New insights on the diversity of Brazilian anuran blood parasites: With the description of three new species of *Hepatozoon* (Apicomplexa: Hepatozoidae) from Leptodactylidae anurans

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ABSTRACT

The Amphibia are considered the most threatened vertebrate class globally, yet in Brazil they are also one of the more diverse and species rich groups. Although, in recent years there has been strong focus on amphibian related research, their parasites have not received the same attention. In Brazil, only a single species of *Hepatozoon*, namely *H. leptodactyli* (Lesage, 1908) Pessoa, 1970, has been described from anuran hosts. The present study aimed to describe three new species of *Hepatozoon* parasitising *Leptodactylus labyrinthicus* and *Leptodactylus latrans* from Mato Grosso State, Brazil. From 66 anurans screened for haemogregarines, four belonging to the Leptodactylidae were found positive for species of *Hepatozoon*. Based on the morphological analysis of peripheral blood gamonts and spleen and liver tissue meronts, three different morphotypes of *Hepatozoon* spp. were identified. Morphotype 1 (M1) and morphotype 2 (M2) in *L. labyrinthicus* and morphotype 3 (M3) in *L. latrans*. Molecular data based on partial 18S rDNA sequences revealed an interspecific divergence, between the species ranging from 0.43% to 1.16%. Phylogenetic analysis recovered isolates from the present study monophyletic with other isolates from Brazilian reptile and anuran hosts, sister to a clade comprising species isolated from African, North American and European reptile and anuran host species. Thus, using morphological and molecular analysis three new species infecting Brazilian Leptodactylidae anurans were identified and described. This study increases the knowledge of Brazilian anurans blood parasites and demonstrates the importance of using integrative approaches for diagnosis of hemoparasites.

1. Introduction

Brazil is one of the most species rich areas for amphibians globally (Segalla et al., 2016). However, amphibians are also considered as the most threatened vertebrate group with many species facing extinction (Wake and Vredenburg, 2008). Amphibians also host a variety of endoparasites and ectoparasites, which in some cases influence their fitness, behaviour, feeding, reproduction, and fertility (Barta and Desser, 1984; Leal et al., 2015; Laison et al., 2003).

With regards to anuran haemoparasites, intracellular apicomplexans of the family Hepatozoidae Miller, 1908 (Apicomplexa: Adeleorina) are commonly reported (Davies and Johnston, 2000; Leal et al., 2015;

Netherlands et al., 2018). The life cycle of species of *Hepatozoon* is characterized by the formation of syzygy followed by sporogonic development in a definitive host or vector (invertebrate host) following a blood meal from an infected vertebrate (intermediate) host (Desser et al., 1995; Smith, 1996). The intermediate host contains intra-erythrocytic or intra-leukocytic gamonts with merogonic development occurring mainly in the liver and spleen (Desser et al., 1995; Smith, 1996).

In addition, certain vertebrates may also serve as the first intermediate host for species of *Hepatozoon* that are transmitted to a second intermediate host following the ingestion of the first (Smith, 1996; Viana et al., 2012). Viana et al. (2012) showed that *Leptodactylus chaquensis*

Cei, 1950 may be the first intermediate host for the crocodilian haemoparasites *Hepatozoon caimani* Carini, 1909, with the presence of cysts occurring in the liver of the frog after ingesting sporulated oocysts from infected *Culex* Linnaeus, 1758 mosquitoes.

Several species of *Hepatozoon* have been described in anurans from Africa, Asia, and Europe (Levine, 1988; Netherlands et al., 2014a, 2014b) and according to Netherlands et al. (2018), there are 45 recognized species of *Hepatozoon* infecting anurans globally. In Brazil the only species of *Hepatozoon* described from an anuran hosts is *Hepatozoon leptodactyli* (Lesage, 1908) Pessoa, 1970 infecting species of *Leptodactylus* based solely on morphological data (Costa et al., 1973). Costa et al. (1973) screened 90 *Leptodactylus latrans* (Steffen, 1815) and *Leptodactylus pentadactylus* (Laurent, 1768) reporting 17 infected with *H. leptodactyli.* Furthermore*,* the leech *Haememteria lutzi* Pinto, 1920 was considered as the possible vector of *H. leptodactyli* (Costa et al., 1973).

However, although there are a number of reports of haemogregarines infecting Brazilian anurans, none were formally described other than *H. leptodactyli* and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1990. Since the advances in molecular diagnosis, two studies on species of *Hepatozoon* in Brazilian anurans were conducted (Leal et al., 2015; Ferreira et al., 2020). Leal et al. (2015) collected 145 *L. chaquensis* and *Leptodactylus podicipinus* from Mato Grosso do Sul State, (Cope, 1862) finding a prevalence of 26.47% and 31.17%, respectively. Moreover, although, 18S rDNA fragments were obtained from the Leal et al. (2015) study, sequence data was isolated only from blood samples of *L. chaquensis* and no species were described. In a recent study using only PCR assays to target a fragment of 18S rDNA, Ferreira et al. (2020) investigated the occurrence of apicomplexan parasites in anurans from south-eastern Brazil, successfully amplifying *Hepatozoon* spp. isolates from a single *L. latrans* and *Rhinella dypctycha* (Cope, 1862).

The present study aimed to describe through morphological, morphometric and molecular diagnosis, three new species of *Hepatozoon* infecting anurans collected at Mato Grosso State, Brazil. Furthermore, this study aimed to increase the knowledge of Brazilian anurans blood parasites and demonstrates the importance of using both molecular and morphological approaches.

2. Material and methods

2.1. Anuran collection

In August of 2018, 66 anurans were captured for a major project of biodiversity of parasites in amphibians (FAPESP 2018/09623–4; FAPESP 2018/00754–9), belonging to eight species, five genera and three families (Table 1). The anurans were collected in the municipality of Nova Xavantina, Mato Grosso State, Brazil (coordinates 14°31′48.03″ S 51°41′43.88″ W). Animals were physically restrained and the blood was collected by puncture of the cervical paravertebral sinus (Zippel et al., 2001) using sterile syringes and needles. During the containment, specimens were examined for ectoparasites, and were subsequently measured and weighed, as well as the sex (male/female) and age of each specimen determined. After the blood collection, three thin blood smears were made on glass slides and the remaining blood sample was stored in EDTA tubes and frozen at −10 °C for further molecular analysis. All applicable international, national, and/or institutional guidelines for the ethical handling 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 A21DF67, A132B2E and A9D77FC).

For histopathological analysis, the anurans were euthanized using 50 mg/kg Tiopentax®, a commercial anaesthetic administered intracerebrally, following the guidelines of Sebben (2007) 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 (Eisen and Schall, 2000).

2.2. Morphological and morphometric analysis

The blood smears were fixed with absolute methanol and stained with 10% Giemsa Methylene Blue Eosin Merck® diluted in distilled water (pH 7.0 for 50min), according to Eisen and Schall (2000). For morphological analysis of the intra-erythrocytic parasite stages, digital images were captured and measured using a compound microscope at $1000 \times$ magnification with the Leica software application suite LAS V3.8 (Leica Microsystems). Measurements are in micrometres (μm) comprising the parasite's length, width and area, with mean and standard deviation (means ± standard deviation) given. Parasitaemia was calculated per 100 erythrocytes, with ~10^4 erythrocytes examined per blood smear following Cook et al. (2009).

The effect of the parasite on the erythrocytes was evaluated by the comparison of infected and non-infected parameters using the nonparametric Mann-Whitney Test, with a significance level of 5%.

