Short communication

First detection of feline hemoplasmas in free-ranging jaguars (*Panthera onca*)

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ABSTRACT

Species of hemoplasmas have been described worldwide, but little information is available for wild felids. Between February 2000 and January 2010, blood samples were collected from 30 jaguars (*Panthera onca*) and 22 domestic cats (*Felis catus*) from the Cerrado, Pantanal and Amazon biomes of Brazil. In all samples molecular tests were performed for *Mycoplasma haemofelis/Mycoplasma haemocanis* (Mhf/Mhc), 'Candidatus Mycoplasma haemominutum' (CMhm) and 'Candidatus Mycoplasma turicensis' (CMt). Twenty-two (73.4%) jaguars and four domestic cats (18.2%) tested positive for infection with at least one feline hemoplasma: 73.4% jaguars from the three areas were positive for CMhm, 13.6% jaguars from the Pantanal and 50.0% from the Amazon were positive for Mhf/Mhc, and 9.1% of individuals from the Pantanal tested positive for CMt. Domestic cats from the Cerrado (28.6%) and the Pantanal (30.0%) were positive for feline hemoplasma. All but one jaguar from the three sites are healthy. One female adult jaguar showed low body weight and dehydration. This is the first record of feline hemoplasmas in free-ranging jaguars. The high prevalence of CMhm suggest the participation of jaguars in the maintenance of this hemoplasma in nature. Although susceptible to Mhf/Mhc and CMt, jaguars did not appear to participate in the maintenance of these agents in the environment. The involvement of domestic cats in the transmission of any of these hemoplasmas cannot be excluded.

Previously classified as *Haemobartonella* and *Eperythrozoon*, the hemotropic mycoplasmas or hemoplasmas are mycoplasmal bacteria that infect erythrocytes and may cause severe hemolytic anemia (Tasker, 2010). Three different species of hemoplasmas have been characterized in felids: *Mycoplasma haemofelis* (originally known as *Haemobartonella felis*), 'Candidatus Mycoplasma haemominutum' and 'Candidatus Mycoplasma turicensis' (Tasker, 2010). Bloodsucking arthropods, such as ticks and fleas are suspected to be involved in the transmission of the hemoplasmas, as are aggressive interactions between hosts (Willi et al., 2007; Tasker, 2010).

Hemoplasma infections have been described in domestic and wild cats worldwide (Willi et al., 2007; André et al., 2011; Krengel et al., 2013; Ghazisaeedi et al., 2014; Santis et al., 2014). The jaguar is the largest feline in the Americas and is globally classified as Near Threatened, mainly due to loss of natural habitat and predatory hunt (IUCN,

2017). The aims of this study were to investigate the occurrence of feline hemoplasma infection in free-ranging jaguars and domestic cats and verify possible transmission between them.

The study area comprises three regions of Brazil: Emas National Park (ENP) (−18,061146 S; −52,941067 W) in the Cerrado biome; Caiman Ecological Refuge (-19,80319 S; −56,27373 W) and Barranco Alto Ranch (-19,57643 S; −56,16144 W) in the Pantanal biome; and Cantão State Park (CSP) (-9,64503 S; −50,13065 W) in the transitional area between the Cerrado and Amazon biomes. Both national parks are surrounded by large scale crop plantations or extensive livestock ranching.

Between February 2000 and May 2009, 29 free-ranging jaguars were captured using trained hounds or metal cage traps. Further, one juvenile jaguar, raised in an indigenous territory near CSP, was also part of this sampling effort (Furtado et al., 2017a,b).

Captured jaguars were anesthetized (9.7 mg/kg intramuscularly of tiletamine-zolazepam) and blood samples were collected by internal femoral vein puncture in vacuum tubes with anticoagulant (EDTA). Physical and oral examinations were conducted. The animals were classified according to tooth wear and body weight as adult (older than 2 years) or juvenile (0-2years). All adult jaguars were fitted with a radiocollar. Following capture, jaguars from the Cerrado and Pantanal study areas were monitored via radiotelemetry and/or camera traps (Furtado et al., 2017a,b). Recaptures of five individuals were performed at intervals of 60 days or more.

