Parasitology

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Hepatozoon milleri sp. nov. (Adeleorina: Hepatozoidae) in *Akodon montensis* (Rodentia: Cricetidae: Sigmodontinae) from southeastern Brazil

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Research Article

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Abstract

Members of the genus *Hepatozoon* (Miller, 1908) are blood parasites found in a wide range of host species, including wild rodents; however, information about the life cycle, distribution and *Hepatozoon* species diversity infecting these mammals are lacking. We studied the parasite stages and DNA sequences of *Hepatozoon* sp. of 11 naturally infected *Akodon montensis*. Thin blood smears, tissue samples and whole blood were obtained for morphology, morphometry and molecular analyses. Seven of the 11 rodents had gamonts on the blood smears. Biological and morphological features of the parasite such as tissue tropism, gamonts and meronts size and morphology, as well as the DNA sequence comparison and phylogenetic analysis, indicated that the *Hepatozoon* sp. detected in this study is distinct from those species previously reported in small rodents. Herein, we propose a new species, named *Hepatozoon milleri* sp. nov. This is the first description of a new *Hepatozoon* species from wild small rodents in Brazil, based on morphological and molecular characteristics.

Introduction

Hepatozoon species are apicomplexan, blood protozoan parasites distributed worldwide and adapted to a wide variety of vertebrates (Smith, 1996). Their complex life cycle is heteroxenous in nature and involves blood-sucking vectors (metastigmatid and mesostigmatid mites, fleas, mosquitoes and lice), in which the sexual cycle occurs with posterior formation of multisporocystic oocysts containing infective sporozoites. Transmission to vertebrate intermediate hosts occurs through the consumption of infected arthropods. After ingestion, the released sporozoites undergo merogony in a variety of organs (lungs, spleen, liver, kidney and skeletal muscle), depending on the vertebrate hosts or the parasite species. Merogony is followed by gamontogony, with the development of gamonts in leucocytes or erythrocytes (Smith *et al.*, 1994; Ewing and Panciera, 2003; Laison *et al.*, 2003; Watkins *et al.*, 2006; Baneth *et al.*, 2007).

Alternative life cycles of some *Hepatozoon* spp. can include more than one vertebrate intermediate host, which acts as a paratenic host, harbouring in the tissues an infective quiescent form of the parasite called the cystozoites (Smith, 1996). Those cyst forms have been demonstrated in the life cycle of *Hepatozoon* species that infect anurans, reptiles and mammals (Desser, 1990; Smith *et al.*, 1994; Laison *et al.*, 2003; Viana *et al.*, 2012). In this predator–prey transmission, the vertebrate intermediate hosts become infected through the ingestion of prey containing cystozoite stages of the parasite (Smith, 1996; Johnson *et al.*, 2008; Viana *et al.*, 2012).

Hepatozoon spp. infection has been detected in a wide range of wildlife species (Criado-Fornelio *et al.*, 2006; Metzger *et al.*, 2008; André *et al.*, 2010; Farkas *et al.*, 2014), including wild rodents (Duscher *et al.*, 2014; Rigó *et al.*, 2016; Wolf *et al.*, 2016; Sousa *et al.*, 2017). However, the knowledge about species diversity, distribution, vectors and life cycle in wild rodent populations remains quite limited.

Balfour (1905) reported a species of haemogregarina (*Leucocytozoon muris*) infecting the leucocytes of a rodent, *Rattus norvegicus*, from Sudan (Wenyon, 1926) for the first time. Miller (1908), in the USA, elucidated the complete life cycle of a haemogregarina from *Rattus rattus* and named it *Hepatozoon perniciosum*. Years later, all known species of *Leucocytozoon* and *Haemogregarina* from mammals were transferred to the genus *Hepatozoon* (Wenyon, 1926), and *H. perniciosum* was considered to be synonymous with *Hepatozoon muris*. In São Paulo State, Brazil, Carini (1910) observed *H. muris* infecting *Mus decumanus* and Carini and Maciel (1915) first reported *Hepatozoon* sp. in a wild rodent, *Akodon fuliginosus*, describing it morphologically and considered as a different species, *Hepatozoon akodoni* (syn. *Haemogregarina akodoni*).

Since then, no other reports of rodent *Hepatozoon* species have been made, mainly in Brazil, primarily because most of the new records of *Hepatozoon* infections in wild rodents

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are based on molecular features, without incorporating morphological information of the protozoan (Maia *et al.*, 2014; Wolf *et al.*, 2016; Sousa *et al.*, 2017). Therefore, unidentified *Hepatozoon* species have been reported in rodent species (Maia *et al.*, 2014; Wolf *et al.*, 2016; Sousa *et al.*, 2017; Kamani *et al.*, 2018) without being named. Those aspects emphasize the importance of including the description of life cycle stages in addition to molecular tools to describe new species or to re-describe recognized *Hepatozoon* organisms.

During a previous study, we investigated the possibility of transmission of *Hepatozoon canis* to dogs by predation of infected rodents, comparing the species found in wild rodents with *H. canis* detected in dogs from the same region (Demoner *et al.*, 2016). Moreover, a new species of *Hepatozoon* infecting wild rodents was detected. Herein, we describe a new rodent *Hepatozoon* species, *Hepatozoon milleri* sp. nov., based on morphometric, morphological and molecular aspects.