2.3. Molecular analysis

DNA was extracted from whole blood and tissue samples following the blood protocol of the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Two PCR assays were performed targeting two different regions of the 18S rDNA of apicomplexan parasites using the HepF300 and Hep900 pair of primers, which amplifies 600 bp (Ujvari and Marques, 2005) and Hemo1 and Hemo2 pair of primers, which amplifies 900 bp (Perkins and Keller, 2001). PCR amplification reactions were carried out in a final volume of 25 μL, containing 1 μL each of 10 pmol primers, 12.5 μL of Master Mix MyFi™ Mix Bioline®, and 5 μL of extracted DNA, with nuclease-free water accounting for the remaining volume. PCR amplification was performed on a Peltier 200 Thermocycler (MJ Research, Watertown, MA) following the conditions of O'Dwyer et al. (2013).

PCR products were subjected to gel electrophoresis at 80 V in a 1.5% agarose gel, stained with Gel Red, 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 using PCR primers 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. A consensus sequence

Table 1
Data on sample number, positivity, and prevalence of anurans collected in 2018, from Mato Grosso State, Brazil, and surveyed for *Hepatozoon* species.

	Family	Genus	Species	N (%)	P (%)
Anuran hosts	Bufonidae	*Rhinella*	*R. diptycha* (Cope, 1862)	13 (19.70)	0
			R. mirandaribeiroi (Gallardo, 1965)	1 (1.52)	0
	Hylidae	*Boana*	*B. raniceps* (Cope, 1862)	6 (9.09)	0
		Scinax	*S. fuscovarius* (Lutz, 1925)	4 (6.06)	0
		Trachycephalus	*T. typhonius* (Linnaeus, 1758)	2 (3.03)	0
	Leptodactylidae	*Leptodactylus*	*L. labyrinthicus* (Spix 1824)	8 (12.12)	2 (25.0)
			L. latrans (Steffen, 1815)	31 (46.96)	2 (6.45)
			L. petersii (Steindachner, 1864)	1 (1.52)	0
Total	3	5	8	66 (100)	4 (6.06)

was created from the forward and reverse assembled electropherograms using Geneious version 7.1.3 (Kearse et al., 2012). For each positive animal, the newly generated sequences from HepF300/Hep900 and Hemo1/Hemo2 contigs were concatenated using Geneious version 7.1.3 (Kearse et al., 2012), forming a long sequence of ~1300 bp.

The sequences from this study were compared with other isolates from haemogregarine parasites available on GenBank. The newly generated sequences of partial 18S rDNA were aligned using Geneious version 7.1.3 (Kearse et al., 2012) with MUSCLE algorithm implemented from within Geneious version 7.1.3 (Biomatters, www.geneious.com) and default settings with related sequences that appeared on Blastn search. Two alignments were made, one with long sequences available on Genbank, with final alignment of 1116 bp, and another with short sequences with 582 bp. The phylogenetic analyses were performed using both alignments using the Bayesian inference (BI) and Maximum Likelihood (ML) methods. For ML method, JModelTest v.2.1.10 (Darriba et al., 2012) was used to identify the best evolutionary model. Based on Akaike information criterion (AIC) the Transitional model (Posada, 2003) with a discrete Gamma distribution (TVM + G) was chosen. Phylogenetic analysis was inferred using PhyML (Guindon and Gascuel, 2003) with 1000 replicate bootstraps (>50%). The BI analysis was carried out using MrBayes implemented from the computational resource CIPRES (Miller et al., 2010), the best BIC score was the General Time Reversible model (GTR + I + Γ) (Tavaré, 1986). The Markov chain Monte Carlo (MCMC) algorithm was run for 10,000,000 generations, sampling 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 isolates from adeleorinid parasites (Haemogregarinidae, Hepatozoidae, Karyolysidae, and Dactylosomatidae) available from GenBank were used to construct both trees. The resultants phylogenetic trees for both BI and ML analysis were edited in FigTree v1.4 (Rambaut, 2012). *Adelina dimidiata* Schneider, 1875, *Adelina grylli* Butaeva, 1996 [GenBank: DQ096835–DQ096836], *Klossia helicina* Schneider, 1875 [GenBank: HQ224955] and *Klossiella equi* [GenBank: MH211602] from the suborder Adeleorina were selected as outgroups following Netherlands et al. (2020). A pair-wise distance (p-distance) matrix was used to compare the interspecific divergence between species of *Hepatozoon* sequences isolated from snakes and anurans hosts (Table 2).

3. Results

Of the 66 captured anurans (Table 1), four specimens of Leptodactylidae were found infected with *Hepatozoon* spp.: one adult female *Leptodactylus labyrinthicus* (Spix, 1824) (73.88 mm snout-vent length and weight of 29 g); one adult male *L. labyrinthicus* (66.72 mm snout-vent length and weight of 23 g); one adult male *L. latrans* (58.23 mm snout-vent length and weight of 13 g); and one adult female *L. latrans* (52.80 mm snout-vent length and weight of 9 g). The total prevalence found was 6.06% (4/66); the prevalence of *L. labyrinthicus* and *L. latrans* were 25% (2/8) and 6.45% (2/31), respectively.

Based on the morphological analysis of peripheral blood gamonts and spleen and liver tissue meronts it was possible to identify three different species of *Hepatozoon* morphotypes. Morphotype 1 (M1) found in one *L. labyrinthicus,* morphotype 2 (M2) in one *L. labyrinthicus*, and morphotype 3 (M3) in two *L. latrans.*

3.1. 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

3.1.1. Hepatozoon formosus Úngari, Netherlands, Silva and O'Dwyer n. sp.

Type-host: Leptodactylus labyrinthicus (Anura: Leptodactylidae).

Type-locality: Municipality of Nova Xavantina, Mato Grosso State, Brazil (coordinates 14°35′47″ S 51°43′9,59″ W).

Site of infection: Peripheral blood erythrocytes, and liver and spleen fragment tissues.

Vector: Unknown.

Etymology: The morphology of gamonts and meronts have shown richness in details and development stages. Thus, the word "formosus" is the Latin translation of the word "beautiful" in English.

Parasitaemia: The parasitaemia of *L. labyrinthicus* was 1.17%.

Prevalence: The total prevalence found was 1.51% (1/66) and 12.5% (1/8) among the *L. labyrinthicus* individuals analysed.

Material deposited: Hapantotypes, one blood smear from *L. labyrinthicus,* one histological slide of the liver, and one histological slide of the spleen, were deposited in the collection of the National Institute of Amazonian Research (INPA), Manaus, Brazil [INPA20].

Gene sequence: The 18S rDNA sequence obtained with 1384 bp from a pepper frog *L. labyrinthicus* liver tissue was deposited in GenBank under accession number [MW584356].

