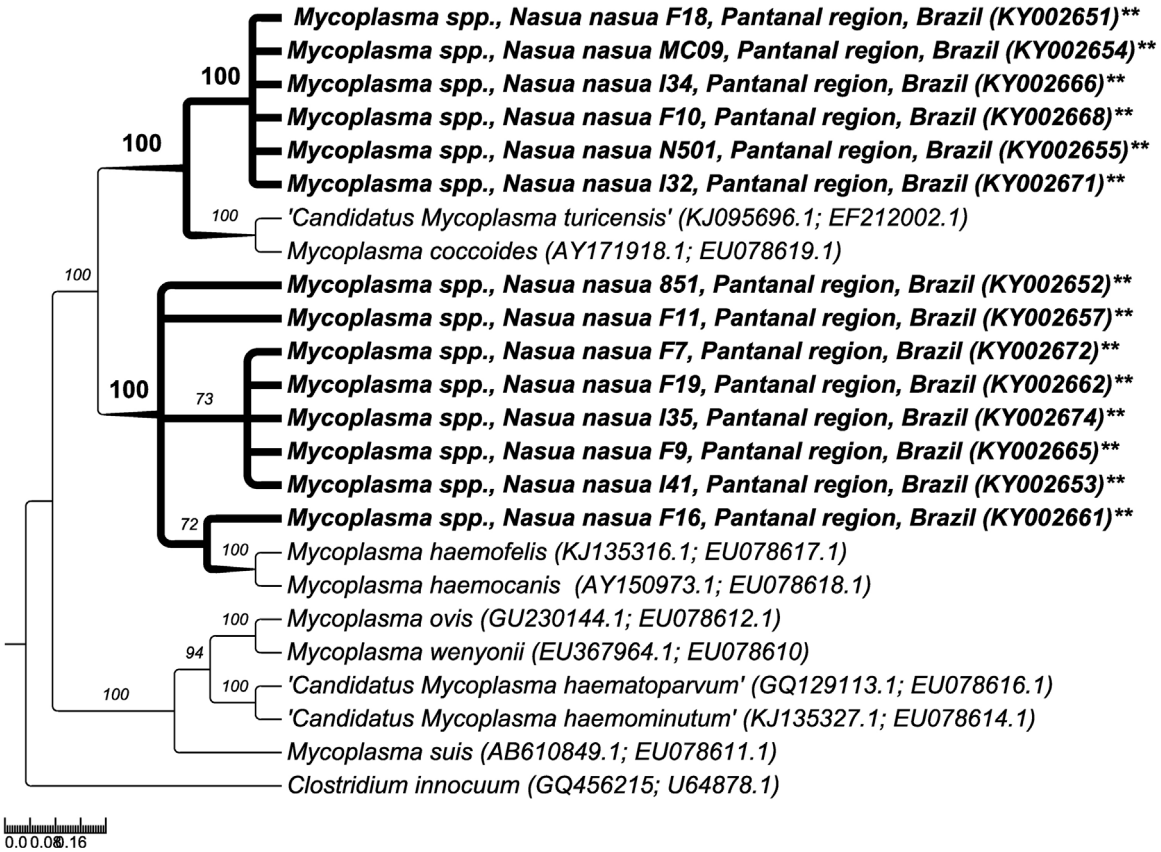

Fig. 5. Phylogenetic tree constructed with 1750pb *Mycoplasma* spp.16S rRNA + *RnaseP* sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Clostridium innocuum* (GQ456215; U64878) as outgroup.

Table 3
Polymorphisms of *Mycoplasma* spp. 16SrRNADNA sequences obtained from *Nasua nasua* blood samples.

Population (Hosts)	N° of 16SrRNA sequences	AS	NVS	H	Hd	Pi	k
N. nasua	24	1247	48	4	0.569	Pi = 0.05270 (SD = 0.00563)	19.44565

N° of 16S rRNA sequences, Number of 16S rRNA hemoplasma sequences obtained from *N. nasua* blood samples; AS, Alignment Size; NVS, Number of Variable Sites; H, Number oh haplotypes; Hd, Haplotype diversity; Pi, Nucleotide Diversity; SD, Standard Deviation; k, Average number of nucleotide differences.