Blood samples were also collected from 22 domestic cats from rural properties surrounding the protected areas. Blood samples were collected by jugular or cephalic vein puncture in vacuum tubes with anticoagulant (EDTA).

All blood samples were separated in aliquots with or without the addition of an equal volume of saturated saline (100 mM Tris, 100 mM EDTA, 2% SDS), and frozen at −20 °C until analysis.

Handling procedures were consistent with the Ethical Principles in Animal Research adopted by the Bioethics Commission of the School of Veterinary Medicine and Animal Science of University of São Paulo (FMVZ-USP) and were approved by permit number 1471/2008. Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio) granted field permits to work in ENP, the Pantanal and CSP (Permits number 14637, 11214 and 11628, respectively).

DNA extraction was performed by pre-incubating the blood with proteinase K (Rubini et al., 2005). DNA was isolated from 200 μl aliquots of the blood using a GFX® Genomic Blood DNA Purification Kit (GE Healthcare), according to the manufacturer's instructions. Each DNA sample was eluted in 100 μl of TE buffer. All DNA samples were tested for *Mycoplasma haemofelis*/Mycoplasma haemocanis (Mhf/Mhc), 'Candidatus Mycoplasma haemominutum' (CMhm) and 'Candidatus Mycoplasma turicensis' (CMt) using a series of PCR and nested PCR protocols according to Fujihara et al. (2007). The PCR amplified fragments of the 16S rRNA gene (616 bp for Mhf/Mhc, 654 bp for CMhm and 559 bp for CMt). Nested PCR was performed using 0.5 μl of each product from the first amplification according to Fujihara et al. (2007). The nested PCR amplified fragments of 272 bp for Mhf/Mhc, 403 bp for CMhm and 319 bp for CMt. Ultrapure water was used as negative control and DNA from an ocelot and a jaguarundi were used as positive controls.

All samples that were PCR negative were tested for the glyceraldehydes-3-phosphate (GAPDH) gene to confirm the integrity of nucleic acids and to discard the presence of PCR inhibitors. This PCR protocol followed instructions by Leutenneger et al. (1999).

Aliquots of 5 μl of each amplified product were analyzed in 2% agarose gel with gel Red (0.05 μl/ml; Uniscience®) by electrophoresis in TAE buffer and visualized under UV transluminator.

Sequencing and phylogenetic analysis were performed with seven positive jaguars samples: two from the Amazon positive for Mhf/Mhc and CMhm (*P. onca* 148 and 149), two from the Cerrado positive for CMhm (*P. onca* 156 and 214), and three from the Pantanal, one positive for Mhf/Mhc (*P. onca* 162), one positive for CMhm (*P. onca* 141), and one positive for Mht (*P. onca* 139); and with four positive domestic cats samples: two from the Pantanal that were positive for CMhm (*F. catus* 80 and 224) and two from the Cerrado, one positive for Mhf/Mhc (*F. catus* 233), and one for CMt (*F. catus* 232).

The PCR products were purified with the *GFX™ PCR DNA and Gel Band Purification Kit®* (GE Healthcare) according to the manufacturer's recommendations. Bidirectional sequencing was performed using a BigDye Terminator 3.1 Cycle Sequencing kit and ABI-3500 sequencer (Applied Biosystems™). The final sequence was obtained with the Cap-Conting application of the Bioedit Sequence Alignment Editor 7.2.5 program (Hall, 1999) and subject to comparison with the GenBank database to confirm the specificity of the sequence at http://www.ncbi.nlm.nih.gov/BLAST. The nucleotide sequences generated for this study were aligned with homologous sequences of feline hemoplasma species

retrieved from GenBank using the CLUSTAL/W method implemented in the program MEGA 6.06 (Tamura et al., 2013). The values of the nucleotide identity were calculated using the Bioedit Sequence Alignment Editor program version 7.2.5 (Hall, 1999).