Table 2
The shaded matrix (upper) shows the p-distance (pair-wise distance) and the non-shaded matrix (lower) shows the percentage of similarity (%) of the nucleotide sequences among the *Hepatozoon* sequences from anurans and snakes available at Genbank (582 nt).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
1. *H. formosus* n. sp. (MW584356)		0.011	0.009	0.009	0.011	0.009	0.009	0.009	0.009	0.071	0.077	0.065	0.066	0.071	0.077	0.065	0.068	0.094	0.084	0.087	0.093	0.031	0.036	0.034	0.016	0.021	0.014	0.049	0.052
2. *Il. longimucleus* n. sp. (MW584361)	98.84		0.002	0.002	0.002	0.002	0.002	0.002	0.002	0.063	0.065	0.057	0.057	0.063	0.066	0.057	0.057	0.086	0.076	0.079	0.088	0.021	0.031	0.029	0.019	0.014	0.007	0.044	0.047
3. *H. latrensis* n. sp. (MW584362)	99.07	99.57		0.000	0.002	0.000	0.000	0.000	0.000	0.063	0.066	0.055	0.057	0.063	0.066	0.055	0.057	0.086	0.076	0.079	0.085	0.021	0.029	0.026	0.018	0.011	0.004	0.042	0.045
4. *H. latrensis* n. sp. (MW584365)	99.07	99.57	100		0.002	0.000	0.000	0.000	0.000	0.063	0.066	0.055	0.057	0.063	0.066	0.055	0.057	0.086	0.076	0.079	0.085	0.021	0.029	0.026	0.018	0.011	0.004	0.042	0.043
5. *H.* sp. (MK503643)	98.84	99.34	99.57	99.57																									
6. *H.* sp.(MK503648)	99.07	99.57	100	100																									
7. *H.* sp.(MK503645)	99.07	99.57	100	100																									
8. *H.* sp.(MK503646)	99.07	99.57	100	100																									
9. *H.* sp.(MK503647)	99.07	99.57	100	100																									
10. *H. catesbianae* (AF130361)	91.99	94.51	94.41	94.41																									
11. *H. theileri* (MG041605)	94.95	94.93	95.03	95.03																									
12. *H. thori* (MG041600)	94.95	94.31	94.20	94.20																									
13. *H. magna* (HQ224960)	94.99	94.63	94.52	94.52																									
14. *H. cf. clamatae* (HQ224962)	94.82	94.51	94.41	94.41																									
15. *H. involucrum* (MG041591)	94.86	94.31	94.20	94.20																									
16. *H. tenuis* (MG041598)	95.03	94.93	95.03	95.03																									
17. *H. ixoxo* (MG041604)	95.03	94.93	94.82	94.82																									
18. *H. sipedon* (JN181157)	93.64	91.61	91.51	91.51																									
19. *H. angeladaviesae* (MG519501)	93.94	93.69	93.58	93.58																									
20. *H. cecilhoarei* (MG519504)	93.90	93.48	93.37	93.37																									
21. *H.* sp. Boiga (AF297085)	93.98	90.35	90.45	90.45																									
22. *H.* sp. (MF97768)	96.89	98.03	97.93	97.93																									
23. *H. ayorgbor* (EF157822)	97.30	97.00	97.10	97.10																									
24. *H. chinensis* (KF939620)	96.47	97.20	97.31	97.31																									
25. *H. domerguei* (KM234646)	97.61	97.88	97.79	97.79																									
26. *H. cuestensis* (MF497770)	97.72	98.65	98.76	98.76																									
27. *H. musa* (MF497767)	98.34	99.28	99.38	99.38																									
28. *H. cevapii* (KC342525)	95.23	96.17	96.27	96.27																									
29. *H. massardi* (KC342526)	95.02	95.96	96.07	96.07																									

3.1.1.1. Description. Gamonts (Fig. 1): only infecting host erythrocytes and varying in the developmental stage (n = 50).

Early-stage gamonts (Fig. 1 A - C): Varying from small to slightly larger, tapering towards one end, rounded and robust to the other end, with or without cytoplasmic vacuoles observed in the tapering end, and no capsule evidenced; measurements of 7.83 $\pm$ 0.98 μm long (7.29–8.50), 3.05 $\pm$ 0.23 μm (2.81–3.33) wide, with an area of 19.08 $\pm$ 1.02 μm^2 (17.87–20.33). Dense nuclei, stained dark-purple and occupying almost the entire parasite; measurements of 4.09 $\pm$ 2.48 μm (2.81–5.70) long, 4.04 $\pm$ 2.18 μm (2.9–4.58) wide, with an area of 14.81 $\pm$ 1.23 μm^2 (11.88–16.76).

Immature gamonts (Fig. 1 D - E): Larger size, with both ends rounded with one end slightly larger, with or without cytoplasmic vacuoles, and no capsule evidenced; measurements of 9.33 $\pm$ 0.16 μm (9.17–9.88) long, 3.43 $\pm$ 0.11 μm (3.15–3.83) wide, with an area of 21.27 $\pm$ 0.74 μm^2 (20.33–22.38). Quadrangular, dense, and reduced nuclei, staining dark-purple; measurements of 5.04 $\pm$ 1.35 μm (4.79–6.07) long, 3.53 $\pm$ 0.56 μm (3.15–3.83) wide, with an area of 16.60 $\pm$ 0.19 μm^2 (14.15–17.16).

Mature gamonts (Fig. F): The largest of the developmental gamonts stages. Robust and elongated, with both ends rounded, thin parasitophorous capsule (sometimes not evidenced), cytoplasm stained whitish without cytoplasmic vacuoles; measurements of 11.93 $\pm$ 0.61 μm (9.82–13.67) long, 4.18 $\pm$ 0.24 μm (3.93–4.67) wide, with an area of 40.20 $\pm$ 3.01 μm^2 (37.76–44.00). Dense and quadrangular nuclei, located centrally of the parasite body, stained dark-purple; measurements of 5.77 $\pm$ 0.23 μm (5.57–5.97) long, 3.91 $\pm$ 0.23 μm (3.33–3.93) wide, with an area of 17.29 $\pm$ 1.29 μm^2 (15.26–18.77).

Effects on the host cell: Early-stage gamonts do not cause displacement of the host cell nucleus or any morphological changes to the host cell. Immature gamonts, in some cases, cause a slight displacement of the host cell nucleus. Mature gamonts cause displacement of the host cell nucleus and slight morphological changes to the erythrocyte. Infected erythrocytes measured 16.85 $\pm$ 1.04 μm (15.39–18.45) long (U = 692.0; p = 0.601) and 12.43 $\pm$ 1.22 μm (10.90–14.89) wide (U = 1121.0; p $\leq$ 0.001) with an area of 145.59 $\pm$ 4.10 μm^2 (133.64–164.44) (U = 826.0; p = 0.033) (n = 20). Non-infected erythrocytes measured 16.84 $\pm$ 0.37 μm (16.33–17.54) long and 9.94 $\pm$ 1.39 μm (8.05–11.59) wide with an area of 136.53 $\pm$ 4.32 μm^2 (125.42–147.16) (n = 50).