Mycoplasma' species is proposed for the hemoplasma detected in 14 coatis and one crab-eating fox, which grouped in a separate branch with high values of clade support, and was closely related to a new *Mycoplasma* genotype detected in a capybara from Brazil (Vieira et al., 2009), based on ML and Bayesian phylogenetic analyses of 16S rRNA gene. The concatenated phylogenetic analysis of 16SrRNA and *RnaseP Mycoplasma* sequences also positioned six sequences obtained from coatis in a distinct branch with a significant value of clade support. Due to the inability to culture hemoplasmas in vitro, the only method to differentiate these species relies on the phylogenetic analysis. Although 16S rRNA and *RnaseP* are the most used target genes in phylogenetic analysis of hemoplasmas (Peters et al., 2008; Neimark et al., 2005), non-ribosomal genes (*dnaK*) have also been used (Harasawa et al., 2015). In addition to this, the 16S rRNA sequences obtained from coatis blood samples comprises only one haplotype (#hap 1), which showed to be different from the others three haplotypes found in the 16SrRNA sequences obtained from coatis closely related to *M. haemocanis/M haemofelis*. Although the *Mycoplasma* sequences obtained from coatis and a crab-eating fox showed to be closely related to the new hemoplasma genotype detected in a Brazilian capybara, these genotypes were not positioned in the same clade, highlighting the circulation of a new genotype of *Mycoplasma* among carnivores in Pantanal region, Brazil.

'*Candidatus* Mycoplasma haemominutum' was the most common feline hemoplasma species found in previous studies involving Iberian lynxes (*Lynx pardinus*) from Spain, Eurasian lynxes (*Lynx lynx*) from Switzerland, lions (*Panthera leo*) from Tanzania, and seven Brazilian neotropic felid species namely Geoffroy's cats (*Oncifelis geoffroyi*), jaguarundis (*Puma yaguaroundi*), margays (*Leopardus wiedii*), ocelots (*Leopardus pardalis*), oncillas (*Leopardus tigrinus*), pumas (*Puma concolor*), and jaguars (*Panthera onca*) (Willi et al., 2007; André et al., 2011). On the other hand, '*Candidatus* M. turicensis' was the most prevalent hemoplasma species among European wild cats (*Felis silvestris silvestris*) (Willi et al., 2007). The only report on hemoplasma infection in a Brazilian wild felid showed a coinfection with *M. haemofelis* and '*Candidatus* M. turicensis' in an ocelot (Willi et al., 2007), whereas '*Candidatus* M. haemominutum' was the only hemoplasma species detected in a wild ocelot in the present study, corroborating to previous reports on the occurrence of hemotrophic mycoplasmas in ocelots maintained in captivity in zoos in Brazil (André et al., 2011). Wild animals may be more susceptible to become infected by hemoplasmas when compared to those animals maintained in captivity, since they are more likely to be exposed to a great diversity of bloodsucking arthropods, multiple pathogens and aggressive interactions (Filoni et al., 2006). Further studies should be perform in order to sample a higher number of wild felids aiming to know the most common hemoplasma species circulating among wild Brazilian felids and the impact of infection on animal health.