Feline hemoplasma nucleotide sequences from the samples in this study have been deposited in the GenBank database under the accession numbers KY780173 to KY780185. A phylogenetic tree was constructed with the program MEGA 6.06 (Tamura et al., 2013) using the *Maximum likelihood* method based on the Kimura 2-parameter model with invariant sites and 1,000 bootstrap replicates.

An animal that had at least one positive result during capture or recapture was considered positive. Combining laboratory diagnoses with data from jaguar monitoring allowed us to characterize the spatial distribution of CMhm in the Pantanal.

We use logistic regression to test the effect of sex and age on jaguar prevalence and to compare prevalence between jaguars and cats. The models were implemented using the software R, version 2.10.1 (R Development Core Team, 2011). A table shows coefficient estimates, their standard error (SE), 95% confidence intervals (CI 95) and *p* values. Coefficients with *p* values < 0.05 were considered to have significant effect on the probability of an individual to test positive.

Among the 30 jaguars analyzed, 11 (36.7%) were male and 19 (63.3%) were female; 20 (66.7%) were adult and 10 (33.3%) were juvenile. All but one jaguar (96.7%, n = 29) were in good physical condition, had appropriate body weight and showed no clinical signs of any apparent disease. One female adult jaguar captured in CSP showed low body weight, dehydration, no upper left canine, no incisors and wear of the lower canines. All domestic cats sampled were in good physical condition. Jaguars were monitored post-capture for periods of 21.7 months (range of 1–91 months).

All the samples analyzed were positive for the endogenous control GAPDH in the DNA extractions. Twenty-two (73.4%) jaguars and four domestic cats (18.2%) tested positive for at least one feline hemoplasma infection. Seven jaguars were concurrently infected with several species: four with Mhf/Mhc and CMhm; one with CMhm and CMt; and one with all three. Jaguars from the three areas were highly exposed to CMhm, but only few individuals from the Pantanal and CSP tested positive for Mhf/Mhc and CMt (Table 1). Two rural properties from the Cerrado (33.3%, n = 6), two from the Pantanal (28.6%, n = 7) and none from the Amazon (0%, n = 3) had domestic cats infected with feline hemoplasmas. Fig. 1 illustrates the occurrence of CMhm in the Pantanal.

Four recaptured jaguars present with the same diagnosis for Mhf/Mhc, CMt and CMhm as on their initial capture two to 40 months later. One jaguar tested negative for CMhm on recapture six months after its being positive on initial capture.

The sequencing followed by the phylogenetic analysis confirmed that the felids were infected with hemoparasites close related to Mhf/Mhc, CMhm and CMt (Table 2, Fig. 2). As the sequences of the 16S

Table 1
Number and percentages of jaguars and domestic cats tested PCR positive for *Mycoplasma haemofelis*, 'Candidatus Mycoplasma Haemominutum' and 'Candidatus Mycoplasma turicensis' from areas of the Cerrado, Pantanal and Amazon, Brazil.

Biome	Species	Examined	N° (%) of animals positive PCR for:		
			Mhf[a]	CMhm[b]	CMt[c]
Cerrado	Jaguar	4	0	3 (75)	0
	Domestic cat	7	1 (14.3)	0	1 (14.3)
Pantanal	Jaguar	22	3 (13.6)	16 (72.7)	2 (9.1)
	Domestic cat	10	1 (10)	2 (20)	0
Amazon	Jaguar	4	2 (50)	3 (75)	0
	Domestic cat	5	0	0	0