Tissue merogony (Figs. 2 and 3): the liver and spleen from *L. labyrinthicus* presented a number of meronts with merozoites of *Hepatozoon formosus* n. sp. The meronts varied in size and morphology, it was observed one macromeront with 4 macromerozoites were lodged in the spleen and micromeronts with 16–35 micromerozoites lodged in the
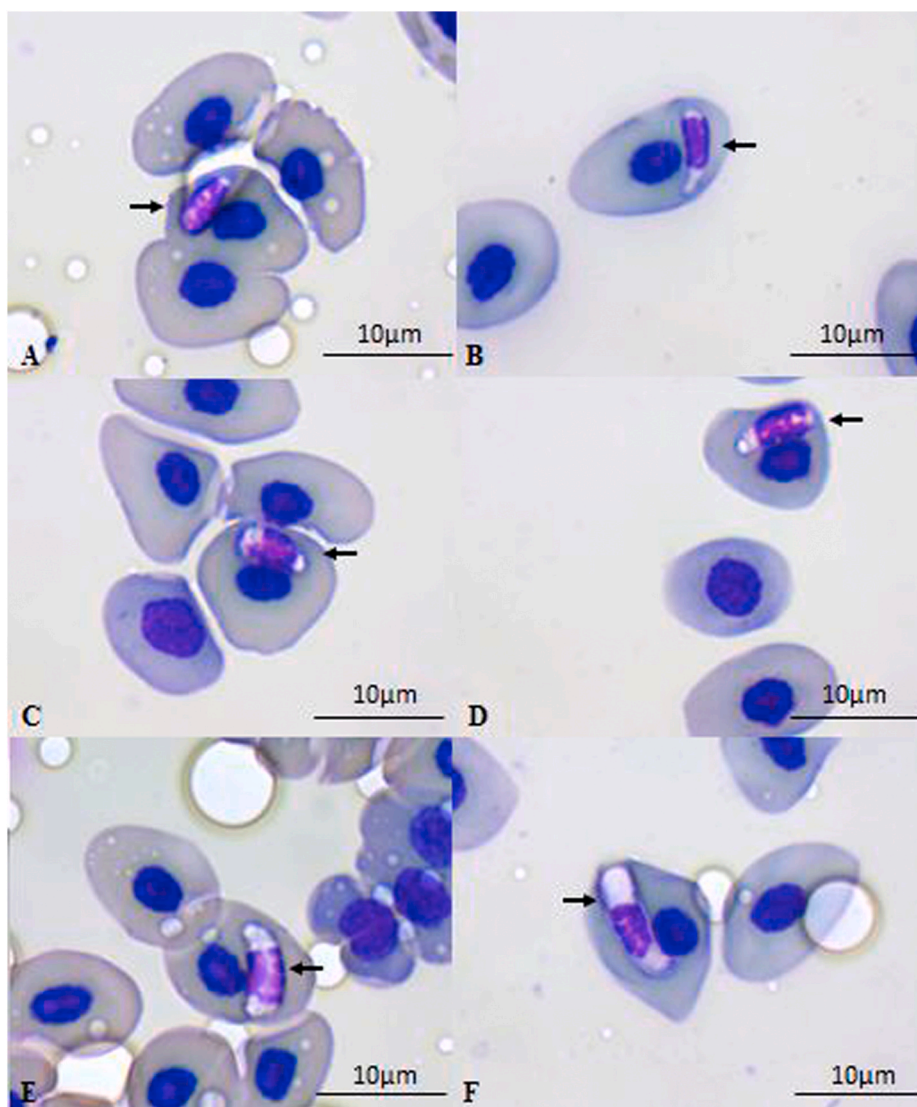

Fig. 1. (A–F). Different gamonts developmental stages of *Hepatozoon formosus* n. sp. in the blood smears of *Leptodactylus labyrinthicus* from Mato Grosso State, Brazil (indicated by the arrows). A-C) Early-stage gamonts; D-E) Immature gamonts; F) Mature gamonts. Scale bar: 10 μm.
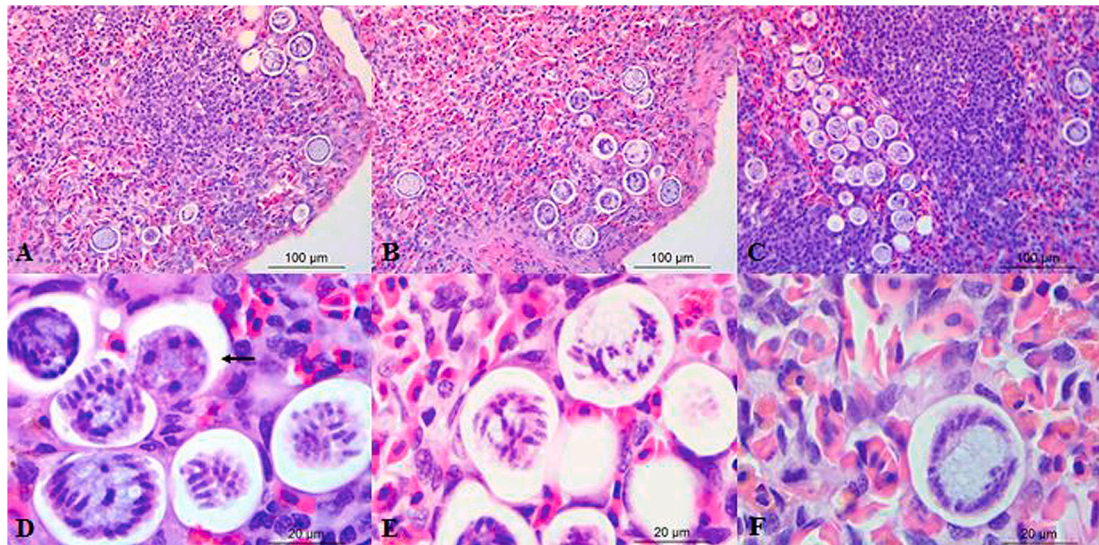
Fig. 2. (A–F). Histological slides of *Leptodactylus labyrinthicus* liver with *Hepatozoon formosus* n. sp. tissue merogony. A-C) An overview showing the amount and diversity of tissue merogonic stages infecting the anurans. D-F). Different developmental stages of meronts with merozoites. D) Macromeronts with four macromerozoites indicated by the arrow. E-F) Stages of micromeronts with micromerozoites. Scale bar: 100 μm and 20 μm.
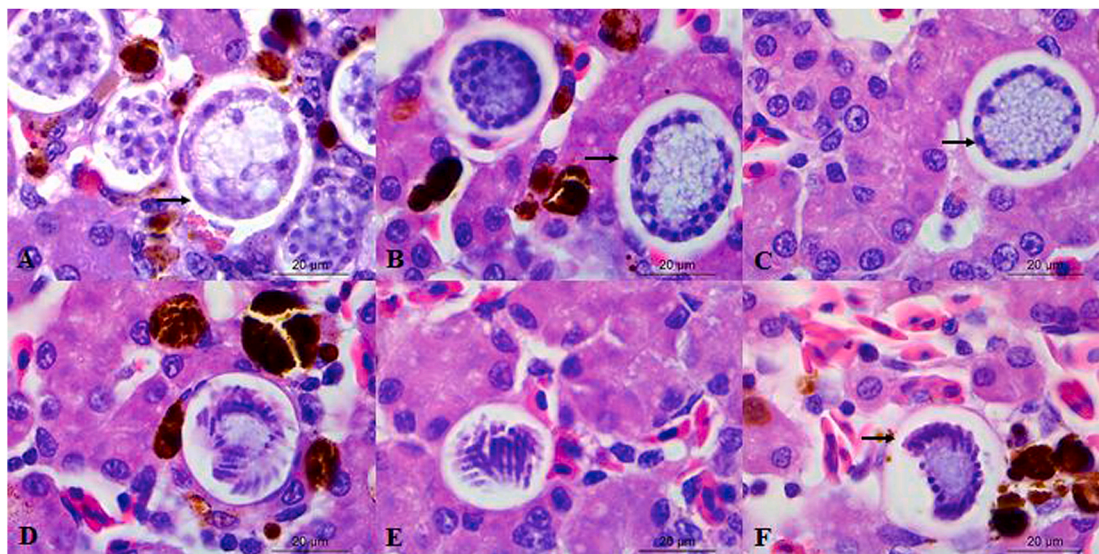

Fig. 3. (A–F). Histological slides of the *Leptodactylus labyrinthicus* spleen with *Hepatozoon formosus* n. sp. micromeronts with micromerozoites in different developmental stages. A, B, C and F) Early stage of micromeronts indicated by the arrow. Scale bar: 20 μm.

liver and spleen.

Macromeront (Fig. 2D arrow): Rounded cyst with thick cell wall (8.08 μm) containing four large macromerozoites with a granular cytoplasm staining pinkish purple, the merozoite nuclei are dense, rounded, and stained dark-purple. meront measured 27.98 μm long, 28.50 μm wide with an area of 600.93 μm². Including wall thickness, the macromerozoites measured 15.30 ± 0.83 μm long $\times$ 7.05 ± 0.71 μm wide, and area of 99.85 ± 1.23 μm².