Material and methods

Rodents' collection and identification

As part of a study on canine hepatozoonosis in order to evaluate the relationships among *Hepatozoon* spp. infecting wild rodents and domestic dogs (Demoner *et al.*, 2016), rodents were live-trapped in three forest fragments in the municipality of Botucatu, São Paulo State, southeastern Brazil, between November 2013 and June 2014. The animals were collected along terrestrial transects using Sherman and Pitfall traps as previously described (Demoner *et al.*, 2016). In total, 67 rodents were captured and were anaesthetized by inhalation of isoflurane, and blood collection was performed by cardiac puncture for use in genetic and morphological identification. A thin blood smear of each rodent was prepared, fixed with methanol, stained with Wright–Giemsa and screened for *Hepatozoon* gamonts. All the ectoparasites attached on the rodents were collected and identified.

This study was performed on blood and tissue samples from 11 *Akodon montensis*, which had their specific identification determined, first by their morphological aspects, especially from the head, and later, confirmed by PCR and sequencing. The sequences obtained from the rodents blood were compared for similarity with the sequences of other rodents available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and presented 99% of similarity with *A. montensis* from Brazil (GU938933 and KJ013083).

Euthanasia of all collected rodents was performed by deep anaesthesia with isoflurane, followed by organ removal (heart, lung, kidney, spleen, liver, and skeletal cardiac and muscle) (Demoner *et al.*, 2016).

Hepatozoon *sp. identification and characterization*

These 11 rodents were found to be infected with a different *Hepatozoon* species by either microscopy or PCR. The parasitaemia was calculated as the percentage of infected leucocytes observed in 200 cells (Aktas *et al.*, 2015). Collected tissues were preserved for molecular identification of *Hepatozoon* spp. and for histological examination. For histological evaluation, formalin-fixed tissue samples were routinely processed and stained with haematoxylin and eosin (Kiernan, 2015). Parasite morphology was analysed by optical microscopy, at objectives of 40× or 100×, and ocular of 10×. For the morphometric study, a computerized image analysis system was used, and measurements of the length and the width of gamonts and tissue stages were obtained using the software Qwin Lite 2.5 (Leica). In order to characterize the *Hepatozoon* species, DNA from rodent blood and tissue samples (approximately 100–200 μL of whole blood and 20–50 mg of skeletal muscle, spleen, liver, lung and kidney) was extracted using the Illustra Blood genomic Prep Mini Spin Kit® and the Illustra Tissue Mini Spin Kit® (GE Healthcare, Buckinghamshire, UK), according to the manufacturer's instructions.

Amplification of the 18S ribosomal RNA gene (a 600 bp fragment) of *Hepatozoon* spp. was performed on the rodent samples using the primer pair HepF300 (5′-GTT-TCT-GAC-CTA-TCA-GCT-TTC-GAC-G-3′) and Hep900 (5′-CAA-ATC-TAA-GAA-TTT-CAC-CTC-TGA-C-3′) (Ujvari *et al.*, 2004). In order to amplify a larger segment of the 18S rRNA of *Hepatozoon* spp. (approximately 1120 bp), a second PCR assay was carried out using the primers 4558 (5′-GCT-AAT-ACA-TGA-GCA-AAA-TCT-CAA-3′) and 2733 (5′-CGG-AAT-TAA-CCA-GAC-AAA-T-3′) (Mathew *et al.*, 2000), for additional phylogenetic analysis. PCR reaction conditions and sequencing were described before by Demoner *et al.* (2016).

To avoid misdiagnosis of *Hepatozoon* monozoic cysts with *Toxoplasma gondii*, a PCR was performed on DNA tissues only from the rodents that were positive to monozoic cyst on the tissues' histology. The PCR was conducted with the primers TOX4 (5′-CGC TGC AGG GAG GAA GAC GAA AGT TG-3′) and TOX5 (5′-CGC TGC AGA CAC AGT GCA TCT GGA TT-3′), which targeted a 529-bp fragment of the *T. gondii* repeated sequence (Homan *et al.*, 2000).

By comparing for similarity with the sequences available in GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the sequences obtained in the present study were identified as *Hepatozoon* sp. and were subjected to multiple alignment analyses (with additional *Hepatozoon* spp. sequences retrieved from Genbank®) using the MUSCLE algorithm in the software Geneious v.7.1.3 (Biomatters, http://www.geneious.com), for phylogenetic analysis. Only the sequences with at least 990 bp overlapping were used for the multiple alignment.

The best evolutionary model for Bayesian inference, based on the Akaike information criterion, was identified using the jModelTest v.2.1.10 (Darriba *et al.*, 2012). The chosen parameter of the substitution model was GTR + G. For phylogenetic reconstruction, MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to construct the phylogenetic tree by Bayesian inference. Markov chain Monte Carlo simulations were run for 10^7 generations in two parallel runs, with sampling of trees at 1000-generation intervals and a burn-in of 25%. Phylogenetic trees were visualized in FigTree v.1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). An alignment with 1007 bp from one *Hepatozoon* sp. sequence obtained in this study and seven *Hepatozoon* sp. sequences, isolated from rodents available on GenBank®, was used to estimate the percentage of nucleotide divergence. This analysis was performed on the MEGA6 software, using the *p*-distance model (Tamura *et al.*, 2013).

Results

General observations

Previously, Demoner *et al.* (2016) collected 67 rodents from five different species and 55.2% were PCR positive for *Hepatozoon* spp. One of the species was *A. montensis*, with 19 animals collected and 17 infected (89.5%) (Demoner *et al.*, 2016). For the present study, we used only the 11 rodents, all identified as *A. montensis*, that presented *Hepatozoon* stages on blood or tissues (Table 1), and were PCR positive. For the species description, we used only the seven rodents that had gamonts on blood smears. Their parasitaemia varied from 9 to 65.5% (mean of 38.6%) (Table 1).

