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ORIGINAL PAPER

Occurrence and Molecular Identification of Hemoparasites in Wild Mammals Kept in Rehabilitation Centers in Brazil

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Abstract

Purpose Hepatozoonosis and piroplasmosis are diseases caused by apicomplexan protozoa that affect different types of animals, including mammals. The present study aimed to evaluate the occurrence of *Hepatozoon* spp. and piroplasms in wild mammals kept in captivity in rehabilitation centers in the states of Minas Gerais and Goiás, Brazil.

Methods For this, blood samples from 152 animals were collected and analyzed by conventional optical microscopy and polymerase chain reaction (PCR). In addition, positive PCR samples were submitted to sequencing for molecular characterization of the specimens found.

Results Microscopic analysis revealed 53 of the 152 animals (28.3%) parasitized by piroplasms. No *Hepatozoon* sp. was observed. On the other hand, using the primers HepF300/HepR900 and Piro1F/Piro5R, both amplifying fragments of the 18S rDNA gene, eight animals (5.2%) were positive for *Hepatozoon* spp. and 40 (26.3%) for piroplasms. From the sequencing of the positive samples *Hepatozoon canis*, *Hepatozoon felis*, *Theileria cervi*, *Theileria equi* and *Cytauxzoon felis* were identified. In addition to the aforementioned hemoparasites, some animals were found parasitized by microfilaria. Such data ratify the presence of hemoparasites in captive wild animals, and are unprecedented in the two geographical regions covered by the present study. 19.7% of mammals harbored ectoparasites of the genera *Amblyomma* and *Rhipicephalus.*

Conclusion Wild mammals are infected by several pathogens that can also infect domestic animals, some of them potentially zoonotic which can directly contribute to mortality and species reduction. Therefore, a deep understanding of the parasites, the hosts and the diseases is extremely necessary so that prevention, control and treatment measures are effectively applied.

Keywords Blood smear · *Hepatozoon* spp. · Piroplasms · Tickborne disease · Diagnosis

Introduction

Hepatozoon spp. and Piroplasmids are apicomplexan parasites that infect a wide variety of vertebrate hosts [1, 2] and are the etiological agents of hepatozoonozis and piroplasmosis, two tick-borne infections with a great economic and veterinary impact in wild animals, including mammals worldwide [3, 4]. Piroplasmosis is caused by four related genera: *Babesia*, *Theileria*, *Cytauxzoon* and *Rangelia* [2] while Hepatozoonosis is often attributed to *H. canis*, *H. americanum* and *H. felis,* although the genus *Hepatozoon* is composed of more than 300 species [10–12].

Ticks of the ixodid family are the vectors responsible for transmitting the etiological agents to the hosts [5, 6]. Mammals can become infected by ingesting the arthropod infected by mature oocysts containing sporozoites of *Hepatoozoon* spp. [4, 7] or by the blood meal of ticks that

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harbor piroplasms [3, 5]. *Hepatozoon* spp. usually infect leukocytes whereas piroplasms infect erythrocytes, triggering the respective diseases in their hosts [8].

The pathology caused by these parasites is usually subclinical [13, 14]. However, both in Piroplasmosis and Hepatozoonosis, an acute disease with variable clinical symptoms can occur depending on the species or strains of the pathogen involved [15]. Among the symptoms fever, apathy, diarrhea, anemia, pale mucosa and hemorrhages are the most common [16]. Severe symptoms can cause direct harm to animals or even lead them to death [3].

The Brazilian territory, due to its large land area, climate and geographical location, has great richness of biodiversity, housing approximately 13% of the world's mammal fauna [17]. Brazilian mammals are located in natural and captive environments such as zoos, conservation programs, scientific or commercial breeding sites, research institutes, screening and rehabilitation centers [18]. The captive environment has the potential to favor the transmission and maintenance of various infectious agents. The intimate contact between the sheltered animals associated with sanitary management and the immunosuppression of some of these animals are factors that may facilitate the installation and spread of diseases in these environments [19].

Hemoparasites were usually identified and classified based on aspects related to morphology, host specificity and route of transmission (vectors) [20]. The optical microscopy was considered the gold standard diagnostic methodology, being practical and routinely used to detect blood parasites infection. Despite that, the tests do have recognized limitations [7]. The techniques based on the analysis of genetic material have proven to be useful in providing detailed and reliable information, being considered as a differential methodology in the identification and characterization of pathogens [7].