Micromeronts (Figs. 2 and 3): Rounded to ovoid meronts in different developmental stages, with wall thickness measuring 6.03 ± 2.02 μm (4.01–9.15). The meronts measured 37.62 ± 1.34 μm (32.86–41.46) long, 36.84 ± 1.28 μm (31.23–39.96) wide with an area of 937.95 ± 33.69 μm² (827.02–1212.90) (n = 15) (including wall thickness). It was possible to observe between 16 and 35 micromerozoites in different processes of development, with early stage meronts showing a layer of micromerozoites, stained purplish, closer to the wall and a mass located in the middle of the cyst stained whitish (arrow-head). Micromerozoites

are slender and long, measuring 7.49 ± 0.72 μm (5.99–9.64) long, 1.93 ± 0.16 μm (1.44–2.17) wide, with an area of 14.77 ± 0.87 μm² (10.35–18.54); with a long, rectangular and dense nuclei, stained dark-purple, measuring 4.32 ± 0.82 μm (3.15–5.39) long, 1.82 ± 0.30 μm (1.40–2.79) wide, with an area of 5.85 ± 0.59 μm² (3.04–8.88) (n = 30).

3.1.1.2. Remarks. Based on morphology and morphometry of peripheral blood and tissue stages from *L. labyrinthicus*, *Hepatozoon formosus* n. sp. does not conform morphologically to *H. leptodactyli* (currently the only recognized species of *Hepatozoon* from Brazilian anurans); or to any other species of *Hepatozoon* described from anurans worldwide.

The gamonts of *H. leptodactyli* occurred both as intracellular and extracellular forms; the immature gamonts are characterized by a crescent or roundish form with no cytoplasmic vacuoles; mature gamonts presented a visible distinct capsule with a curved pole and ranged in size from 5.1 μm long $\times$ 2.3 μm wide to 21 μm long $\times$ 8.3 μm wide. Furthermore, gamonts of *H. leptodactyli* often cause hypertrophy to the

host cell, displacement of host nucleus, and multiple gamonts have been reported to parasitize a single cell. Whereas for *H. formosus* n. sp. only erythrocytic gamonts were observed; immature gamonts are smaller, tapering towards one end and rounded to the other end, and in some cases, vacuoles are present; cause no displacement of the host cell nucleus or morphological changes to the host cell; mature gamonts are smaller compared to *H. leptodactyli* and possess a capsule (not frequently observed) with both ends of the gamont rounded. Mature gamonts also cause hypertrophy of the host cell and slight displacement of the host cell nucleus, but no multiple infections were observed.

Tissue stages of *H. leptodactyli* were also reported in liver and spleen tissue. The micromeronts measured 13 μm long × 16 μm wide containing between 16 and 32 micromerozoites, with average of 2.5 μm long × 3 μm wide. Macromeronts measured 14 μm long × 14.7 μm wide containing between 2 and 8 macromerozoites measuring an average of 18 μm long × 8 μm wide. Macromeront and micromeronts of *Hepatozoon formosus* n. sp. from the current study are also reported. Macromeront measured 27.98 μm long × 28.50 μm wide with 4 macromerozoites measuring 15.30 μm long × 7.05 μm wide. Micromeronts measured an average of 37.62 μm long × 36.84 μm wide possessing between 16 and 35 micromerozoites measuring 7.49 μm long × 1.93 μm wide.

Hepatozoon formosus n. sp. most closely resembles the *Hepatozoon* sp. reported by Leal et al. (2015) in two Brazilian anurans (*L. chaquensis* and *L. podicipinus*). As in the present study, these authors observed early stage gamonts with the nuclei occupying almost the entire parasite. However, the authors also reported extraerythrocytic gamonts with varying morphological characteristics, measuring 9.7 μm long × 4.5 μm wide from *L. chaquensis* and 13.95 μm long × 4.6 μm wide from *L. podicipinus,* with nuclei occupying the entire host cell or rounded dislocated by one end of the parasite; the nuclear chromatin ranging from fragmented to dense and stained from blue-gray to pink (Leal et al., 2015); all of these characteristics differed from those observed in *Hepatozoon formosus* n. sp.

3.1.2. Hepatozoon longinucleus Úngari, Netherlands, Silva and O'Dwyer n. sp.

Type-host: Leptodactylus labyrinthicus (Anura: Leptodactylidae).
Type-locality: Municipality of Nova Xavantina, Mato Grosso State, Brazil (coordinates 14°35′47″ S 51°43′9,59″ W).
Site of infection: Peripheral blood erythrocytes.
Vector: Unknown.
Etymology: During morphological analysis of the mature gamonts, a unique characteristic was observed. The gamont nucleus is long, occupying ¾ of the parasite's length. This character has not been observed in other species of *Hepatozoon* from anurans globally. The word "longinucleus" is a combination of the Latin words "longus" and "nucleus".
Parasitaemia: The parasitaemia of *L. labyrinthicus* was 0.2%.
Prevalence: The total prevalence found was 1.51% (1/66) and 12.5% (1/8) among the *L. labyrinthicus* individuals analysed.
Material deposited: Hapantotype, one blood smear from *L. labyrinthicus* deposited in the collection of the National Institute of

Amazonian Research (INPA), Manaus, Brazil [INPA22].

Gene sequence: The 18S rDNA sequence obtained with 1396 bp from a pepper frog *L. labyrinthicus* blood was deposited in GenBank under accession number [MW584361].

3.1.2.1. Description. Gamonts (Fig. 4): Elongated gamonts only found infecting host erythrocytes with a thin parasitophorous capsule (in some cases not evidenced), both ends rounded with one slightly tapered and more curved than the other, with cytoplasm stained purplish-blue; gamonts measured 12.58 ± 0.62 μm (13.27–11.50) long and 4.17 ± 0.41 μm (4.36–2.40) wide, with an area of 42.24 ± 5.94 μm^2 (43.35–31.37). Thin and elongated nucleus with dense chromatin stained purple, slightly displaced to one side of the parasite and occupying ¾ of the parasite body; measuring 9.21 ± 0.32 μm (10.74–7.50) long and 2.18 ± 0.54 μm (2.94–1,71) wide, and area of 19.16 ± 1.04 μm^2 (20.98–16.78) (n = 15).

Effects of host cell: Gamonts cause displacement of the host cell nucleus. In some cases, the parasite causes morphological changes in the host cell (hypertrophy). Infected erythrocytes measured 17.65 ± 2.74 μm (16.16–19.67) long (U = 344.0; p = 0.016) and 12.97 ± 1.40 μm (10.98–13.24) wide (U = 278.0; p ≤ 0.001) with an area of 158.82 ± 3.20 μm^2 (142.85–170.97) (U = 168.0; p ≤ 0.001) (n = 15). Non-infected erythrocytes measured 16.93 ± 1.07 μm (18.49–15.43) long and 11.03 ± 0.40 μm (9.58–11.90) wide with an area of 140.42 ± 5.21 μm^2 (133.18–151.72) (n = 50).

3.1.2.2. Remarks. Based on gamont morphology and its elongated nucleus observed in the peripheral blood of *L. labyrinthicus, Hepatozoon longinucleus* n. sp. does not resemble *Hepatozoon formosus* n. sp., *H. leptodactyli* or any other currently recognized species of *Hepatozoon* from anurans globally.