[a] Mycoplasma haemofelis.
[b] Candidatus Mycoplasma haemominutum.
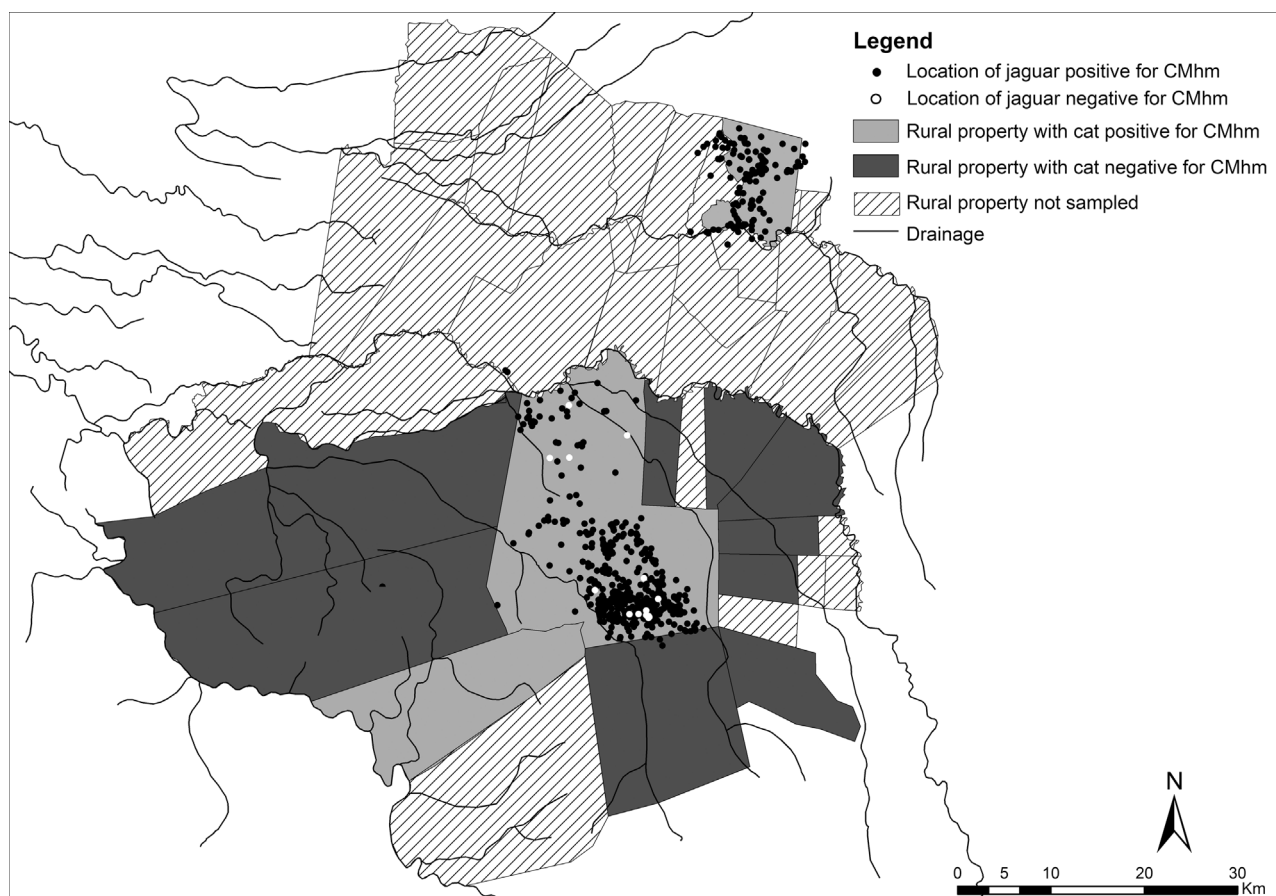[c] Candidatus Mycoplasma turicensis.

Fig. 1. Locations obtained through radio-telemetry of free-ranging jaguars positive/negative for 'Ca. Mycoplasma haemominutum' (CMhm) and rural properties with domestic cats positive/negative for the hemoparasite in the Pantanal.

rRNA genes of Mhf and Mhc analysed in this study have 99% identity, we can not distinguish between these two bacteria. It would be necessary to use the RNAase P gene for this differentiation. However, according to Nascimento et al. (2012), *M. haemocanis* and *M. haemofelis* are different mycoplasmal species infecting dogs and cats, respectively. Also found by Ravagnan et al. (2017) and Willi et al. (2007). We are encouraged to suggest that the virus found in this work is Mhf, but analysis with the RNAse P gene should be performed for confirmation.