The found occurrence of *Mycoplasma* among wild rodents sampled

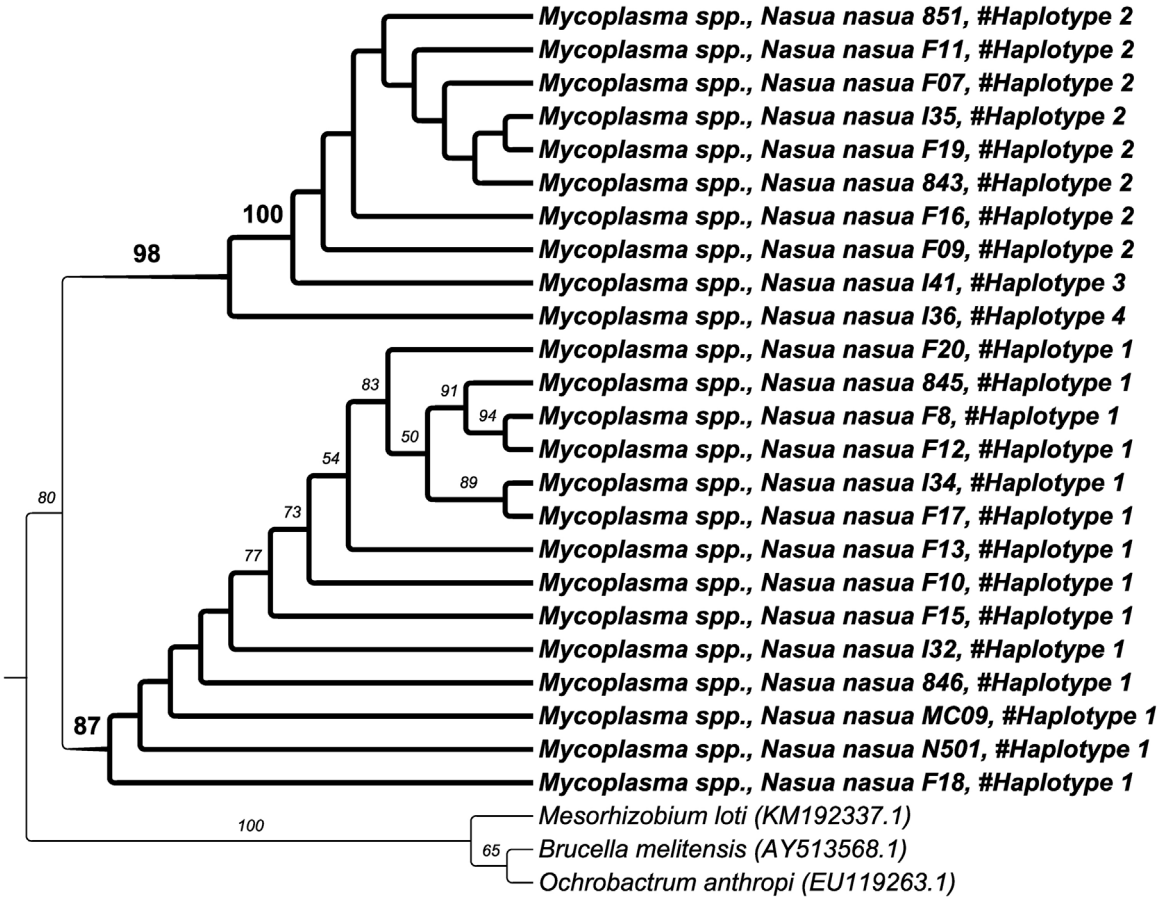

Fig. 6. Tree distribution of four haplotypes found among the hemoplasmas sequences obtained from coatis blood samples, based on the polymorphisms analysis of 16S rRNA alignment.

in the present study was lower (1/110) than that found in a previous recent study involving wild rodents (4/32) in the Pantanal, Brazil (Gonçalves et al., 2015). However, the occurrence of *Mycoplasma* infection found among wild rodents in Pantanal biome in the above mentioned study was significantly lower than that found in the others three Brazilian biomes (Cerrado, Amazon Forest, and Atlantic Forest) (Gonçalves et al., 2015). Additionally, high occurrence of hemoplasmas was found among capybaras (64%), synanthropic and laboratory rodents (*Rattus novergicus*) (63.5%) from urban areas in the state of Paraná, Brazil (Vieira et al., 2009; Conrado et al., 2015). A closer contact among rodents from urban areas with different host species and arthropods could explain the differences observed in prevalence rates. In spite of that, a high diversity of *Mycoplasma* genotypes has been detected in rodents from Japan, Hungary and Brazil (Vieira et al., 2009; Sashida et al., 2013; Conrado et al., 2015; Gonçalves et al., 2015; Hornok et al., 2015). A genotype closely related to *M. haemomuris* was detected in a wild rodent (*O. mamorae*) in the present study. The same genotype has been already detected in wild rodents from the same biome Pantanal (Gonçalves et al., 2015), suggesting that *M. haemomuris,* besides being the most common hemoplasma species found in synantropic rodents (Conrado et al., 2015; Hornok et al., 2015) seems to be the main hemotrophic mycoplasma species parasitizing wild rodents in the Pantanal biome in Brazil.