With regards to *H. leptodactyli*, extracellular gamonts, intracellular early-stage and intracellular mature gamonts were reported with measurements ranging from between 5.1 μm long × 2.3 μm wide to 21 μm long × 8.3 μm wide; the nuclei is located in median or subterminal portion varying from granules and threads to compact oval or rounded; a visible capsule and curved pole were observed, and red staining cytoplasmic inclusions are visible when stained with Giemsa. In comparison to *Hepatozoon longinucleus* n. sp., mature gamonts are in some cases folded within a capsule, all intracellular measuring 12.58 μm long × 4.17 μm wide, with a relatively long nuclei occupying 2/3 of parasites length, furthermore no red inclusions were observed, the parasite's cytoplasm stained purplish-blue and the nuclei purplish.

The main difference between *H. formosus* n. sp. and *H. longinucleus* n. sp. is gamont morphology. *Hepatozoon formosus* n. sp is characterized by the presence of different gamont stadium of infection, ranging from early to mature stages, as compared to *Hepatozoon longinucleus* n. sp. of which only mature stages were observed. However, these differences could be related to the recent infection of *H. formosus* n. sp. in its host, as compared to *H. longinucleus* n. sp. and its host. Mature gamonts of *H. formosus* n. sp. are robust and elongated, with both ends rounded,
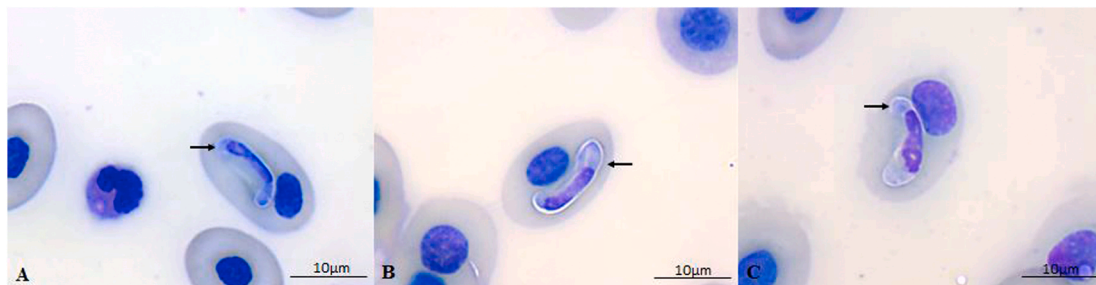

Fig. 4. (A–C). Gamonts of *Hepatozoon longinucleus* n. sp. in the blood smears of *Leptodactylus labyrinthicus* from Mato Grosso State, Brazil (indicated by the arrows). Scale bar: 10 μm.

containing a dense and quadrangular nucleus, located centrally to the parasites body. While, *H. longinucleus* n. sp. is characterized by elongated gamonts with one end slightly tapered and more curved than the other, cytoplasm stained purplish-blue and an elongated nucleus slightly displaced to one side of the parasite and occupying ¾ of the parasite's body.

Compared to other haemogregarines reported in Brazilian frogs, Sousa and Filho (1974) observed a haemogregarine-like parasite that was not identified to genus level, with varying morphology ranging from small gamonts (7.7 μm long × 1.7 μm wide) to larger gamonts (9.8 μm long × 3.4 μm wide). Leal et al. (2015) also reported extraerythrocytic and intraerythrocytic gamonts with a variety of sizes and shapes belonging to species of *Hepatozoon*. Parasite morphometrics and measurements cited above does not compare to *H. longinucleus* n. sp., differing in size, shape and structure of mature gamonts, and position and size of the nucleus.

3.1.3. Hepatozoon latrensis Úngari, Netherlands, Silva and O'Dwyer n. sp.

Type-host: two specimens of *Leptodactylus latrans* (Anura: Leptodactylidae).

Type-locality: Municipality of Nova Xavantina, Mato Grosso State, Brazil (coordinates 14°35′47″ S 51°43′9,59″ W).

Site of infection: Peripheral blood erythrocytes, liver, and spleen fragment tissues.

Vector: Unknown.

Etymology: This new species of *Hepatozoon* was found in two anuran hosts, belonging to the species *L. latrans.* Therefore, the name "latrensis" refers to the host species, translated into Latin nomenclature.

Parasitaemia: The parasitaemia found in the anurans was 0.5% and 18.21%.

Prevalence: The total prevalence found was 3.03% (2/66) and 6.45% (2/31) among *L. latrans* analysed.

Material deposited: Hapantotypes, one blood smear from *L. latrans,* one histological slide of the liver, and one histological slide of the spleen, were deposited in the collection of the National Institute of Amazonian Research (INPA), Manaus, Brazil [INPA21].

Gene sequence: The 18S rDNA gene sequences obtained, with 1403 bp and 1343 bp, from the two pepper frog *L. latrans* blood were deposited in GenBank under accession number [MW584362 and MW584365].

3.1.3.1. Description. Gamonts (Fig. 5): Observed infecting host erythrocytes, robust with both ends rounded, parasitophorous capsule evident, cytoplasm staining whitish-purple with cytoplasmic vacuoles visible in some cases; gamonts measuring 9.88 ± 0.56 μm (9.06–10.74) long and 4.35 ± 0.41 μm (3.27–5.02) wide, with an area of 37.46 ± 3.00 μm^2 (34.91–43.95). Nuclei rounded to quadrangular in shaped, staining purplish-blue, large in width, almost the same as the parasite, slightly displaced to one of the extremities and occupying 1/3 of the parasite's body; measuring 4.17 ± 0.57 μm (3.13–5.02) long and 3.37 ± 0.60 μm (2.67–4.02) wide, with an area of 8.87 ± 1.54 μm^2 (7.02–9.13) (n = 15).

Effects of host cell: The parasite causes displacement of the host cell nucleus to its extremities, morphological changes in the host cell related to the cell structure, and hypertrophy. Infected erythrocytes measured

16.35 ± 0.45 μm long (U = 860.5; p ≤ 0.001) and 10.02 ± 0.31 μm wide (U = 344.0; p = 0.003), with an area of 133.93 ± 0.51 μm^2 (U = 915.0; p ≤ 0.001) (n = 15). Non-infected erythrocytes measured 14.10 ± 0.28 μm long and 10.74 ± 1.01 μm wide, with an area of 113.86 ± 0.78 μm^2 (n = 50).

Tissue merogony (Fig. 6A–F): Histological sections of liver and spleen revealed the presence of micromeronts with micromerozoites, macromeronts with macromerozoites, and a single dizoic cyst.

Macromeronts (Fig. 6 A and B): Ovoid meronts measuring 23.19 ± 1.23 μm (21.38–26.50) long, 19.66 ± 0.85 μm (15.64–22.11) wide and 347.32 ± 35.78 μm^2 (307.01–479.29) area, with an irregular shaped wall measuring 7.95 ± 1.28 μm (5.30–9.87) (n = 2). Each macromeront contained between 3 and 5 visible macromerozoites, with cytoplasm granular and staining purplish-pink, in some cases vacuoles also visible. Macromerozoites measured 18.25 ± 0.15 μm (18.04–18.70) long and 5.70 ± 0.37 μm (5.03–5.84) wide, with an area of 69.74 ± 0.45 μm^2 (69.31–69.80). Nuclei are rounded, compact and dense, staining dark-purple, measuring 2.17 ± 0.48 μm (1.98–2.30) long and 2.10 ± 0.15 μm (1.99–2.40) wide, with an area of 4.53 ± 0.84 μm^2 (4.35–5.01) (n = 8).