Adults were significantly more exposed to CMhm than juveniles ($p = 0.016$) (Table 3). We were unable to analyze the effect of age and sex on infection with CMt due to low prevalence rates. Jaguars were significantly more exposed to CMhm than domestic cats ($p < 0.001$).

This is the first report of Mhf/Mhc, CMhm and CMt in free-ranging jaguars. The high exposure of jaguars in good physical conditions to

CMhm without any apparent alterations in their movements (based on post-capture monitoring) suggests that the species could participate in the maintenance of the agent in nature and act as an asymptomatic carrier of this hemoplasma. As reported by different authors (Willi et al., 2007; Tasker, 2010; Aquino et al., 2014; Santis et al., 2014) CMhm was the most commonly detected hemoplasma and has already been described in captive jaguars from Brazil (André et al., 2011). Free-ranging felids are more infected with feline hemoplasmas than captive animals (Willi et al., 2007).

Coinfection with Mhf/Mhc and CMhm observed in five jaguars in our study has previously been reported for domestic and wild felids (Willi et al., 2006; Sykes et al., 2007; Willi et al., 2007; Aquino et al., 2014; Santis et al., 2014). One of the coinfected jaguars was the de-hydrated animal with missing or worn teeth captured in the CSP; which

Table 2
Number of analysed sequences and DNA identity of hemoplasmas from free-ranging jaguars and domestic cats from the Cerrado, Pantanal and Amazon in Brazil.

