



Description of a New Species *Hepatozoon quagliattus* sp. nov. (Apicomplexa: Adeleorina: Hepatozoidae), infecting the Sleep Snake, *Dipsas mikanii* (Squamata: Colubridae: Dipsadinae) from Goiás State, Brazil

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Abstract

Purpose Species of *Hepatozoon* Miller, 1908 (Hepatozoidae) are blood protozoans with a cosmopolitan distribution and are reported to parasitize a range of vertebrate hosts including mammals, birds, reptiles, and amphibians. The present study aimed to describe a new species of *Hepatozoon* (Apicomplexa: Adeleorina: Hepatozoidae) found infecting the sleep snake *Dipsas mikanii* (Schlegel, 1837) (Squamata: Colubridae: Dipsadinae).

Methods The snake was collected in 2017 at the municipality of Britânia, Goiás State, Brazil. Blood smears were made in order to find blood gametocytes and PCR was performed targeting the 18S rRNA gene.

Results Microscopy screening of blood smears revealed the presence of intraerythrocytic gamont stages of *Hepatozoon* sp. in the peripheral blood with a parasitemia of 0.25%. Furthermore, meronts and monozyotic cysts were observed in histological sections of the liver from the infected individual. The interspecific divergence of 18S rRNA sequences fragments isolated from *D. mikanii* had differences (2.39–11.3%) as compared to other sequences of species of *Hepatozoon* from snakes.

Conclusions Based on morphological and molecular data, a new species of *Hepatozoon* infecting *D. mikanii* from Brazil is described.

Keywords Hemoparasite · Haemogregarine · Reptile · Snake · PCR · 18S rRNA

Introduction

Haemogregarines are a group of adeleorinid coccidia (Apicomplexa: Adeleorina) subdivided into four families: Dactylosomatidae Jakoeska and Nigrelli, 1955; Karyolysidae Labbé, 1984; Haemogregarinidae Léger, 1911, and Hepatozoidae Miller, 1908. Hepatozoidae includes only species of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina: Hepatozoidae) with a cosmopolitan distribution, reported

parasitizing the erythrocytes or leukocytes of reptile, amphibian, bird, and mammal vertebrate hosts [1].

Hepatozoon spp. are heteroxenous with a life-cycle involving an obligatorily vertebrate and invertebrate (hematophagous) host. Up until 1996, more than 120 species were identified globally [1]. According to Úngari et al. [2], the number of species has increased with at least 18 additional species described. Species of *Hepatozoon* are commonly reported in snake hosts, with transmission occurring either via ingestion of an infected invertebrate vector, by the ingestion of an intermediate vertebrate host serving as prey or through vertical transmission [3–5].

The description of *Hepatozoon* spp. in the recent past was mainly based on morphological characteristics [1, 6, 7], that alone are not always reliable in identifying species of *Hepatozoon* due to the gamont plasticity and low host specificity [8, 9]. Currently, with advances in molecular tools and techniques, it is possible to distinguish between species of

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Hepatozoon and compare their phylogenetic relationships [10].

Therefore, the main goal of this study was to describe a new species of *Hepatozoon* infecting the Brazilian sleep snake, *Dipsas mikanii* (Schlegel, 1837) using morphological and molecular characterisation. This is the first report of haemogregarine parasites infecting the snake *D. mikanii*.

Material and Methods

Snake Collection

In August of 2017, reptiles were collected as part of a larger project on the biodiversity of parasites in Brazilian reptiles, a single individual of the snake *Dipsas mikanii* was collected from the municipality of Britânia, Goiás State, Brazil (14°57'08,4" S 51°06'30,7" W) and examined. The blood collection was by puncture of the cervical paravertebral sinus [11, 12] using sterile and disposable syringes and needles. The sex (male/female) and age of the specimen was estimated. After the blood collection, three blood smears were made and the remaining blood sample was stored in EDTA tubes and frozen at -10°C for further molecular work. For histopathological analysis, the sleep snake was euthanized using 50 mg/kg Tiopentax[®], a commercial anaesthetic administered intracerebrally, following the guidelines of Sebben [13] and the Animal Ethics Committee of Veterinary Medicine. The liver, spleen, heart, and kidney were fixed in 4% buffered neutral formalin and stained with haematoxylin–eosin [14].

All applicable international, national, and institutional guidelines for the care and use of animals were followed (IBAMA license #60640-1; CEUA-UNESP #1061). The access to the genetic data was authorized by the Brazilian Ministry of Environment (Sisgen A627064).

Morphological and Morphometric Analysis

Blood smears were fixed with absolute methanol and stained with 10% Giemsa Methylene Blue Eosin Merck[®] diluted in distilled water (pH 7.0 for 50 min) [15]. For morphological analysis of mature gamonts, digital images were taken at 1000× magnification, using a light microscope and the Leica software application suite LAS V3.8 (Leica Microsystems).

Measurements in micrometres (μm) comprised the parasite's length and width, with mean and standard deviation (means \pm standard deviation). Parasitaemia was calculated as the percentage of infected erythrocytes observed in 10,000 cells [16].