Micromeronts (Fig. 6 C - E): Rounded early stages meronts of development measuring 15.08 ± 0.93 μm (15.23–16.98) long, 14.99 ± 1.53 μm (13.99–17.86) wide, 212.22 ± 28.17 μm^2 (195.17–298.15) area, with meronts encased in an irregular wall measuring 4.01 ± 1.20 μm (3.24–7.48) (n = 5). A whitish-purple to pink stained mass, likely from where merozoite formation is initiated (B and E), also visible. Micromeronts were only observed in early stages of development and could not be measured or counted, however, possible early stages nuclei formation visible, with chromatin forming dense rounded structures staining dark-purple.

Dizoic cyst (C): Ovoid cyst with thick wall (7.09 μm) containing two slender and long cystozoites with cytoplasm staining purplish-pink. Cystozoite nuclei rounded, compact and staining dark-purple. Cyst measured 17.17 μm long and 23.11 μm wide, with an area of 297.07 μm^2 (measurements including wall); respective cystozoites measured 11.25 and 12.07 μm long, and 2.26 and 3.01 μm wide, with an area of 27.08 and 30.07 μm^2 respectively; cystozoites nuclei measured 2.04 and 2.19 μm long and 1.21 and 1.33 μm wide, with an area of 3.08 and 3.71 μm^2.

3.1.3.2. Remarks. Based on gamont and meront morphology and morphometrics observed in the peripheral blood and tissue of *L. labyrinthicus, Hepatozoon latrensis* n. sp. does not conform to *H. formosus* n. sp., *H. longinucleus* n. sp., *H. leptodactyli* or any of other species of *Hepatozoon* from anurans worldwide.

Hepatozoon leptodactyli share certain characteristics with *Hepatozoon latrensis* n. sp., such as nucleus size and the presence of a visible capsule. However, *H. latrensis* n. sp. differs from *H. leptodactyli* in average size of mature gamonts stage measuring 9.88 μm long × 4.35 μm wide, the absence of red inclusions in the cytoplasm, both ends of the parasite are equal and rounded, with a round shaped nuclei positioned towards one end of the gamont occupying in some the entire width of the parasite's body and staining purplish-blue.
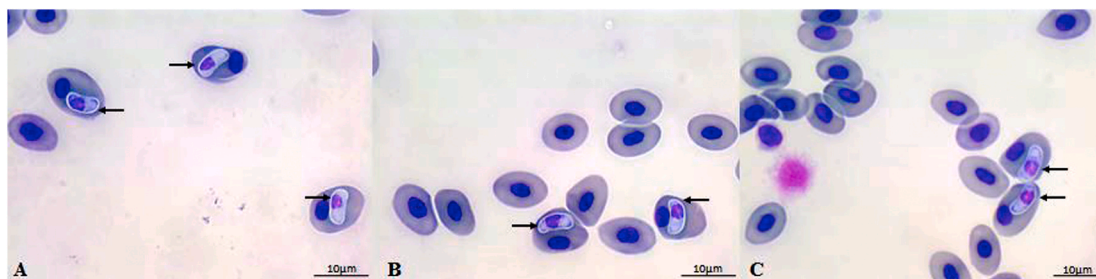

Fig. 5. (A–C). Gamonts of *Hepatozoon latrensis* n. sp. in the blood smears of two *Leptodactylus latrans* from Mato Grosso State, Brazil (indicated by the arrows). Scale bar: 10 μm.
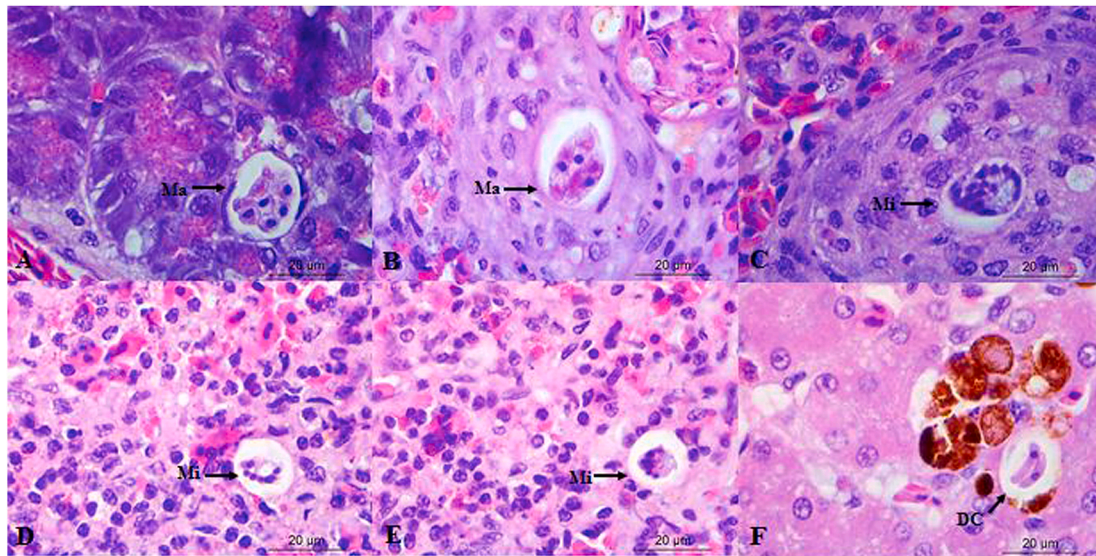
Fig. 6. (A–F). Histological sections of two *Leptodactylus latrans* anurans livers with *Hepatozoon latrensis* n. sp. tissue merogony. Histological slides of the liver from one anuran specimen showing macromeronts with macromerozoites (A and B); micromeronts with micromerozoites (C–E) and dizoic cyst with cystozoites (F). D-E) Micromeronts with merozoites of the liver from the second anuran specimen. Macromeronts (Ma), micromeronts (Mi), and dizoic cyst (DC) are indicated by arrows. Scale bar: 100 µm and 20 µm.

With regards to the tissue merogony of *H. leptodactyli* high parasitaemia was reported in the lung, intestine, heart, and brain; the micromeronts average size was 13 µm wide × 16 µm long, with between 16 and 32 micromerozoites visible measuring an average of 3 µm long × 2.5 µm wide; rounded macromeronts of *H. leptodactyli* measured an average of 14 µm long × 14.7 µm wide with 2–8 macromerozoites measuring 18 µm long × 8 µm wide. Moreover, the meronts from *H. latrensis* n. sp. were observed in different site of infection (spleen and liver); macromeronts of *H. latrensis* n. sp. are larger and oval (not rounded as in *H. leptodactyli*), measuring 23.19 µm long × 19.66 µm wide with 3–5 macromerozoites measuring 18.25 µm long and 5.70 µm wide.

Hepatozoon formosus n. sp. and *H. longinucleus* n. sp. differ in comparison to *H. latrensis* n. sp. Mature gamonts of the later species being smaller in size (9.88 ± 0.56 µm long × 4.35 ± 0.41 µm wide with 37.46 ± 3.00 µm² area). *Hepatozoon formosus* n. sp. measures 11.93 ± 0.61 µm long, 4.18 ± 0.24 µm wide with an area of 40.20 ± 3.01 µm² and *H. longinucleus* n. sp. measures 12.58 ± 0.62 µm long × 4.17 ± 0.41 µm wide with an area of 42.24 ± 5.94 µm². Moreover, *H. formosus* n. sp. have a square-shaped nucleus extending the width of the parasite and *H. longinucleus* n. sp. contains a long-tapered nucleus, compared to the centrally placed rounded nucleus of *H. latrensis* n. sp.

Regarding the *Hepatozoon* from Brazilian snakes, *Hepatozoon musa* described from the snake *Philodryas nattereri* Steindachner, 1870 from Ceará State, north-eastern Brazil have mature gamonts, measuring 16.568 µm long × 3.095 µm wide and area of 59.710 µm², characterized by considerable curvature at both ends and nuclei typically placed centrally, differing from the mature gamonts of *H. latrensis* n. sp., which are smaller, nucleus is displaced to one end and both ends of the parasite are rounded and not curved.