Based on this, this work aimed to evaluate the occurrence and the species of *Hepatozoon* spp. and Piroplasms infecting wild mammals from the southeastern and central-western Brazil, by microscopy and molecular techniques.

Materials and Methods

Study Locations

The study was conducted in Wild Animal Screening Centers (CETAS) supervised by IBAMA, located in the states of Goiás and Minas Gerais, and in the Wild Animals Teaching and Research Laboratory (LAPAS), linked to the Federal University of Uberlândia (UFU), Minas Gerais, Brazil.

Sampling

For the collection of blood samples, veterinarians responsible for the screening and research centers, submitted the animals to physical and chemical restraints, strictly following the semiological rules [21]. For chemical containment, several compounds were used according to the sedation protocols of each of the centers such as Xylazine Hydrochloride (Virbac, France) associated with Ketamine (Virbac, France) or Titelamine-Zolazepam Hydrochloride (Virbac, São Paulo, Brazil) or Isoflurano (Cristália, Minas Gerais, Brazil).

From each animal 1–3 mL of blood were obtained by puncturing their jugular, cardiac or cephalic veins with sterile needles of different calibers: 13 × 0.45 mm (26G) or 0.7 × 30 mm (22G) for small animals and 25 × 0.8 mm (21G) for medium and large animals. The samples were collected in vacuum tubes containing EDTA and stored at -20 °C until the moment of the execution of the molecular techniques.

After collecting the venous blood, a puncture was performed in the marginal vein of the ears (right and left) or tail, to make blood smears which were subsequently fixed and stained by the May-Grunwald-Giemsa method [22]. Two blood slides were made per animal. All slides were fully scanned in an optical microscope (Olympus CH20i, Olympus Optical Co) under the 100 × objective with immersion oil to detect and identify the evolutionary forms of the parasites. To further increase the reliability of the results all analyzes were performed by two different researchers. In case of divergence, a third researcher was invited to analyze the slide to resolve all doubts.

Molecular Analysis

DNA Extraction and Polymerase Chain Reaction (PCR)

DNA was extracted using the commercial kit QIAmp DNA Mini Kit® (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Negative controls (only reagents) were used in each extraction round.

For the detection of *Hepatozoon* spp. the primers HepF300 and HepR900 [23] which amplify a genomic fragment of approximately 600 base pairs of the 18S rDNA gene were selected.

The reactions directed to the piroplasms were carried out with the primers Piro1F and Piro5R, which also amplify a fragment of the 18S rDNA gene (1684 bp) according to the conditions established by Kawabuchi *et al.* [24].

Bands of interest were visualized through the agarose gel electrophoresis technique at 2.0% (P/V) stained with GelRed®.

Negative controls (only reagents) and positive controls (DNA from *Hepatozoon* spp. and Piroplasms, previously isolated and sequenced for confirmation) were used in each PCR reaction.

Sequencing

The PCR amplicons were purified with ExoSAP-IT (Amersham Biosciences, Piscataway, Nova Jersey, USA). Reactions were performed in a Mastercycler pro thermocycler (Eppendorf, Brazil) using the Big Dye terminator V.3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, United States). Products were read in an ABI 3130 Genetic Analyzer automatic sequencer (Applied Biosystems, Foster City, CA, United States). The quality of partial sequences was assessed and the joining of fragments sequenced in their respective intersection areas was obtained using SeqMan Pro®. Nucleotide alignment was performed manually with ClustalX [25] based on the homologous sequences available on GenBank.

Two phylogenetic trees, one for the 18S rDNA sequences from *Hepatozoon* and another for the 18S rDNA sequences from Piroplasms were inferred by the Bayesian method with the Mrbayes 3.2.5 software. The reversible general time model was used in addition to invariant sites and a range of distribution rates between sites. [26]. The first 20% of the trees represented the burn-in and were removed, and the remaining trees were used to calculate the posterior Bayesian probability (BPP).