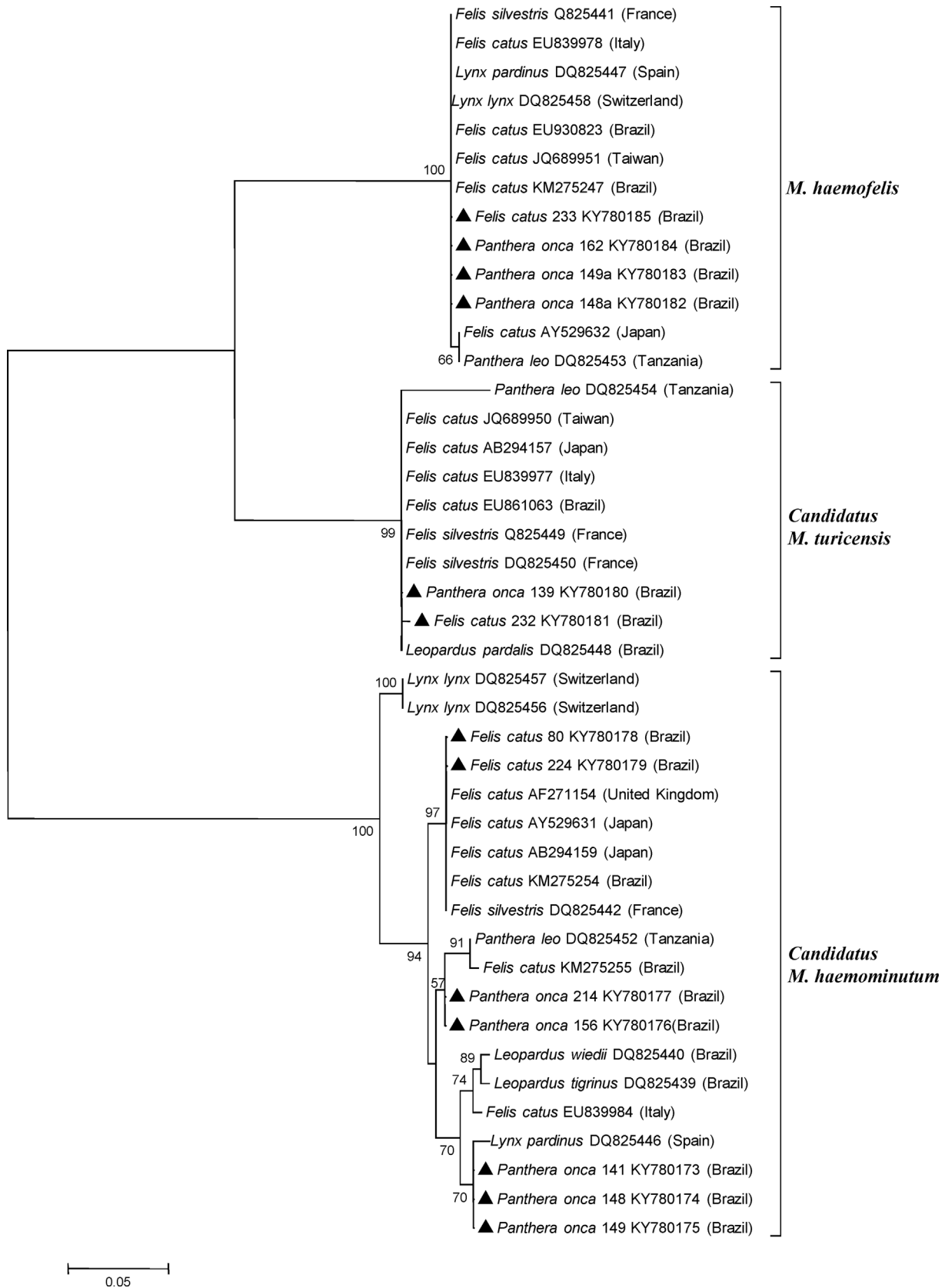| Hemoplasma	Species (number of sequenced samples)	Biome	Closest Genbank entry (by BLAST) (% similarity)
Mycoplasma haemofelis	Jaguar (3)	Amazon, Pantanal,	*Mycoplasma haemofelis* from Brazilian cat– EU930823 (100%)
	Domestic cat (1)	Cerrado	*Mycoplasma haemofelis from Iberian lynx– DQ825447 (100%)*
'Candidatus Mycoplasma haemominutum'	Jaguar (3)	Pantanal, Amazon	'Ca. Mycoplasma haemominutum' from Iberian lynx– DQ825446 (99.6-98.7%)
'Candidatus Mycoplasma haemominutum'	Jaguar (2)	Cerrado	'Ca. Mycoplasma haemominutum' from Lion– DQ825452 (98.7%)
'Candidatus Mycoplasma haemominutum'	Domestic cat (2)	Pantanal	'Ca. Mycoplasma haemominutum' from Brazilian cat–KM275254 (100%)
'Candidatus Mycoplasma turicensis'	Jaguar (1)	Pantanal	'Ca. Mycoplasma turicensis' from Brazilian ocelot –DQ825448 (100%)
			'Ca. Mycoplasma turicensis'from European wildcat– DQ825450 (100%)
'Candidatus Mycoplasma turicensis'	Domestic cat (1)	Cerrado	'Ca. Mycoplasma turicensis' from Brazilian ocelot –DQ825448 (99.6%)
			'Ca. Mycoplasma turicensis' from Brazilian cat–EU861063 (99.6%)

Fig. 2. Phylogenetic tree of 254 bp nucleotide sequences of the 16S rRNA gene of *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis' using the *Maximum likelihood* method based on the Kimura 2-parameter model with invariant sites. Triangle indicates the sequences from this study. Bootstrap values greater that 50 are shown at the nodes. The bar represents the substitution number per site.

Table 3

Estimated parameters by logistic regression testing the effect of sex (coefficient β₁) and age (coefficient β₂) on the diagnosis of *Mycoplasma haemofelis* and 'Candidatus Mycoplasma haemominutum' for jaguars; and the effect of species (coefficient β₁) on the diagnosis of *Mycoplasma haemofelis*, 'Candidatus Mycoplasma turicensis' and 'Candidatus Mycoplasma haemominutum'. Using as references categories: female for sex, juveline for age and domestic cats for species.