The effect of the parasite on the erythrocytes was evaluated by the comparison of infected and non-infected

parameters using the non-parametric Mann–Whitney Test. The significance level was 5%.

Molecular Analysis

DNA extraction from whole blood was carried out using the Illustra blood genomicPrep Mini Spin Kit (GE Healthcare), following the manufacturer's instructions. An 18S rDNA fragment (600 bp) was amplified using the primers HepF300 (5'-GTTTCTGACCTATCAGCTTTTCGACG-3') and Hep900 (5'-CAAATCTAAGAATTTACCTCTGAC-3') [17]. PCR reaction was carried out in a final volume of 25 μL , including 1 μL of each primer (5 pmol), 12.5 μL of the MyFi[™] Mix Bioline[®] Master Mix, and 5 μL of extracted DNA, with nuclease-free water accounting for the remaining volume; under conditions established by O'Dwyer et al. [18]. Amplifications were performed on a Peltier 200 Thermocycler (MJ Research, Watertown, MA), with initial denaturation at 94 $^{\circ}\text{C}$ for 3 min, followed by 35 cycles of 94 $^{\circ}\text{C}$ for 45 s, 50 $^{\circ}\text{C}$ for 60 s, and 72 $^{\circ}\text{C}$ for 60 s, followed by a final extension at 72 $^{\circ}\text{C}$ for 7 min.

DNA was extracted from liver tissue, following the blood protocol of the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA, USA). Partial 18S rRNA gene fragments (1120 bp) were amplified using the primer set 4558 (5'-GCT AATACATGAGCAAATCTCAA-3') and 2733 (5'-CGG AATTAACCAGACAAAT-3') [19]. For PCR amplification, reactions were carried out in a final volume of 25 μL , containing 1 μL of each primer (10 pmol), 12.5 μL of the MyFi[™] Mix Bioline[®] Master Mix, and 5 μL of the extracted DNA, with nuclease-free water accounting for the remaining volume. PCR amplification was performed on a Peltier 200 Thermocycler (MJ Research, Watertown, MA), with initial denaturation at 94 $^{\circ}\text{C}$ for 3 min; followed by 40 cycles of 94 $^{\circ}\text{C}$ for 1 min, 56 $^{\circ}$ for 1 min, and 72 $^{\circ}\text{C}$ for 90 s; and final extension for 7 min at 72 $^{\circ}\text{C}$, with 4 $^{\circ}\text{C}$ hold.

The amplified PCR products were subjected to electrophoresis at 80 V in a 1.5% agarose gel, stained with GelRed, and observed using an ultraviolet transilluminator. The products of interest were purified by adding 2 μL of ExoSAP-IT[®] (Affymetrix, Santa Clara, CA, USA) to 5 μL of PCR product according to the manufacturer's recommendations. Amplicons were then sequenced in both directions using the PCR primer sets on a 3500 Genetic Analyzer capillary sequencer (Applied Biosystems) and after BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3.1 (Applied Biosystems) according to the manufacturer's recommendations.

Resultant sequences were assembled and chromatogram-based contigs generated using Geneious version 7.1.3 [16]. The two resultant sequences from the present study of 589 bp (HepF300/Hep900) and 891 bp (4558/2733) were identical for the overlapping sections. The longer sequence fragment (891 pb) was aligned with haemogregarine

sequences downloaded from GenBank, using the MUSCLE algorithm implemented from within Geneious version 7.1.3 (Biomatters, www.geneious.com). Phylogenetic analyses were performed using Bayesian inference (BI) and maximum likelihood (ML). For maximum-likelihood analysis, JModelTest v.2.1.10 [20] was used to identify the best evolutionary model. Based on Akaike information criterion (AIC) the TVM+G model was selected. The phylogenetic analysis was inferred using PhyML v.3.0 [21] with 1000 replicate bootstraps (> 50%).

For Bayesian inference (BI), MrBayes analysis was carried out using computational resource CIPRES [22]. The Bayesian analysis was run with the nucleotide substitution model GTR+I+G. To search with the Markov chain Monte Carlo method, chains were run with 10,000,000 generations, saving one tree every 1000 generations. On the burn-in, the first 25% of generations were discarded, and the consensus trees were estimated using the remaining trees. Bayesian posterior probabilities' (BPP) cut-off was considered > 50%.

The resultant phylogenetic trees for both BI and ML analysis were edited in FigTree v1.4 [23]. *Klossia equi* (MH211602), *Adelina dimidiata* (DQ096835), and *Adelina grylli* (DQ096836) were used as out-groups. A pair-wise distance (*p* distance) matrix was used to compare the interspecific divergence between species of *Hepatozoon* sequences isolated from snake hosts performed on MEGA7 [24].

Results

The Brazilian free-living sleep snake *D. mikanii* was found infected with a species of *Hepatozoon*. The parasitized individual with a parasitaemia of 0.25% was an adult male measuring 48.3 cm (snout–vent length) and weighing 42 g. Mature gamonts were observed infecting the snake peripheral blood erythrocytes, and meronts and monozoic cysts were observed in histological sections of the snake's liver (Figs. 1, 2). The association of the morphological and

morphometric results together with the molecular and phylogenetic analysis (Fig. 3; Table 1, 2; Supplementary materials 1, 2, 3) revealed a new species of *Hepatozoon*.