Although bloodsucking arthropods are suspected to be the vectors of hemoplasmas, the real role of these ectoparasites in the transmission cycles remains inconclusive (Woods et al., 2005). Supporting that hypothesis, '*Candidatus* M. haemominutum' and *M. haemofelis* were detected in fleas (*Ctenocephalides felis*) collect from cats in the USA (Lappin et al., 2006), although experimental attempts of hemoplasmas transmission between felines via fleas were inconclusive (Woods et al., 2005). In addition, *M. haemofelis* and "*Candidatus* M. haemominutum" DNA was detected in dog brown tick, *R. sanguineus,* collected from lions in Tanzania (Fyumagwa et al., 2008). The tick *R. sanguineus* s.l. is considered the main vector of *M. haemocanis* (Seneviratna et al., 1973). Despite of that, even in the localities where this tick species is well distributed and high rates of infestation are documented in Brazil (Labruna and Campos Pereira, 2001) the occurrence of hemotropic mycoplasmas in dogs was generally low (Ramos et al., 2010; Alves et al., 2014; Valle et al., 2014; Soares et al., 2016). In fact, there is only one study about the ability of *R. sanguineus* to transmit *M. haemocanis* to dogs (Seneviratna et al., 1973). The others reports were based only in positive statistical correlations between *M. haemocanis* infection and *R. sanguineus* infestation rates (Soares et al., 2016). Keeping that in mind, the role of the tick *R. sanguineus* in *M. haemocanis* epidemiology remains unclear. The lice *Polypax spinulosa* and *Polyplax serrata* are known to be able to transmit *M. coccoides* to rodents (Eliot, 1936; Berkenkamp and Wescott, 1988). Recently, *Mycoplasma* phylotypes were detected in fleas (*Synosternus cleopatrae*) collected from rodents (*Gerbillus andersoni*) from Israel, suggesting the possible participation of fleas as vectors of *Mycoplasma* spp. to rodents (Cohen et al., 2015). The possible arthropod vector of hemoplasmas among wild animals in Brazil remains unknown. In the present study, none of the ticks or fleas collected from wild animals showed to be positive for *Mycoplasma* spp. The participation of other arthropods or other transmission routes, such as aggressive interactions or predation among Brazilian wildlife should be better investigated.

In conclusion, the present study revealed that wild animals in southern Pantanal region, Brazil, are exposed to different species of hemotropic hemoplasmas; some of them are known to cause clinical diseases in domestic animals. Domestic dogs, some crab-eating foxes and *N. nasua* seemed to share the same *Mycoplasma* spp. species, closely related to *M. haemocanis/M. haemofelis*. The role of domestic dogs as a source of *Mycoplasma* infection to wild animals should be better investigated. On the other hand, wild felines and rodents appear to be infected by specific host species of *Mycoplasma* spp. The real role of arthropods in the hemoplasmas transmission cycles remains unknown. Finally, probably a new genotype of *Mycoplasma* was detected among wild coatis and crab-eating foxes in the present study. Therefore, future studies are much needed in order to elucidate the main and alternatives routes of *Mycoplasma* spp. transmission and to estimate the impact of hemoplasma infection among the Brazilian wildlife from Pantanal biome.

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