By morphological examination, three different parasite stages were found in the infected rodents: gamonts in the peripheral

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Table 1. *Hepatozoon milleri* positivity, in *Akodon montensis*' tissues and blood by both PCR and microscopy

Identity	Blood			Liver			Spleen			Muscle			Lungs			Kidney		
Akodon montensis	PCR	Gam.	Paras. (%)	PCR	Histol.		PCR	Histol.		PCR	Histol.		PCR	Histol.		PCR	Histol.	
					Mer.	Cyst.		Mer.	Cyst.		Mer.	Cyst.		Mer.	Cyst.		Mer.	Cyst.
R1	+	+	34	+	+	–	+	–	–	+	–	–	–	–	–	–	–	–
R2	+	+	39	+	+	–	+	–	+	–	–	–	+	–	+	–	–	–
R3	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–
R5	+	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–
R7	+	+	9	+	+	–	+	–	+	–	–	–	+	–	+	+	–	+
R8	+	+	44	+	+	–	+	–	+		–	–	–	–	–	+	–	+
R13	+	+	62	+	+	–	+	–	+	+	–	–	–	–	–	+	–	+
R20	+	+	17	+	+	–	+	–	–		–	–	–	–	–	–	–	–
R23	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	–	–	–
R24	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
R31	+	+	65.5	+	–	–	+	–	–	+	–	–	–	–	–	–	–	–
Total	8	7	38.6 (mean)	10	6	0	10	0	4	3	0	0	2	0	2	3	0	3

Gam., gamont; Paras., parasitaemia; Histol., histology; Mer., meront; Cyst., cystozoite.

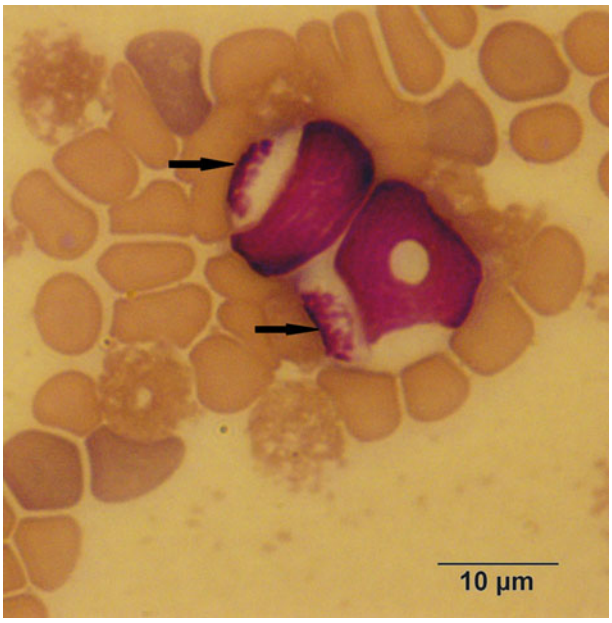

Fig. 1. Gamonts of *Hepatozoon milleri* sp. nov. in the peripheral blood of an *Akodon montensis*. Giemsa stain.

blood (within mononuclear leucocytes or extracellular/free parasites) (Fig. 1); mature and immature meronts (exclusively in the liver, within hepatocytes) (Fig. 2); and cystozoites (monozoic cysts – cysts with one zoite inside) in the lung, spleen or kidney (Fig. 3) (Table 1). The gamonts were elongated and oval, with the cytoplasm pale blue stained. Its main characteristic was the nucleus that was large, lightly condensed and very granular, occupying almost half of the gamont cytoplasm, centrally located or displaced to one extremity of the gamont. The meronts were in different stages of development. The immature meronts were round-to-oval in shape, containing amorphous material enclosed within a thick wall (Fig. 2A). As it became more developed, a basophilic-stained material was formed at the periphery of the cell, and would originate the merozoites (Fig. 2B). The mature meronts were globular shaped, with a thick membrane enclosing the well-defined and elongated merozoites (with a single nucleus) formed around the residual body, whose number varied from 20 to 30 (Fig. 2C). The cystozoites were ovoid shaped, with the cytoplasm staining white surrounding a single zoite, which was curved and mononuclear (Fig. 3).

Only two of the 11 infected rodents were infested by a few mesostigmatid mites that were morphologically identified as *Androlaelaps rotundus*, a non-blood-sucking mite, discarding its role as possible *Hepatozoon* vector.

Molecular and phylogenetic analysis

The sequences obtained from the 11 PCR-infected animals were all identical, on blood and tissues. Based on the PCR and sequencing, a *Hepatozoon* sp. sequence, with lengths ranging from 1000 to 1050 base pairs (bp), was obtained from the blood and tissues of infected *A. montensis* specimens. BLAST analysis of the obtained sequence showed nucleotide sequence similarities (97–99%) with other *Hepatozoon* sequences detected in wild rodents from various parts of the world [Genbank accession numbers: FJ719817 and FJ719819 (*Hepatozoon* sp. in *Abrothrix olivaceus* and *Abrothrix sanborni*, respectively, from Chile), AB181504 (*Hepatozoon* sp. in *Bandicota indica* from Thailand), AY600625 and JX644997 (*Hepatozoon* sp. detected in bank voles from Europe)].