Species Description

Phylum Apicomplexa Levine, 1970.

Class Conoidasida Levine, 1988.

Subclass Coccidia Leuckart, 1879.

Order Eucoccidiorida Léger, 1911.

Suborder Adeleorina Léger, 1911.

Family Hepatozoidae Wenyon, 1926.

Genus *Hepatozoon* Miller, 1908.

Hepatozoon quagliattus Úngari, Netherlands, Silva and O'Dwyer sp. nov.

Taxonomic Summary

Type-host: sleep snake *Dipsas mikanii* (Schlegel, 1837) (Squamata: Colubridae: Dipsadinae).

Type-locality: Municipality of Britânia, Goiás State, Brazil (18°53'09.4" S 48°15'29.5" W).

Site of infection: Blood erythrocytes and liver.

Vector: Unknown.

Etymology: The species, *Hepatozoon quagliattus* sp. nov., is named after Prof. André Luiz Quagliatto Santos, to honour his contribution to the parasites of Brazilian wildlife. Professor Quagliatto has dedicated his work to studying and preserving Brazilian wildlife, especially in regards to reptiles.

Parasitemia: The parasitemia was 0.25%.

Material deposited: Hapantotypes, one blood smear from *D. mikanii* and one histological slide of the liver were deposited in the collection of the Nacional Institute of Amazonian Research (INPA), Manaus, Brazil [INPA23].

Gene sequence: The 18S rRNA gene sequences obtained from the blood and liver of a sleep snake *D. mikanii* were deposited in GenBank under accession numbers [MW591556 / MW591599].

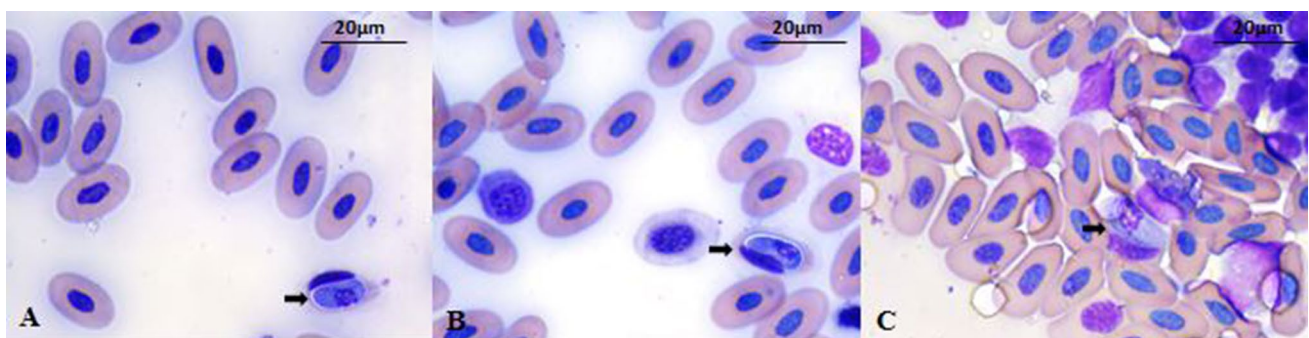


Fig. 1 a–c Intraerythrocytic mature gamonts (arrows) of *Hepatozoon quagliattus* sp. nov. from blood smears of the sleep snake *Dipsas mikanii*, collected in August 2017 in the municipality of Britânia, Goiás State, Brazil. scale bar: 20 µm

Table 1 Measurements (mean \pm standard deviation) of the developmental stages of *Hepatozoon quagliattus* sp. nov. followed by the infected and non-infected host cells

Hemoparasite	<i>N</i>	PL (μm)	PW (μm)	PA (μm^2)	NL (μm)	NW (μm)	NA (μm^2)
Mature gamonts	25	13.58 \pm 0.62	6.22 \pm 0.41	72.24 \pm 5.94	6.21 \pm 0.32	4.18 \pm 0.54	19.16 \pm 1.04
Meronts without membrane	4	4.96 \pm 0.32	4.96 \pm 0.49	19.58 \pm 1.35			
Meronts with membrane	4	9.24 \pm 0.58	10.95 \pm 0.79	74.82 \pm 1.20			
Monozoic cyst	2	9.81 \pm 0.72	13.04 \pm 1.02	92.92 \pm 0.85			
Cystozoite	2	10.35 \pm 0.81	2.14 \pm 0.30	20.80 \pm 0.12	2.15 \pm 0.50	1.49 \pm 0.32	4.87 \pm 0.41
Host cell	<i>N</i>	Length (μm)		Width (μm)		Area (μm^2)	
Infected	25	20.17 \pm 1.23a		10.67 \pm 1.45b		168.72 \pm 0.85c	
Non infected	50	18.38 \pm 0.54		9.54 \pm 0.39		131.39 \pm 1.78	

PL parasite length, PW parasite width, PA parasite area, NL parasite's nuclei length, NW parasite's nuclei width, NA parasite's nuclei area.