Collection and Identification of Ticks

During the clinical evaluation the entire body surface of the mammals was examined thoroughly for the presence of ticks. The ectoparasites were collected manually and were kept in airtight glass flasks containing 100% isopropyl alcohol until the moment of identification. The ticks were identified from morphological criteria and dichotomous keys for nymphs and adults according to Barros-Battesti *et al.* [27] and Martins *et al.* [28].

Results

General Results

This study included blood samples from 152 animals, being 85 (55.9%) males and 67 (44.0%) females distributed in 8 orders, 14 families and 22 species. Of these, 31 (20.3%), were puppies (0 to 6 months old), 30 (19.7%) young (6 months to 1 year old) and 91 (59.8%) adults (older than 1 year old). All animals included in the study were captives. For the purposes of this study captive animals were those that had been sheltered in CETAS for more than four months or those that had been there for more than two months, but for various reasons, they would not be reintegrated into their original habitat.

The taxonomic information of these animals, as well as the respective positivity detailed in the topics below, are compiled in Table 1.

Microscopic Positivity for *Hepatozoon* spp. and Piroplasms

By microscopic analysis 28.3% (43/152) of the total blood smears assessed, were positive for piroplasms. Different morphologies of the evolutionary intra-erythrocyte forms of piroplasms were observed, such as rings, drops and commas, with defined contour and the presence of nucleus surrounded by tri-laminated membrane (Fig. 1). No evolutionary forms of *Hepatozoon* spp. were observed in the samples.

Concomitant Infections

Microfilaria were also found in blood smears of two Porcupine (*Coendou prehensilis*), belonging to the Erinaceomorpha order (Fig. 2) (Table 1).

Molecular Analysis

To confirm the microscopic diagnostic, all blood samples ($n = 152$) were tested for the presence of parasites by PCR amplification. Of these, eight (5.2%) had a band profile compatible with *Hepatozoon* spp. and 40 (26.3%) with piroplasms.

Of the 48 PCR positive samples, only seven were sequenced. Of these, two were identified as *Hepatozoon felis* (MT458170 and MT458171) coming from a Tyra and a Puma and two as *Hepatozoon canis* (MT458172 and MT458173) originating from a Tapir and a Fox (Fig. 3). The other three haplotypes were identified as Piroplasms, being *Theileria cervi* (MT458053) from a stag deer, *Cytauxzoon felis* (MT458054) from an Ocelot and *Theileria equi* (MT458055) from a Tapir (Fig. 4). None of the *Hepatozoon spp.* and Piroplasms sequences obtained, herein, was completely similar to those available from Genbank, therefore being deposited in the database.

Tick Infestation

In total, 423 ticks were collected from 30 animals (19.7%) being 360 adults and 63 nymphs, belonging to the genera *Amblyomma* and *Rhipicephalus*. The species *Amblyomma nodosum*, *A. parvum*, *A. ovale, A. sculptum*, *A. longirostre*, *A. dubitatum*, *Rhipicephalus microplus* and *R. sanguineus* were identified (Figure S1).

Table 1 Taxonomy of wild mammals kept in rehabilitation centers of southeastern and central-western Brazil, and their positivity for hemoparasites, especially *Hepatozoon* spp. and Piroplasms by optical microscopy and PCR