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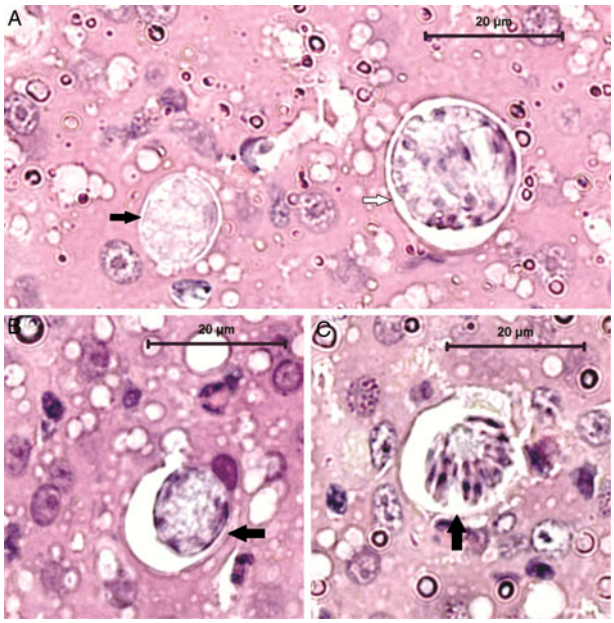

Fig. 2. Histological detection of *Hepatozoon milleri* sp. nov. meronts in the liver of an infected *Akodon montensis*. (A) Two immature meronts, with different developmental stages, containing amorphous material. (B) Further developed meront characterized by basophilic-stained material at the periphery of the cell, which probably gives rise to the merozoites. Haematoxylin and eosin stain, bar = 20 µM. (C) Mature meront. Note numerous well-defined merozoites arranged around the residual body. Haematoxylin and eosin stain, bar = 20 µM.

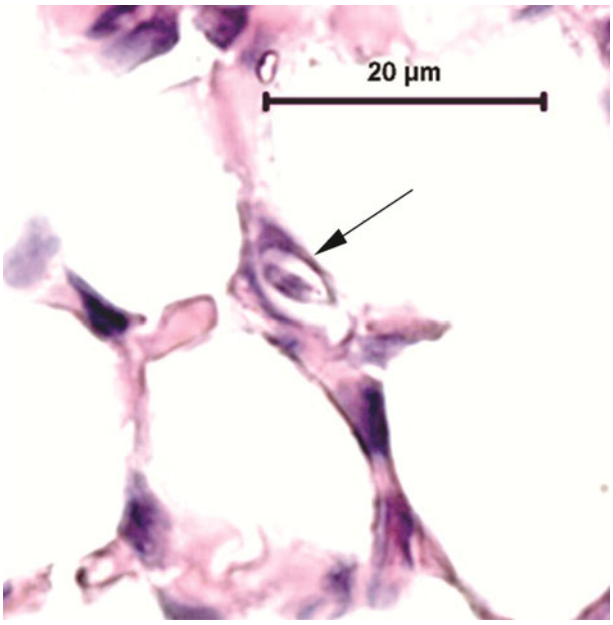

Fig. 3. Cystozoite of *Hepatozoon milleri* sp. nov. within a monozoic cyst in the spleen of an infected *Akodon montensis*. Haematoxylin and eosin stain, bar = 20 µM.

The phylogenetic tree based on 992 bp from the 18S rRNA fragment indicated that the sequence from the *Akodon*-derived *Hepatozoon*, considered as *H. milleri* sp. nov., grouped into a major clade containing *Hepatozoon* spp. sequences detected in wild rodents in several regions of the world, while sequences from carnivores, reptiles, amphibians and marsupials formed distinct clades (Fig. 4). The nucleotide divergence among *H. milleri* sp. nov. and *Hepatozoon* spp. in rodents around the world ranged from 0.69 to 2.68% (Table 2). The new sequence reported in this study clustered with *Hepatozoon* sequences previously isolated from wild rodents in Chile (FJ719817; FJ719819) with nucleotide divergence of 0.69 and 0.89%. The *p*-distance between *H. milleri* sp. nov. and *Hepatozoon* spp. from rodents in Brazil were of 0.6% (KP757838), 1.0% (KX776349; KX776353) and 3.7% (KX776335; KX776337). However, the sequences (KP757838; KX776349; KX776353; KX776335; KX776337) were too short (≈600 bp) to be included in the phylogenetic analysis.

The tissues of the four animals that presented monozoic cysts in their tissues were PCR negative for *T. gondii*.

Considering the morphological and molecular features, we described the new *Hepatozoon* species in *A. montensis* from Brazil, named *H. milleri* sp. nov.

Description

Hepatozoon milleri sp. nov.
(Figs 1–3).

Parasite morphology

Gamonts: Elongated with an oval shape, measuring 10.96 ± 0.87 µM (range 9.42–13.09) long by 4.9 ± 0.51 µM (3.88–6.38) wide ($n = 36$). Slightly condensed and granular nucleus, occupying almost half of the cytoplasm, centrally located or displaced to one extremity of the gamont, measuring 5.58 ± 1.02 µM (3.71–7.13) long by 3.8 ± 0.51 µM (2.52–4.75) wide (Fig. 1).

Immature meronts: Round-to-oval in shape, containing amorphous material enclosed within a thick wall (Fig. 2A) and measuring 17 ± 3.49 µM (11.52–25.39) long by 14.43 ± 2.17 µM (9.83–18.83) wide ($n = 42$). More developed meronts contained basophilic-stained material at the periphery of the cell, presumed to be merozoite formation (Fig. 2A and B).