Statistic ^aU = 1169.0; $p \leq 0.001$

^bU = 1188.0; $p \leq 0.001$

^cU = 1241.0; $p \leq 0.001$

Morphological and Morphometric Analysis

Gamonts (Fig. 1): Gamonts were found infecting only the host's erythrocytes. Mature gamonts are characterised as full-bodied and elongated with thin parasitophorous vacuole (PV); however, in some cases, the PV is not evidenced; both ends rounded; barely noticeable, but slightly curved at one end with cytoplasm stained purplish blue. Gamont nucleus ovoid in shape with dense chromatin stained dark purple, and slightly displaced to one side of the parasite. Gamonts measured (mean \pm standard deviation) 13.58 \pm 0.62 μm long and 6.22 \pm 0.41 μm wide, with an area of 72.24 \pm 5.94 μm^2 ; parasite nucleus measured 6.21 \pm 0.32 μm long and 4.18 \pm 0.54 μm wide, with an area of 19.16 \pm 1.04 μm^2 ($n = 25$) (Table 1).

Effects of host cell: Gamonts cause displacement of the host cell nucleus with morphological changes. Mean cell length, width and area measures were higher in infected cells ($p \leq 0.001$). In some cases, the parasite causes the rupturing of the erythrocyte membrane, destroying it in the process. Infected erythrocytes measured 20.17 \pm 1.23 μm long and 10.67 \pm 1.45 μm wide with an area of 168.72 \pm 0.85 μm^2 ($n = 15$). Non-infected erythrocytes measured 18.38 \pm 0.54 μm long and 9.54 \pm 0.39 μm wide with an area of 131.39 \pm 1.78 μm^2 ($n = 50$) (Table 1).

Tissue developmental stages: From the histological analysis (liver, heart, spleen, and lung sections), four meronts and two monozoic cysts were observed in the snake's liver sections (Fig. 2).

Meronts: Characterised by a thick-walled and robust capsule. Meronts measured (excluding capsule) 4.96 \pm 0.32 μm long and 4.96 \pm 0.49 μm wide with an area of 19.58 \pm 1.35 μm^2 ($n = 4$); measurements including the membrane capsule measured 9.24 \pm 0.58 μm long and

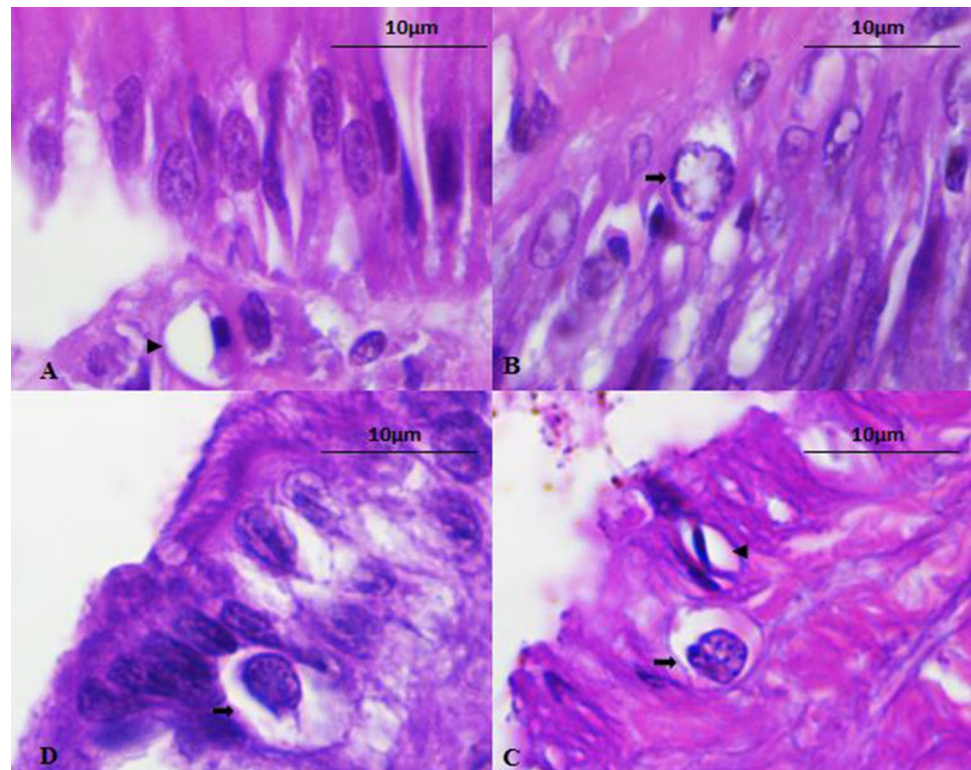
10.95 \pm 0.79 μm wide with an area of 74.82 \pm 1.20 μm^2 ($n = 4$) (Table 1).

Monozoic cysts: Characterised by a thick-wall encapsulating a single macromerozoite. Monozoic cysts measured 9.81 \pm 0.72 μm long and 13.04 \pm 1.02 μm wide with an area of 92.92 \pm 0.85 μm^2 . Cysts including the membrane measured 10.35 \pm 0.81 μm long and 2.14 \pm 0.30 μm wide with an area of 20.80 \pm 0.12 μm^2 . Nuclei measured 2.15 \pm 0.50 μm by 1.49 \pm 0.32 μm with an area of 4.87 \pm 0.41 μm^2 (Table 1).