Order	Family	Species (*n**)	Positivity	
			Microscopy (*n***)	PCR (*n***)
Pilosa	Myrmecophagidae	*Myrmecophaga tridactyla* (21)	Piroplasm (6)	Piroplasm (2)
		Tamandua tetradactyla (11)	Piroplasm (5)	Piroplasm (2)
Rodentia	Caviidae	*Hydrochoerus hydrochaeris* (2)	–	Piroplasm (1)
	Erinaceinae	*Coendou prehensilis* (10)	Microfilaria (2)	Piroplasm (7)
		Sphiggurus spinosus (1)	Piroplasm (1)	–
Marsupialia	Didelphidae	*Didelphis albiventris* (26)	Piroplasm (5)	*Hepatozoon* spp. (1) and Piroplasm (4)
Artiodactyla	Tayassuidae	*Pecari tacaju* (1)	Piroplasm (1)	–
	Cervidae	*Mazama gouazoubira* (6)	Piroplasm (1)	Piroplasm (1)
		Ozotoceros bezoarticus (1)	–	Piroplasm (1)
Carnívora	Canidae	*Chrysocyon brachyurus* (6)	–	*Hepatozoon* spp. (1) and Piroplasm (1)
		Lycalopex vetulus (5)	Piroplasm (1)	*Hepatozoon* spp. (1) and Piroplasm (3)
		Cerdocyon thous (10)	Piroplasm (5)	Piroplasm (4)
	Procyonidae	*Procyon cancrivorus* (2)	Piroplasm (1)	Piroplasm (2)
		Nasua nasua (2)	–	Piroplasm (2)
Carnivora	Felidae	*Leopardus pardalis* (3)	Piroplasm (1)	*Hepatozoon* spp. (2) and Piroplasm (3)
		Puma concolor (12)	Piroplasm (2)	*Hepatozoon* spp. (1)
	Mustelidae	*Eira Barbara* (1)	–	*Hepatozoon* spp. (1) and Piroplasm (1)
Perissodactyla	Tapiridae	*Tapirus terrestres* (2)	Piroplasm (1)	*Hepatozoon* spp. (1) and Piroplasm (1)
Primates	Cebidae	*Callithrix penicillata* (23)	Piroplasm (11)	Piroplasm (4)
	Atelidae	*Alouatta caraya* (5)	Piroplasm (2)	Piroplasm (1)
Cingulata	Dasypodidae	*Dasypus novemcinctus* (1)	–	–
		Cabassous tatouay (1)	–	–

*n** number of collected and analyzed samples per species

*n*** number of positive samples in each analysis

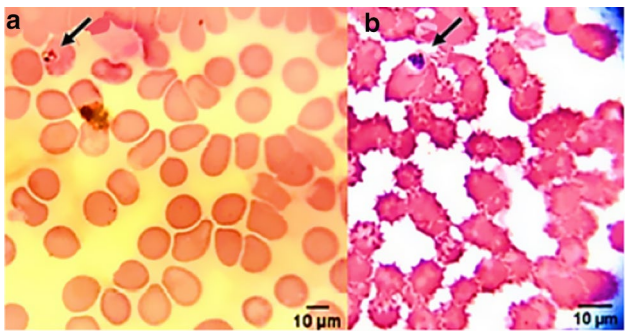

Fig. 1 Blood extensions of *Didelphis albiventris* (**a**) and *Myrmecophaga tridactyla* (**b**) observed by optical microscopy under a 100× objective. Arrows point to cells parasitized by Piroplasms

Detailed information on the above-mentioned ticks (species, evolutionary stage and number of specimen found) and their respective hosts can be accessed in supplemental Table(S1).

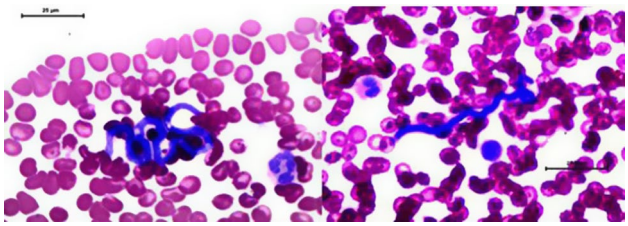

Fig. 2 Microfilaria (in blue) observed in wild rodent (*Coendou prehensilis*) blood extensions under a 400X objective

Discussion

The incidence and diversity of tick-borne infections have increased in recent years due to factors such as the improvement of diagnostic tools; increased contact of humans with wildlife and vectors due to urbanization and consequent loss of habitat, and environmental changes, especially climate changes [29–31].

While microscopy showed positive results only for Piroplasms, PCR was able to detect *Hepatozoon* spp. and