Mature meronts: Globular shaped, with a thick membrane enclosing the well-defined and elongated merozoites (with a single nucleus) in numbers of 20–30 (Fig. 2C), formed around the residual body. Meronts measured 20.17 ± 3.26 µM (15.7–26.94) long by 16.42 ± 2.96 µM (12.88–21.57) wide ($n = 13$) and merozoites were 5.63 ± 1.37 µM (3.11–7.9) long by 1.58 ± 0.27 µM (1.26–2.06) wide ($n = 14$).

Cystozoites (monozoic cysts): Ovoid shaped, with the cytoplasm staining white surrounding a single zoite, which is curved and mononuclear (Fig. 3). Cysts measured 5.4 ± 0.82 µM (4–6.93) long by 1.76 ± 0.28 µM (1.18–2.58) wide ($n = 28$).

Taxonomic summary

Phylum Apicomplexa Levine, 1970.

Family Hepatozoidae Wenyon, 1926.

Genus *Hepatozoon* Miller, 1908.

Type host: *Akodon montensis* (Rodentia: Cricetidae: Sigmodontinae).

Other hosts: Unknown.

Vector: Unknown.

Type locality: The specimens were captured in forest fragments from the municipality of Botucatu, São Paulo State, southeastern Brazil (22°53′09″S, 48°26′42″W).

Type material: Blood smear and histology slide from Akodon montensis are deposited at INPA ('The National Institute of Amazonian Research'), Av. André Araújo, 2936 – Petrópolis, Manaus – AM, Brazil, 69067-375. 1x blood smear is deposited under accession number INPA 15a and 1x histology slide is deposited under accession number INPA 15b.

Other material: DNA samples are conserved at the Parasitology Department, IBB, Unesp, São Paulo, Brazil.

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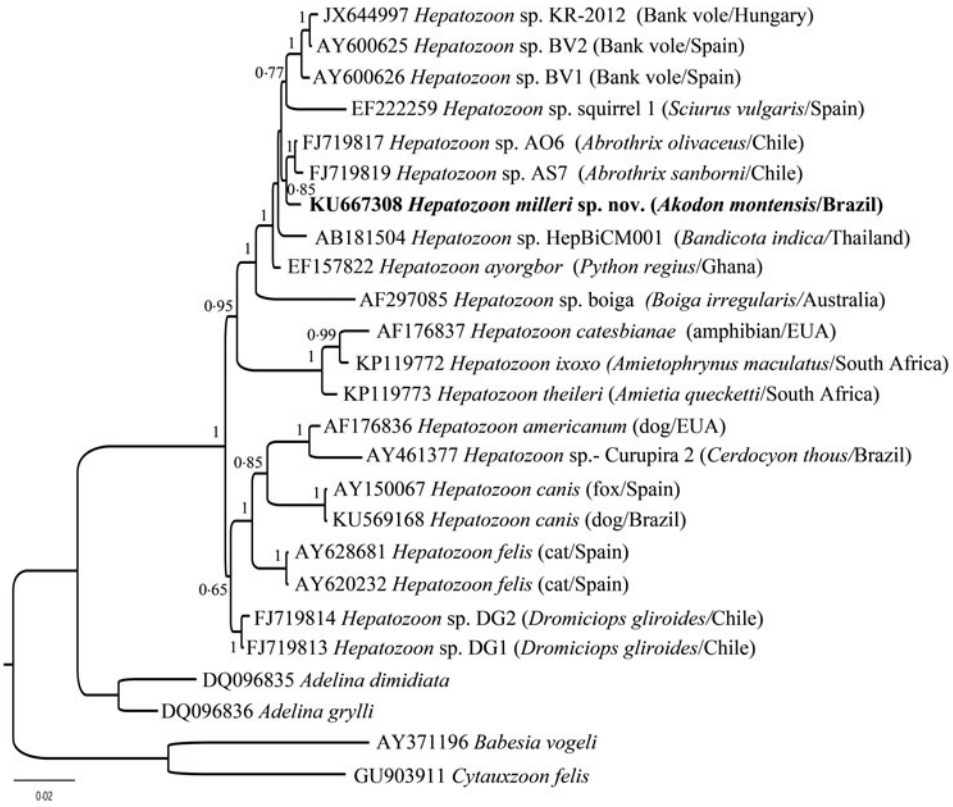

Fig. 4. Bayesian inference (BI) tree based on the 18S rRNA gene partial sequences (992 bp) of *Hepatozoon milleri* sp. nov. isolated from *Akodon montensis* in south-eastern Brazil and sequences available in the GenBank database, using GTR + G evolutionary model. *Babesia vogeli*, *Cytauxzoon felis*, *Adelina dimidiata* and *Adelina grylli* were used as outgroups. Numbers at the nodes indicate posterior probabilities under BI. Posterior probabilities lower than 50 are not shown. New sequence identified in the study is indicated in bold.