Remarks

With regards to species of *Hepatozoon* described from Brazilian snakes, there are currently 35 recognised species, of which 14 have been described from the subfamily Diapsadinae (Supplementary material 1). To date, there have been no reports on the occurrence of species of *Hepatozoon* in *D. mikanii*. Furthermore, *Hepatozoon musa* Borges-Nojosa, Borges-Leite, Maia, Zanchi-Silva, da Rocha Braga and Harris, 2017 described from *Philodryas nattereri* Steindachner, 1870 is the only species of *Hepatozoon* described from the Diapsadinae using morphological and molecular tools. In comparison to *H. quagliattus* sp. nov., gamonts of *H. musa* are longer and thinner, measuring 18.95 \pm 0.93 μm long and 3.76 \pm 0.34 μm wide with an area of 77.60 \pm 8.50 μm^2 [25]. In addition, to the morphometric differences of *Hepatozoon quagliattus* sp. nov., gamonts of *H. musa* are curved at both ends with a centrally placed nucleus. Molecular data support the morphological findings with an interspecific divergence of between 7.2 and 7.5% among isolates of *H. musa* and the new species. Gamonts of *Hepatozoon philodryasi* Carini, 1910 described from the Patagonia green racer *Philodryas patagoniensis* (Girard, 1858) are smaller in size compared to *H. quagliattus* sp. nov. measuring 11.00–13.00 μm long

Fig. 2 a–d Histological slides of liver fragments with merons (arrows) and monozoic cysts with cystozoite inside (head arrow) of *Hepatozoon* sp. nov., from a sleeping snake *Dipsas mikanii* collected in 2017 in the city of Britânia, State of Goiás, Brazil. scale bar: 10 μ m



and 2.00–3.50 μ m wide [26]. Furthermore, gamonts of *H. philodryasi* do not alter host cell morphology. *Hepatozoon carinicauda* Pessôa and Cavalheiro 1969 described from

Helicops carinicaudus (Wied-Neuwied, 1825) have larger gamonts measuring 23.00–25.00 μ m long by 5.00–6.00 μ m wide as compared to the new species (Table 2).

Table 2 Morphometric data on blood and tissue stages of *Hepatozoon* species from Brazilian snakes (Diapsadinae)

Species	Host	Morphometric data ^a blood stage	Morphometric data (μ m) ^a tissue stage
<i>Hepatozoon quagliattus</i> sp. nov. (this study)	<i>Dipsas mikanii</i> (Schlegel, 1837)	G: 13.58 μ m long \times 6.22 μ m wide, 72.24 μ m ² area	M: 9.24 μ m long \times 10.95 μ m wide, 74.82 μ m ² area MC: 10.35 μ m long \times 2.14 μ m wide, 20.80 μ m ² area
<i>Hepatozoon musa</i> Borges-Nojosa, Borges-Leite, Maia, Zanchi-Silva, da Rocha Braga and Harris, 2017	<i>Philodryas nattereri</i> Steindachner, 1870	G: 18.95 μ m long, 3.76 μ m wide, 77.60 μ m ² area	–
<i>Hepatozoon philodryasi</i> Carini, 1910	<i>Philodryas patagoniensis</i> (Girard, 1858)	G: 11.00–13.00 μ m long, 2.00–3.50 μ m wide	–
<i>Hepatozoon carinicauda</i> Pessôa and Cavalheiro 1969	<i>Helicops carinicaudus</i> (Wied-Neuwied, 1825)	G: 23.00–25.00 μ m long, 5.00–6.00 μ m wide	–
<i>Hepatozoon sipedon</i> Smith, Desser and Martin, 1994	<i>Nerodia sipedon sipedon</i> (Linnaeus, 1758)	G: 19.00 μ m long, 3.70 μ m wide	Ma: 49.00 μ m long \times 40.30 μ m wide Mi: 55.10 μ m long \times 44.60 μ m wide
<i>Hepatozoon terzii</i> (Sambon and Seligman, 1907)	<i>Boa constrictor</i> Linnaeus, 1758	G: 10.00–16.20 μ m long, 2.50–3.70 μ m wide	M: 26.00 μ m long \times 21.30 μ m wide DC-TC: 19.00 μ m long \times 13.80 μ m wide
<i>Hepatozoon strigatus</i> (Pessôa, 1967)	<i>Thamnodynastes strigatus</i> (Günther, 1858)	G: 14.00–18.00 μ m long, 6.00–8.00 μ m wide	Mi: 55.00 μ m in diameter

M meronts, MC monozoic cyst, Mi micromeronts, Ma macromeronts, DC dizoic cyst, TC tetrazoic cyst, G gametocytes

^aLength \times width (μ m); area (μ m²)