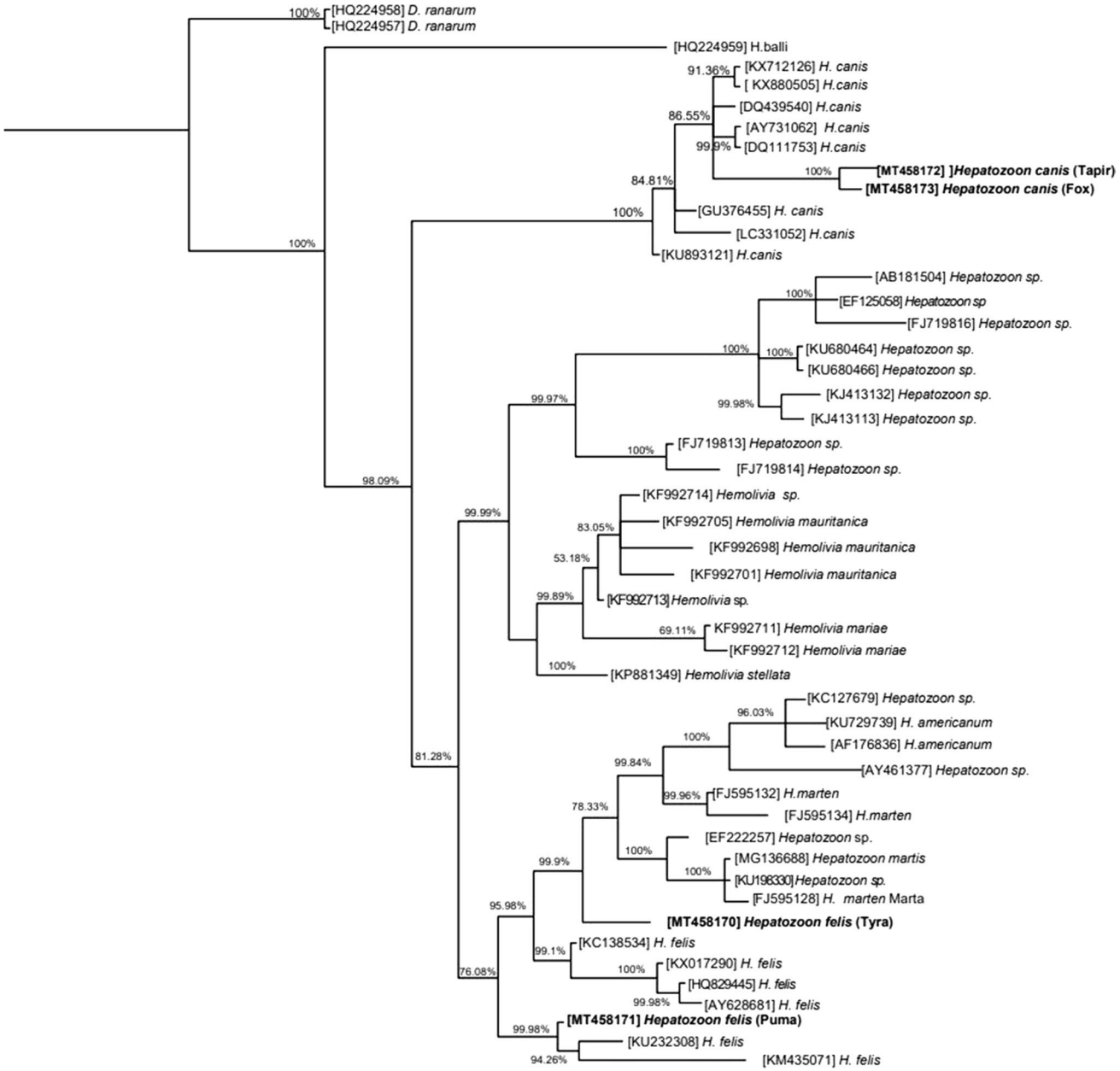

Fig. 3 Phylogenetic relationships among *Hepatozoon* spp. from different geographical regions and different hosts inferred using partial 18S rRNA gene sequencing and the Bayesian clustering method. Bootstrap analysis were based in 1000 replicates

Piroplasms in the blood samples analyzed in this study. The infection rates obtained by both techniques corroborate the literature, which presents values between 1.5 and 91% of prevalence for *Hepatozoon* spp. [10; 12; 32;33] and between 5.2% and 96.7% for Piroplasms, exclusively Babesia spp., *Theileria* spp. and *Cytauxzoon* spp. [11, 34–38] in wild mammals.