Table 2. Distance matrix among partial 18S rDNA sequences of *Hepatozoon* spp. isolated from rodents

Sequences	1. KU667308[a]	2. FJ719817	3. FJ719819	4. AB181504	5. AY600625	6. AY600626	7. JX644997	8. EF222259
1. KU667308 *Hepatozoon milleri* sp. nov.[a]		7	9	14	15	16	17	27
2. FJ719817 *Hepatozoon* sp.	0.69%		2	13	12	14	14	24
3. FJ719819 *Hepatozoon* sp.	0.89%	0.19%		15	14	14	16	26
4. AB181504 *Hepatozoon* sp.	1.39%	1.29%	1.49%		18	20	20	29
5. AY600625 *Hepatozoon* sp.	1.49%	1.19%	1.39%	1.78%		6	2	27
6. AY600626 *Hepatozoon* sp.	1.58%	1.39%	1.39%	1.98%	0.59%		8	29
7. JX644997 *Hepatozoon* sp.	1.68%	1.39%	1.59%	1.98%	0.19%	0.79%		29
8. EF222259 *Hepatozoon* sp.	2.68%	2.38%	2.59%	2.88%	2.68%	2.88%	2.88%	

Upper triangle shows the number of nucleotide difference, while the lower triangle shows the percentage of nucleotide difference among the sequences.

DNA sequence: The 18S rRNA gene sequence has been deposited in the Genbank under the accession number KU667308.

ZooBank registration: In accordance with section 8.5 of the International Code of Zoological Nomenclature (ICZN), details of the new species are submitted to ZooBank with the Life Science Identifier (LSID) urn:lsid:zoobank.org:act: EE4F5009-0860-4D1A-B787-E55FCEAA7C94.

Etymology: The name *H. milleri* sp. nov. is homage to Dr William Whitfield Miller, who described the genus *Hepatozoon* as intraleucocytic haemogregarines in laboratory rats.

Remarks:

The morphometric data of others rodents *Hepatozoon* species and *H. milleri* sp. nov. are summarized in Table 3. The gamonts morphometry were similar among the species. In the original description of *H. muris* (Miller, 1908), the gamonts were slightly longer than the *H. muris* studied by Carini and Maciel (1915), and *H. milleri* from the present study. The morphometry of the gamonts of *H. muris* described by Miller (1908) was 12 × 6 μM, whereas the gamonts of the *H. muris* described by Carini (1910) measured 10–13 μM × 4–6 μM. The gamonts of *H. akodoni* described by Carini and Maciel (1915) measured approximately 10 × 3.5 μM, being smaller than the *H. muris* gamonts. The gamonts described herein measured 10.9 × 4.9 μM on average and, despite being smaller than *H. muris*, are wider than *H. akodoni* described by Carini and Maciel (1915). By contrast, *Hepatozoon* sp. from *Clethrionomys glareolus* (syn. *Myodes glareolus*) (Johnson *et al.*, 2007) and *H. muris* of *R. norvegicus* (Harikrishnan *et al.*, 2011) were much smaller than the other reported species.

In most of the *Hepatozoon* species reported, the morphometry of the gamont nucleus was not recorded, but in general, they were ovoid and centrally located, slightly granular. The nucleus of *H. milleri* was larger than the nucleus of *H. akodoni* (Carini and Maciel, 1915) and the one studied by Johnson *et al.* (2007).

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Table 3. Measurements of developmental stages of *Hepatozoon milleri* sp. nov. and other rodents *Hepatozoon*

Hepatozoon spp.	Intermediate host	Country	Gamont (μM)	Gamont nucleus (μM)	Meront (μM)	Merozoites (μM)	Reference
H. milleri	*Akodon montensis*	Brazil	10.9 × 4.9	5.6 × 3.8	20.2 × 16.4	5.6 × 1.6	Present study
Hemogregarina balfouri	*Jaculus jaculus*	Sudan	Erythrocytes 5.5–7 × 1.4–2.8	N/A	22.4 × 16.8	N/A	Balfour (1905)
H. muris	*Rattus norvegicus*	USA	12 × 6	N/A	25 × 30	N/A	Miller (1908)
H. muris	*Mus decumanus*	Brazil	10–13 × 4–6	N/A	15–30	N/A	Carini (1910)
H. akodoni	*Akodon fuliginosus*	Brazil	10 × 3.5	4 × 2.5	N/A	N/A	Carini and Maciel (1915)
H. erhardovae	*Clethrionomys glareolus*	Finland	N/A	N/A	Lungs 19 × 14	N/A	Laakkonen *et al.* (2001)
Hepatozoon sp.	*Sigmodon hispidus* and *Peromyscus leucopus*	USA	7.5 × 12	3.0 × 7.5	Liver (only *S. hispidus*) 20–35 (mean 23.5)	USA	Johnson *et al.* (2007)
H. muris	*Rattus norvegicus*	India	8	N/A	24–26 × 22	N/A	Harikrishnan *et al.* (2011)

N/A, data not available.

The main characteristic of the *H. milleri* gamont nucleus is its sparsity and large size, occupying half of the gamont cytoplasm. This characteristic was observed in all blood smears from all positive animals. In contrast, the nucleus of *H. muris* gamonts were central and condensed (Miller, 1908). Moreover, the cells parasitized by *H. akodoni* gamonts had their nucleus morphology changed, becoming more fragmented (Carini and Maciel, 1915), a characteristic that was not observed in the present study.