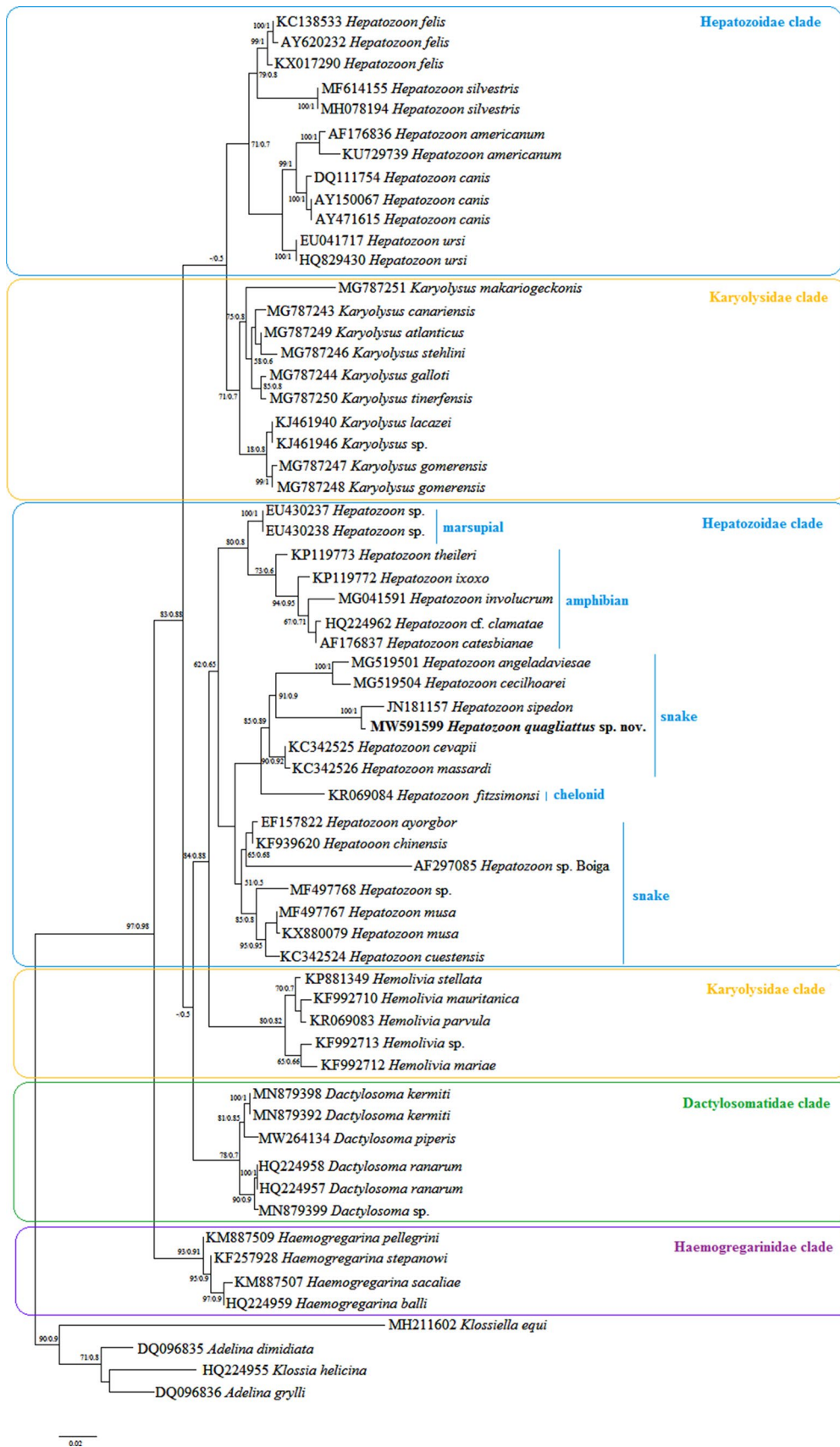


Fig. 3 Consensus phylogram of haemogregarines based on 18S rRNA sequences (492 nt). The topology trees were inferred by Bayesian (BI) and maximum-likelihood (ML) methods (represented by ML tree). The isolates *Adelina dimidiata* (DQ096835), *Adelina grylli* (DQ096836), and *Klossia helicina* (HQ224955) were used as an out-group

Through molecular analysis, *Hepatozoon quagliattus* sp. nov. have shown nucleotide identity with *Hepatozoon sipedon* Smith, Dessler and Martin, 1994 infecting the northern water snake, *Nerodia sipedon sipedon* (Linnaeus, 1758). Thus, comparing the gamonts morphology of both species, *H. sipedon* has longer and slender gamonts, with central roughly ovoid nuclei, and the morphometric analysis revealed 19.00 μm long and 3.70 μm wide [27], differing from the present study (Table 2).

With regards to the other developmental stages, meronts and monozytic cysts were observed in the host's liver and spleen tissues, which presented unique characteristics with reduced size, but with great membrane thickness, measuring almost the size of the meront itself. In comparison to species of *Hepatozoon* from other snakes, the meront's capsule size is thicker than the other species. Nevertheless, morphometric analyses have shown total measurements significantly smaller as compared with other species from snakes. Macromeronts of *H. sipedon* measures 49.00 \pm 4.00 μm long by 40.30 \pm 5.50 μm wide, and micromeronts measure 55.10 \pm 3.10 μm long by 44.60 \pm 4.00 μm wide, both meronts measuring larger than *Hepatozoon quagliattus* sp. nov. Another well-defined species is *Hepatozoon terzii* (Sambon and Seligman, 1907). According to Paperna and Lainson [28], the meronts of *H. terzii* found in the snake *Boa constrictor* Linnaeus, 1758, measured 26.00 \pm 5.70 μm long by 21.30 \pm 4.90 μm wide and dizoic and tetrazoic cysts measured 19.00 \pm 4.00 μm long by 13.80 \pm 2.30 μm wide, both larger than meronts of *Hepatozoon quagliattus* sp. nov. (Table 2).