Currently, the aforementioned methods are the most commonly used to investigate hemoparasites in mammals [39, 40], and have advantages and disadvantages. Microscopy is a low-cost technique that requires little infrastructure and, in addition to the diagnosis itself, it is capable of providing subsidies for morphometric analysis. However, the subjectivity inherent to the technique, associated with the low parasitemia presented by some animals, and the intermittent pattern of some infections, such as hepatozoonosis [40], can limit its methodological potential [42; 43]. On the other hand, despite the higher cost, PCR has great sensitivity, even in low parasitemia, provides quantitative and qualitative information and is an excellent option if there is no information on the initial infection [43]. However, it also suffers interference from factors such as the low efficiency

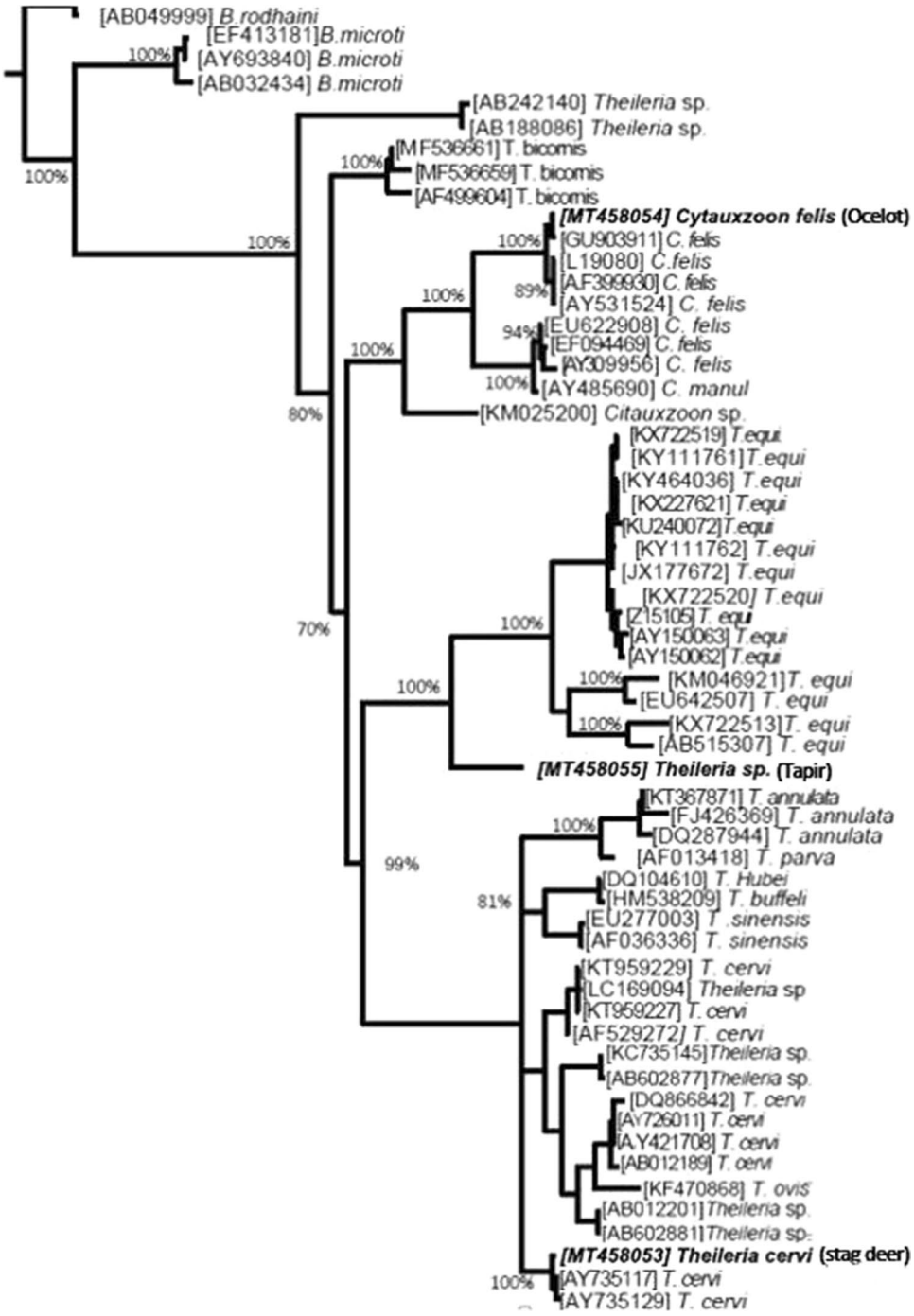

Fig. 4 Phylogenetic relationships among Piroplasms from different geographical regions and different hosts inferred using partial 18S rRNA gene sequencing and the Bayesian clustering method. Bootstrap analysis were based in 1000 replicates

of the DNA extraction process, small amount of target DNA in the samples and small sample volume, which can affect the success of the technique [16]. These factors endorse the divergence of results achieved by each of the applied techniques, individually, and in the comparison between them, and reinforce the idea that the combination of two or techniques is the most favorable scenario for obtaining reliable results and complementary information about the infection and the parasites.