Organisms	Parameters	Estimative	Standard Error	*p value*	CI 95%[+]
Mycoplasma haemofelis[*]	Intercept	−1.322	0.563	0.019	[−2.577; −0.308]
	β₁ (male)	−0.981	1.190	0.410	[−4.031; 1.105]
'Ca. Mycoplasma haemominutum'	Intercept	−0.706	0.718	0.326	[−2.282; 0.648]
	β₁ (male)	1.451	1.265	0.252	[−0.812; 4.600]
	β₂ (adult)	2.445	1.014	0.016	[0.582; 4.686]
Mycoplasma haemofelis	Intercept	−2.303	0.742	0.002	[−4.137; −1.072]
	β₁ (jaguar)	0.693	0.889	0.435	[−0.953; 2.708]
'Ca. Mycoplasma haemominutum'	Intercept	−2.303	0.741	0.002	[−4.137; −1.072]
	β₁ (jaguar)	3.314	0.849	< 0.001	[1.830; 5.294]
'Ca. Mycoplasma turicensis'	Intercept	−3.044	1.023	0.003	[−5.931; −1.481]
	β₁ (jaguar)	0.405	1.258	0.747	[−2.003; 3.513]

[*] Model including age effect not identifiable.
[+] 95% Confidence Interval.

was also PCR positive for *Hepatozoon* spp. and *Cytauxzoon felis* (Furtado et al., 2017a,b). We cannot discard the hypothesis that these clinical signs may be related to the coinfection of hemoparasites. On the other hand, the other female jaguar captured in CSP was also PCR positive for the same hemoparasites but did not present with any clinical signs.

CMt was detected only in two jaguars coinfected with other hemoplasmas, as also described by other authors (Aquino et al., 2014; Santis et al., 2014) who reported the occurrence of this hemoplasma only in association with CMhm. Probably these two hemoplasmas have similar modes of transmission (Willi et al., 2007).

Despite the small number of domestic cats sampled and their low exposure to hemoplasmas, our results demonstrate the circulation of Mhf/Mhc and CMhm in the Pantanal, and Mhf/Mhc and CMt in surroundings of ENP. Feline hemoplasmas in Brazil have been reported in domestic cats from different regions (Macieira et al., 2008; Biondo et al., 2009; Aragão de Souza et al., 2013; Miceli et al., 2013; Aquino et al., 2014; Santis et al., 2014).

The mode of transmission of feline hemoplasmas has not yet been identified (Willi et al., 2007; Tasker, 2010). However, considering the high occurrence of hemoplasmas in tropical areas and in free-ranging felids, Willi et al. (2007) suggest the involvement of hematophagous arthropods. In the current study, jaguars that tested positive for hemoplasmas were parasitized by different species of ticks (Furtado et al., 2017a); however, we did not test the ticks for hemoparasites, and therefore cannot make inference about their role in hemoplasma transmission. But the high exposure of jaguars to CMhm and the occurrence map of the agent in the Pantanal suggest that these hemoparasite could be transmitted through vectors, since jaguars are solitary and it is unlikely that cats and jaguars come in close contact.

The hemoplasma infection showed to be persistent in four of five recaptured jaguars. This agrees with Tasker (2010), who stated that infections with CMhm tend to be positive for months or years after the infection. For one jaguar results changed from positive to negative between capture and recapture, suggesting that either the infection cleared (Willi et al., 2007), or that low levels of hemoplasma in the blood went undetected by the PCR (Tasker, 2010).

The higher exposure of jaguars to CMhm compared to domestic cats reinforces the hypothesis that wild felids could represent an important reservoir of the agent in nature. The higher incidence of CMhm in adults than in juveniles agree with findings in domestic cats (Tasker, 2010; Ghazisaeedi et al., 2014), since adult animals have had more time being exposed to the pathogen. Also, the results suggest that jaguars are susceptible for Mhf/Mhc and CMt. As domestic cats were shown to be susceptible for the same feline hemoplasmas, their involvement in the transmission of any of these agents cannot be ruled out.

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