Meronts in various stages of maturity were observed in this study. The earliest meronts were similar in size and appearance to immature meronts that have been previously described in small rodents infected with *Hepatozoon* spp. (Miller, 1908; Carini, 1910; Johnson *et al.*, 2007; Breshears *et al.*, 2009). On the other hand, the morphology and size range of mature meronts, detected in Brazilian wild rodents, are not consistent with previous descriptions of developed meronts in rodents from other regions. For example, Miller (1908) detected larger mature meronts (mean of 25 × 30 μM, some reaching 28 × 35 μM) of *H. muris* in infected laboratory rats, containing a small number of merozoites (12–20), whereas we found meronts, which at maturity measured 20 × 16 μM on average, containing from 20 to 30 merozoites. The developed meronts of *H. muris* described by Carini (1910) were smaller (diameter of 15–30 μM) and enclosed numerous merozoites (25–50). Unfortunately, Carini and Maciel (1915) did not observe tissue meronts of *H. akodoni* in the rodent organs. Johnson *et al.* (2007) and Breshears *et al.* (2009) studied *Hepatozoon* sp. in *Sigmodon hispidus* in the USA. The infected wild rodents harboured mature meronts greater (20 × 30 μM) than those reported in our study and had merozoites radially arranged in numbers of 50 approximately, in contrast to the meronts reported here, which had laterally or radially arranged merozoites.

Cystozoites (monozoic cysts) were detected in the lungs, spleen and kidneys of some *Hepatozoon*-infected rodents. Laakkonen *et al.* (2001) detected monozoic and dizoic cysts of *Hepatozoon erhardovae* in the lungs of *C. glareolus* in Europe and cystic forms of *Hepatozoon griseisciuri* have been recorded in the lungs of the grey squirrel *Sciurus carolinensis* (Desser, 1990). Conversely, Johnson *et al.* (2007) did not observe cyst formation in tissues from naturally *Hepatozoon*-infected wild rats from the USA. Among *Hepatozoon* spp., the cyst form differs mainly in size (10–21 μM long by 2–17 μM wide) than it does in morphology, which is typically round-to-oval shaped containing one (monozoic) or two (dizoic) curved mononuclear zoites (Laakkonen *et al.*, 2001; Laison *et al.*, 2003; Baneth *et al.*, 2007; Johnson *et al.*, 2008).

Discussion

Based on gamonts morphology, morphological and morphometrical characteristics of the blood and tissue stages and finally, molecular and phylogenetic analyses, the protozoa reported in the present study is a new species, described as *H. milleri* sp. nov. Although there are several *Hepatozoon* species reported in rodents, most of them were described many years ago, with some studies dating over 100 years. Additionally, those descriptions were based only on morphology, usually of only one developmental stage, the figures were drawn by hand, and measurements were not standardized. Since the authors did not deposit voucher specimens at that time, it is not possible to study those old *Hepatozoon* species and compare with ours.

Hepatozoon spp. infections have been reported in a wide variety of vertebrates and are found in several mammalian hosts in Brazil (Criado-Fornelio *et al.*, 2006; Metzger *et al.*, 2008; André *et al.*, 2010; Farkas *et al.*, 2014). However, data on *Hepatozoon* spp. infecting Brazilian wild rodents are still lacking. The morphological features of the parasite stages suggested that the *A. montensis* specimens in the present study were infected with a new species of *Hepatozoon*, whose gamonts have a large and sparse nucleus that occupy half of the gamont cytoplasm, being different from the previously reported species. The possibility that this nucleus characteristic could be the result of nuclear material fragmentation due to an artefact was discarded, as all the smears were done immediately after blood collection, as well as dried and fixed. In addition, we observed the same gamont characteristic in all infected animals.

Balfour (1905) reported *L. muris* (syn. *H. muris*) infecting the *R. norvegicus* leucocytes (Wenyon, 1926). A few years later, Miller (1908) elucidated the life cycle of *H. muris* (*H. perniciosum*) in laboratory white rats and in its vector, the mite *Lelaps echidninus*, and observed gamonts formation in lymphocytes of infected rodents (Miller, 1908). Remarkable was that *H. muris* was very pathogenic for the vertebrate host, and that was why Miller (1908), at first, named it *H. perniciosum*.

With respect to *H. muris*, the reports from Carini (1910) and from Harikrishnan *et al.* (2011) were quite different from the original report (Miller, 1908) and may represent different species (Table 3). The gamonts from *H. muris* described by Miller (1908) were larger (12 × 6 μM) than the ones from Carini (1910) (10–13 μM × 4–6 μM) and Harikrishnan *et al.* (2011) (8 μM). Also, the meronts had different sizes and number of merozoites, being the ones from Miller (1908) larger (25–28 × 30–35 μM) with

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a small number of merozoites (12–20), in contrast with those from Carini (1910) which were smaller (15–30 μM) but with numerous merozoites (25–50). The meronts described by Harikrishnan *et al.* (2011) had intermediate size (24–26 × 22 μM) with no description of the merozoites number.

Developmental stages of *H. milleri* sp. nov. presented morphological and morphometrical differences from the other reported species. Variations in the tissue tropism of *Hepatozoon* spp. within the host species are quite common and thereby, influence the location of the tissue merogony (Smith, 1996). Histological examination of tissues from *Hepatozoon*-infected *A. montensis* revealed *Hepatozoon* meronts only in the liver. Nevertheless, as we worked with only seven specimens, we cannot assure that merogony occurs exclusively in the liver. Johnson *et al.* (2007) observed that the merogony of *Hepatozoon* sp. in *S. hispidus* and *Peromyscus leucopus* from North America was limited to the liver. Additionally, *H. muris* in laboratory white rats (Miller, 1908) developed meronts in the liver and Carini (1910) observed meronts of *H. muris* either in the liver or in the lungs of infected *R. norvegicus*. Unlike those descriptions, merogony of *H. erhardovae* reported in *C. glareolus* from Europe occurred exclusively in the lungs (Laakkonen *et al.*, 2001). Furthermore, the predilection site of tissue merogony of *H. griseisciuri* infecting the grey squirrel *S. carolinensis* in the USA was the lungs (Davidson and Calpin, 1976). The descriptions of the meronts were scarce.