In many parts of the world, information on *Hepatozoon* spp. in domestic and wild canids and felids it has been most attributed to *H. canis* and *H. felis,* respectively [11, 12, 45, 46]. For Piroplasms, host specificity is also widely discussed in the literature [46], and although the results obtained in this work corroborate this condition [11, 35, 38, 48–51], with *H. canis* and *H.felis* infecting canids and felids, respectively, *C. felis* parasitizing a felid (ocelot), *T. cervi* a stag deer and a tapir hosting *T. equi*, it is noteworthy that his condition is

still unclear, especially since not all hosts of these parasites have been fully elucidated [51]. Also, it does not necessarily extend to all species, it may be simply assigned to a single species, a limited number of species or specific evolutionary lineages [53, 54]. Complementing, in evolutionary terms, for a parasite to be highly host-specific, ideally, its host must be widespread, abundant or easily infected. Otherwise, a parasite may go extinct [54].

As can be seen in the phylograms (Figs. 3, 4) isolates of *Hepatozoon* spp. and Piroplasms are grouping regardless the geographical origin, indicating that the genetic patterns of this species are not restricted to a single geographical region. In this sense, Paludo *et al.* [55] and Rubini *et al.* [56] revealed genetic similarity between *H. canis* found in dogs in Brazil, Japan and Sudan. Partial sequences of the 18S gene of *H. felis* isolates from wild cats in Brazil [14] were closely related to those from domestic cats in Spain [57]. Also, a *H. felis* sequence obtained by Aktas *et al.* [58] in Turkey proved to be 99% compatible to that from an isolate found in a Leopard in Korea [59]. For Piroplasms, Nagore *et al.* [60] reported the encounter of *T.equi* isolates with similar gene sequences in different regions of Spain and Switzerland. These findings suggest a current or recent gene flow among populations, indicating geographic range shifts of domesticated animals, most driven by the increase in human travel and the introduction of non-endemic wildlife [55, 62].

Similar to other vector-borne protists, molecular diagnostic approaches for *Hepatozoon* spp. and piroplasmids rely mainly on the amplification of 18S rRNA gene fragments. This gene has been widely used to characterize and classify previously a wide range of hemoparasites including other hemogregarines and piroplasms [63–65]. However, because its relative conserved nature, it shows certain nodes to be unresolved [66–68]. Extending the range of available molecular markers to plastid or mitochondrial genes will greatly improve the understanding the diversity of intergeneric relationships between individual clades of hemoparasites [7] helping to provide information on parasite transmission dynamics, which represents an important issue for endangered species [68].

The microscopic analysis carried out in this study revealed that some mammals harbored other hemoparasites in addition to *Hepatozoon* spp. and Piroplasms. The visualization of microfilariae in the blood smears of two porcupines, corroborates the results by Thoisy *et al.* [69] who report more than 40% of representatives of this animal group parasitized by this hemoparasite. Magi *et al.* [70] and Moronin *et al.* [71] also reported the encounter of microfilariae in the blood of foxes and wolves, strengthening the concept that wild mammals can act as a reservoir for different zoonotic infections.

Some animals whose hemoparasites were isolated and sequenced, in the present study, were also co-parasitized by ticks. From the crossing of the data, some inferences could be established. In maned wolf (*Chrysocyon brachyurus)* and tyra (*Eira barbara*) that housed *H. canis* and *H. felis* respectively, ticks of the genus *Amblyomma* were identified. This result supports the suggestion of vectors of this genus as possible transmitters of *Hepatoozoon* spp. in Brazil, especially in the case of *Amblyomma ovale* [72].