Hepatozoon strigatus (Pessôa, 1967) described from the Brazilian snake *Thamnodynastes strigatus* (Günther, 1858) contains gamonts with a prominent recurved tail (14.00–18.00 μm long by 6.00–8.00 μm wide) and nucleus occupying the second quarter and extend to the first quarter of the gamont, differing from *Hepatozoon quagliattus* sp. nov. Regarding tissue merogony, only data for micromeronts in the liver and lung are reported, measuring up to 50 μm in diameter and containing hundreds of micromerozoites (Table 2) [29].

Therefore, the meronts and monozytic cysts of *Hepatozoon quagliattus* sp. nov., encapsulated within a thick-walled membrane, differ in comparison to *Hepatozoon* spp. described from other Brazilian snakes. In addition to the difference in gamont size, the main characteristics separating *Hepatozoon quagliattus* sp. nov., from other species of

Hepatozoon described from Brazilian snakes, are the effects caused to the host cell, including hypertrophy (enlargement) of the host cell and displacement of the host cell nucleus (Table 2).

Molecular and Phylogenetic Analysis

Bayesian inference (BI) and the maximum-likelihood (ML) method resulted in identical topologies with supported nodes values in the clades (Fig. 3). The phylogenetic analysis included isolates of adeleorinid parasites (Haemogregariniidae, Hepatozoidae, Karyolysidae, and Dactylosomatidae) available from GenBank (Supplementary material 2). From the trees, the Haemogregariniidae Clade forms a sister group to the large well-supported clade comprising species from the Dactylosomatidae, Karyolysidae, and Hepatozoidae. Species of *Dactylosoma* form a monophyletic group, sister to a large-clade comprising species of *Hemolivia*, *Karyolysus*, and *Hepatozoon*. Species of *Hepatozoon* were polyphyletic forming two separated clades according to their vertebrate host species. Species of *Hepatozoon* isolated from large mammals formed a sister group to the Karyolysidae clade comprising species of *Karyolysus*. With regards to the reptile, anuran and marsupial Hepatozoidae clade, *Hepatozoon quagliattus* sp. nov. [GenBank number] grouped with *Hepatozoon sipedon* Smith, Dessler and Martin, 1994 [JN181157], *Hepatozoon angeladavesae* Cook, Netherlands, van As and Smit, 2018 [MG519501], and *Hepatozoon cecilhoarei* Cook, Netherlands, van As and Smit, 2018 [MG519504], sister to a clade comprising species of *Hepatozoon* from anuran hosts (Fig. 3). The interspecific divergence between *H. sipedon* and *Hepatozoon quagliattus* sp. nov. was 2.4%. In comparison, the species of *Hepatozoon* from snakes varied between 88.7 and 93.7% as compared to the isolate from the present study. Moreover, the pair-wise distance among *Hepatozoon* species in snakes and the new species varied from 0.024 to 0.099.

Discussion

Brazil is considered rich in reptile biodiversity [30]. According to Costa and Bérnils [31], Brazil is listed as the third-highest biodiversity hotspot for reptiles, currently with 795 recognised species, of which 405 species are snakes. The genus *Dipsas* Laurenti, 1768 (Squamata: Colubridae: Dipsadinae) comprises 53 species worldwide and 14 of them occur in Brazil [32, 33]. One of the species, *D. mikanii* (Schlegel, 1837), is nocturnal, with terrestrial or semi-arboreal habits [34, 35]. Interestingly, this neotropical snake feeds primarily on land snails and slugs, specialising on slugs of the Veronicellidae [36].

In the literature, there are some reports of greater abundance of *D. mikanii* snakes during the rainy season, which must be related to the period of greater availability of prey. This snake show activity stimulated during wetter periods (under or after rains) to search for molluscs, and at times of drought, it is possible that they remain at rest [37, 38]. However, leeches that remain a potential vector for species of *Hepatozoon* [5] share similar body morphology with slugs. Also, the dipsadinae snake *Atractus reticulatus* (Boulenger, 1885) has been reported to readily feed on earthworms and leeches from the savannahs and grasslands of southern Brazil [39].

Species of *Hepatozoon* are considered the most commonly reported haemoparasites found in snakes [7]. In Brazil, species of *Hepatozoon* from snake hosts are also commonly reported. Pessoa et al. [40] conducted a large-scale study on the prevalence of *Hepatozoon* species in Brazilian snakes, screening approximately 2100 snakes, belonging to 24 genera and 45 species, with a prevalence of up to 70% depending on the host species. However, there are no previous reports of haemogregarines infecting *D. mikanii*.