Extracellular parasites were also detected in the blood smears of the infected rodents in the present study, which is not uncommon because previous studies have demonstrated occasionally free gamonts in infected rodents, probably due to the rupture of host cells (Miller, 1908; Carini, 1910; Laird, 1951; Davidson and Calpin, 1976; Rigó *et al.*, 2016).

Most of the recent reports on *Hepatozoon* species in wild rodent populations have focused mainly on molecular methods without demonstrating the blood parasite forms (Criado-Fornelio *et al.*, 2006; Allen *et al.*, 2011; Maia *et al.*, 2014; Wolf *et al.*, 2016; Sousa *et al.*, 2017; Kamani *et al.*, 2018). Therefore, gamonts comparison among *Hepatozoon* spp. infecting mammals from previous studies is difficult.

Molecularly, we identified a novel species of *Hepatozoon* in the *A. montensis* specimens. Sequences of *Hepatozoon* species have been recorded in wild and domestic rodents from Europe (Criado-Fornelio *et al.*, 2006; Rigó, *et al.*, 2016), Africa (Maia *et al.*, 2014; Kamani *et al.*, 2018), North America (Johnson *et al.*, 2007) and South America (Merino *et al.*, 2009; Demoner *et al.*, 2016; Wolf *et al.*, 2016; Sousa *et al.*, 2017).

Sousa *et al.* (2017) have captured 110 wild rodents in southern Pantanal, Brazil, being 77 *Thichomys fosteri*, 25 *Oecomys mamorae* and eight *Clyomys laticeps*. No gamonts were detected in the blood smears, nevertheless, 11 (44%) *O. mamorae* and 13 *T. fosteri* (16.9%) were positive for 18SrRNA *Hepatozoon* spp.-PCR (Sousa *et al.*, 2017). In contrast, in the present study, the *H. milleri* parasitaemia was very high, varying from 9 to 65.5% (mean of 38.6%). In relation to the other *Hepatozoon* sequences from other countries, the nucleotide difference varied from 1.39% with a wild rodent from Thailand (AB181504) and reached 2.68% with the sequence isolated from a squirrel in Spain (EF222259).

The nucleotide divergence among *H. milleri* sp. nov. and *Hepatozoon* spp. from rodents in Brazil was of 0.6% (KP757838) and 1.0% (KX776349; KX776353) and among the Chilean rodents was 0.69 and 0.89%, respectively (FJ719817 and FJ719819). These low divergences amidst sequences isolated in rodents from South American demonstrate that these parasites are haplotypes of the species described herein. Nevertheless, since Merino *et al.* (2009), Wolf *et al.* (2016) and Sousa *et al.* (2017) did not describe the *Hepatozoon* species, as they had only its molecular data, we decided to describe it providing the morphological data of the parasites.

Sousa *et al.* (2017) observed different *Hepatozoon* haplotypes and a π value higher through the rodent population than the found amidst other host species; and suggested that there is some degree of *Hepatozoon* genetic diversity among the population of wild rodents from Brazil. As two of the sequences they have found (KX776335, KX776337) presented a nucleotide divergence of 3.7% in relation to *H. milleri* sp., they are probably different species.

It has been shown in several studies that differences superior to 1.0% correspond to species-level differences in haemogregarines when the slow-evolving 18S rRNA marker is used (Barta *et al.*, 2012; Cook *et al.*, 2015; Borges-Nojosa *et al.*, 2017). This was confirmed by Netherlands *et al.* (2018) that named three new *Hepatozoon* species from anurans based on morphological and molecular data, with the new species showing interspecific divergence that varied from 1.0 to 2.0%.

With respect to ectoparasites, Sousa *et al.* (2017) in Brazil detected ticks (*Amblyomma* spp.) and fleas (*Polygenis* sp.) on rodents, but could not determine if they were infected by *Hepatozoon* spp. or not. Kamani *et al.* (2018), studying *Hepatozoon* spp. in rodents and their ectoparasites in Nigeria, found two species of ticks (*Rhipicephalus sanguineus* sensu lato and *Haemaphysalis leachi*), two species of fleas (*Xenopsylla cheopis* and *Ctenophthalmus* spp.) and one species of Mesostigmata, gamasid mites (*Haemolaelaps* spp.), all negative for *Hepatozoon* spp. DNA. In our study, we did not find ticks infesting the rodents, and unfortunately, we could not identify possible vectors of *H. milleri* sp. nov. as the only ectoparasites recovered from *Hepatozoon*-infected rodents were non-haematophagous mites.

In conclusion, in the present study, we described a new *Hepatozoon* species, *H. milleri* sp. nov., from wild small rodents in southeastern Brazil. This is the first description of a new *Hepatozoon* from wild rodent species in Brazil, based on morphological and molecular studies. Further investigation should include the identification of possible vectors, the geographical distribution of the protozoan, as well as the host range, which might clarify some epidemiological aspects of this new parasite.

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Conflict of interest. None.

Ethical standards. All animal procedures were conducted in accordance with the guidelines of the Ethical Committee for Animal Research at the Instituto de Biociências/UNESP (CEUA – Comissão de Ética no Uso de Animais – protocol 431) and under a permanent scientific collection license issued by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO 36283-3).

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