Regarding Piroplasms, in the tapir (*Tapirus terrestris*) parasitized with an isolate corresponding to *T. equi*, six specimens of *Amblyomma sculptum* were found. This ectoparasite is the main vector of the bacteria *Rickettsia rickettsia*, in Brazil. *R. ricketsia* is the causative agent of Brazilian Spotted Fever, a human disease of high lethal power [73]. *A. sculptum* is part of a complex of six species known as *Amblyomma cajennense lato* sensu and although it is not part of the list of *T. equi* vectors, it is a strong candidate, due to the biological similarities that shares with the others species of the complex, which are notably known to transmit the hemoparasite in question [74].

In the cervid (*Mazama gouazoubira*) parasitized by *T. cervi*, nine *R. microplus* have been identified. This is the tick species with the greatest economic and medical veterinary relevance in the country, as it is the vector of *Babesia bovis* and *Babesia bigemina*, two piroplasms responsible for bovine babesiosis [75]. Although there is still no evidence in the literature to ratify this species as a transmitter of *T. cervi*, Silveira *et al.* [48] reported that most of the cervids positive for *T. cervi* were infested by *R. microplus*. In addition, this Ixodidae is responsible for the transmission of other species such as *T. equi* [74] reinforcing the idea of a possible establishment of a new parasitic relationship.

The identification of the ticks collected from the mammals included in this study, reiterated the preferential relationship between some ectoparasites and hosts, previously addressed in the literature [76]. In addition to raising questions about the possible roles of some species in the transmission of hemoparasites reported here.

In our context, the ectoparasite *A. ovale* was restricted to carnivores. Usually, the immature stages of this species of tick are found in rodents and marsupials, and the adult stages parasitize mainly wild and domestic carnivores [77]. *Amblyomma nodosum* and *A. sculptum* were identified in anteaters, also as reported by Garcia *et al.* [78]. The species *Amblyomma longirostre* has been identified in porcupine in our study. The literature reports that these animals normally harbor adult stages of the ectoparasite, while larval stages are more common in birds [79].

As presented and discussed above, there is abundant evidence of hemoparasites infections in wild mammals worldwide, in some circumstances displaying high prevalences. It is estimated that the wild fauna constitutes a reservoir still unknown of bio agents, many of these with zoonotic potential [80], and the inherent factors associated with the

process of rehabilitation and conservation of these animals such as, the immunosuppression caused by stress at the time of capture/transport, changes in diet and climate change can also contribute for the installation of diseases that can spread resulting in outbreaks and death [19, 82]. Furthermore, a wide variety of potential vectors can favor the maintenance of sylvatic parasitic life cycles in their geographical area enhancing its transmission to other mammals, including domestic ones, especially in periurban and urban environments [82].

Based on this, deep investigations of the main hemoparasites that infect wild animals, regardless of whether free living or those sent to rehabilitation centers are urgently required. Information from these surveys is mandatory for understanding the epidemiology of diseases, as well as for the implementation of medical, sanitary and health interventions.

Conclusions

Hemoparasites stand out due to the great importance of medical and veterinary research. The results obtained in this study pointed out, for the first time, the occurrence of *Hepatozoon* spp. and piroplasms (*Theileria* spp. and *Cytauxzoon* spp.) in wild mammals kept in rehabilitation centers in Minas Gerais and Goiás, Brazil. The circulating agents in this kind of animals and their vectors, are poorly known, especially in Brazil. Our findings, therefore, add relevant information that may contribute to the design of new studies and programs for effective prevention, diagnosis and control of these pathogens.

There was no agreement of results between the two techniques used in this work. Although PCR has provided more accurate results, we strongly encourage the association of two or more detection techniques for more reliable results.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11686-021-00492-3.

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Author Contributions NMNF: Conceptualization, Methodology, Writing the original draft and Supervision. TSA: Methodology, Investigation and Formal analysis. MGL: Methodology and Formal analysis. MBL: Resources. AQS: Resources. MCC: Conceptualization, Supervision, Project administration, Funding Acquisition and Writing -Review and Editing.

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Declarations

Conflict of interest The authors hereby declare previous originality check, no conflict of interest and open access to the repository of data used in this paper for scientific purposes.

Ethical approval This study was approved by the Ethics Committee on the Use of Animals, from the Federal University of Uberlândia (CEUA / UFU), Uberlândia, MG, Brazil, under the protocol 036/16. And, also, by the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA), a federal agency that regulates the procedures for handling wild animals in the country (License number 52561–2).

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