In the current study, the infected *D. mikanii* had a low parasitaemia with only 25 mature gamonts counted from the three blood smears prepared. The low parasitaemia could be related to the chronic and cyclic merogonic phases, which maintain the infection for long periods of time [41]. For example, De Biase et al. [42] reported a long-term infection of species of *Hepatozoon* in a Brazilian snake with the parasitaemia decreasing after 8 years. Santos et al. [43] also observed a tendency to parasitaemia decrease without complete elimination. According to Tenter et al. [44], species of *Hepatozoon* infections may be similar to *Toxoplasma gondii*, with a persistent infection throughout the hosts' life.

According to Conradie et al. [45], the infection duration of *Hepatozoon theileri* (Laveran, 1905) and *Hepatozoon ixoxo* Netherlands, Cook and Smith, 2014 in its frog host may be related to gamont morphology and the parasites effect on the its host cell. *Hepatozoon theileri* and *Hepatozoon quagliattus* sp. nov. share several characteristics as both are rather large, displace the host cell nucleus, cause erythrocyte distortion, and possess a thin or non-evident capsule. Using electron microscopy, these authors provide the possible relationship between certain characteristics observed in *H. theileri* and the infection period within the host. These characteristics include the absence of a dense capsule and high pathogenicity to the host cell, which, instead of preserving the host's erythrocyte, utilise all the resources from the infected cell before the gamont moves on to a new host cell [45]. When compared to *H. ixoxo*, the dense PV encasing the gamont possibly enables the parasite to remain for long periods of time in the same erythrocyte, thus associated with longevity.

In the past, haemogregarines were identified and new species were described based solely on the morphological comparison of the developmental stages in the vertebrate host, the host species, or the geographic location [5]. However, morphological analysis alone is not always effective in differentiating between species, due to the low host specificity of certain species and plasticity of gamont morphology [8, 9]. Thus, molecular tools have greatly assisted in the classification or identification of species and genera [10].

Based on the 18S rRNA gene, the closest relative of *Hepatozoon quagliattus* sp. nov., with an interspecific divergence of 2.4%, is *H. sipedon* (JN181157) described infecting the northern water snake, *Nerodia sipedon sipedon* (Linnaeus, 1758) [3]. This proximity was supported in the ML/BI phylogenetic analyses, with *Hepatozoon quagliattus* sp. nov. and *H. sipedon* forming a monophyly sister to *H. angeladaviesae* and *H. cecilhoarei*.

Hepatozoon sipedon and *Hepatozoon quagliattus* sp. nov. were identified in different host species with a great geographic distance, being *N. sipedon sipedon* reported from Canada. Moreover, *N. sipedon sipedon* is an aquatic snake from the family Colubridae and subfamily Natricinae, feeding on small fish, frogs, worms, leeches, salamanders, small birds, and mammals.

To date, the life-cycle of only a few species of *Hepatozoon* from snake hosts has been successfully elucidated. Due to the lack of biological data for this group, the life history and transmission mechanisms of *Hepatozoon quagliattus* sp. nov. in *D. mikanii* are difficult to conclude. Since *D. mikanii* do not feed on other vertebrate animals, such as frogs and fish, transmission via an intermediate host is unlikely to occur, as in the case of the genetically close related species *H. sipedon* (based on the 18S rRNA gene). *Hepatozoon sipedon* is characterised by an infection pathway requiring three hosts: an intermediate vertebrate host (snake), a second intermediate vertebrate host (anuran), and a final or definitive invertebrate host/vector (mosquitoes) [3]. These findings are in contrast to the hypothesis that host diet and phylogenetic relationships represent the life-history trends of species of *Hepatozoon* [40, 45, 46]. Furthermore, although unlikely, it is possible that *D. mikanii* may accidentally prey on leeches confused for slugs (due to morphological similarities); however, leeches have not yet been reported as vectors for any species of *Hepatozoon* nor have there been any reports of leeches feeding on *D. mikanii*. The most likely transmission scenario is accidental ingestion of mites or ticks feeding on *D. mikanii*.

Therefore, with the combination of morphological and molecular tools, it is possible to clearly differentiate between *H. sipedon* and the new species here described as *Hepatozoon quagliattus* sp. nov.

Conclusions

Although Brazil is a hotspot for snake species, there is a lack of information on the biodiversity of haemoparasites infecting these hosts, and this number is even lower if related to the use of the right techniques. Thus, the present study reports the first haemogregarine infecting the sleep snake *Dipsas mikanii*, with the description of a new species, *Hepatozoon quagliattus* sp. nov., based on molecular and morphological diagnostics.

Future work should include molecular characterisation with variable markers, such as mitochondrials, to increase the knowledge of phylogenetic position on species of *Hepatozoon* infecting snakes. Notwithstanding, electron microscopy screening may provide further insights into the cytopathogenicity of *Hepatozoon quagliattus* sp. nov. Furthermore, studies focusing on elucidating life-cycle, describing possible vectors, and determining routes of transmission are important for future research on *Hepatozoon quagliattus* sp. nov.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed (IBAMA license 60640-1; CEUA-UNESP 1061).

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