3.2. Molecular and phylogenetic analysis

From the four positive specimens, four newly generated sequences were obtained. Amplicons of 1384 and 1396 bp derived from *H. formosus* n. sp. [MW584356] and *H. longinucleus* n. sp. [MW584361], respectively, isolated from the blood of *L. labyrinthicus*, and two sequence fragments of 1403 and 1343 bp sharing 100% identity were derived from *H. latrensis* n. sp. isolated from the blood *L. latrans* [MW584362/ MW584365].

From the two alignments provided, 582 bp and 1116 bp, the phylogenetic analyses using BI and ML methods from each one alignment resulted in identical tree topologies (Fig. 7, Fig. 8). In the present study's phylogenetic analyses from both alignments, species of *Dactylosoma* isolated from anurans were recovered as the earliest diverging clade of the ingroup, sister to the monophyletic clade of *Haemogregarina* from chelonids and all other haemogregarines (Karyolysidae and Hepatozoidae) analysed. Isolates from *Hemolivia* clustered together; separate from monophyletic *Karyolysus* spp. and polyphyletic *Hepatozoon* spp. Isolates from *Hepatozoon* isolated from large mammals formed a monophyly sister to the Karyolysidae Clade comprising species of *Karyolysus*.

With regards to the phylogenetic analysis based on the alignment comprising shorter sequence fragments (Fig. 7), the species of *Hepatozoon* from the present study *H. formosus* n. sp. [MW584356], *H. longinucleus* n. sp. [MW584361], and *H. latrensis* n. sp. [MW584362/ MW584365] grouped within other *Hepatozoon* isolates from Brazilian anurans [MK503646, MK503647, MK503645, MK503648 and MK503643]. The isolates from Brazilian anurans formed a sister group to a clade comprising species of *Hepatozoon* from Brazilian snakes, *Hepatozoon musa* Borges-Nojosa et al., (2017) [MF497767], *Hepatozoon* sp. [MF497768], and *Hepatozoon cuestensis* O'Dwyer et al., (2013) [MF497077].

Concerning the phylogenetic analysis with longer sequences (Fig. 8), the species of *Hepatozoon* from the present study grouped in the same clade but different branches with four isolates from snakes: *Hepatozoon domerguei* Maia et al. 2014 reported in two Madagascar snakes [KM234646 and KM234648]; *Hepatozoon ayorgbor* Sloboda et al. 2007 reported in a snake from Gana [EF157822]; *Hepatozoon chinensis* Wu et al. 2013 reported in a snake from China [KF939620] and *Hepatozoon* sp. Boiga from Australia [AF297085]. Furthermore, in the both phylogenetic trees, species of *Hepatozoon* isolated from Brazilian anurans were recovered as distantly related to other species of *Hepatozoon* from North American, European and African anurans.

With regards to gene similarity and pair-wise distance data from the tree (Table 2), the three new species from the present study have shown an interspecific divergence ranging from 0.43% to 1.16%. *Hepatozoon formosus* n. sp. reported an interspecific divergence of between 0.93 and 8.01% and *H. longinucleus* n. sp. showed a divergence of between 0.43%– 8.39% in comparison with other species of *Hepatozoon* from reptile and
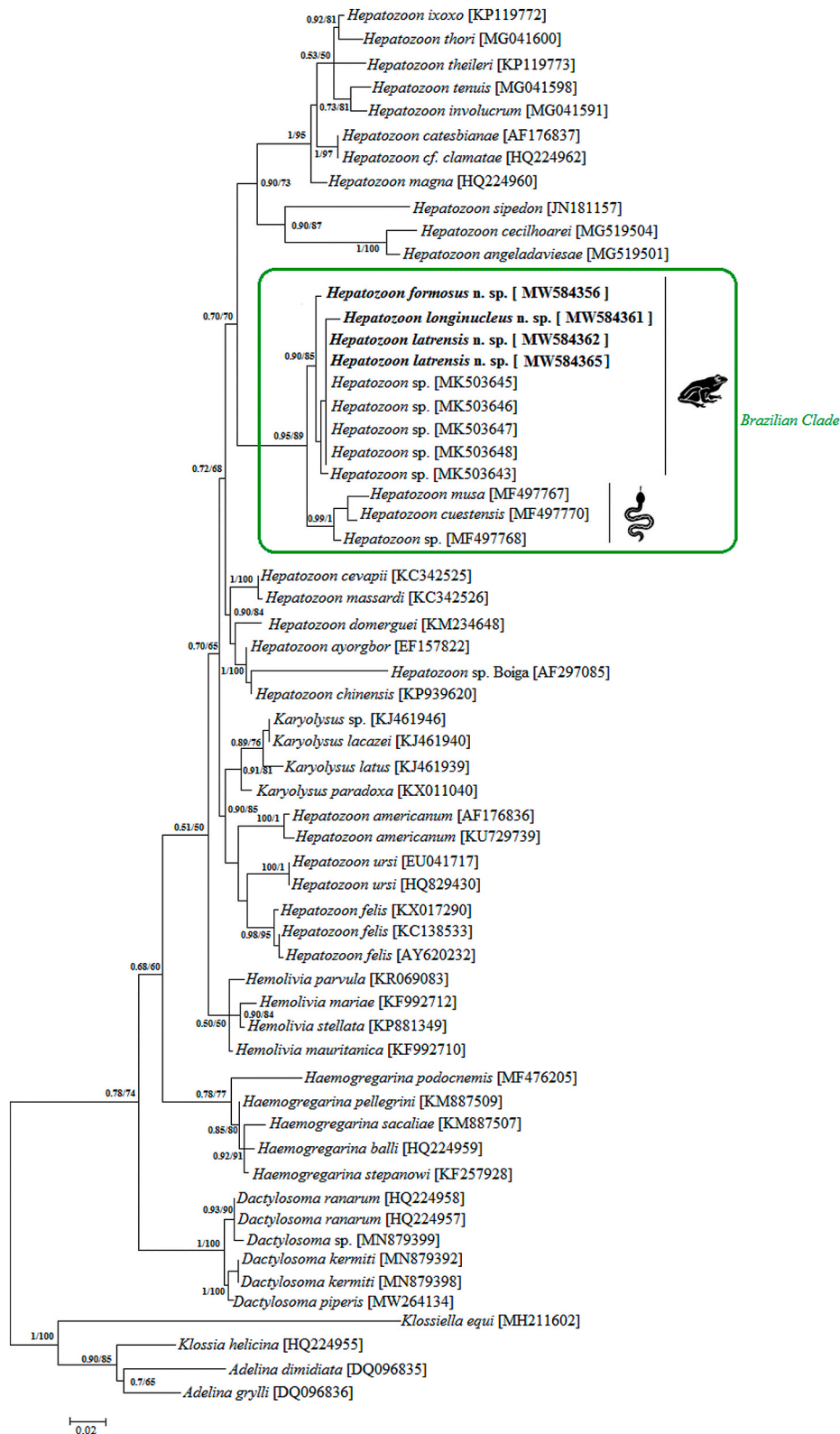
Fig. 7. Consensus phylogram of haemogregarines based on 18S rDNA sequences (582 nt). The topology trees were inferred by Bayesian (BI) and Maximum Like-lihood (ML) methods (represented by ML tree). The isolates *Adelina dimidiata* (DQ096835), *Adelina grylli* (DQ096836), *Klossia helicina* (HQ224955), and *Klossiela equi* (MH211602) were used as an out-group.
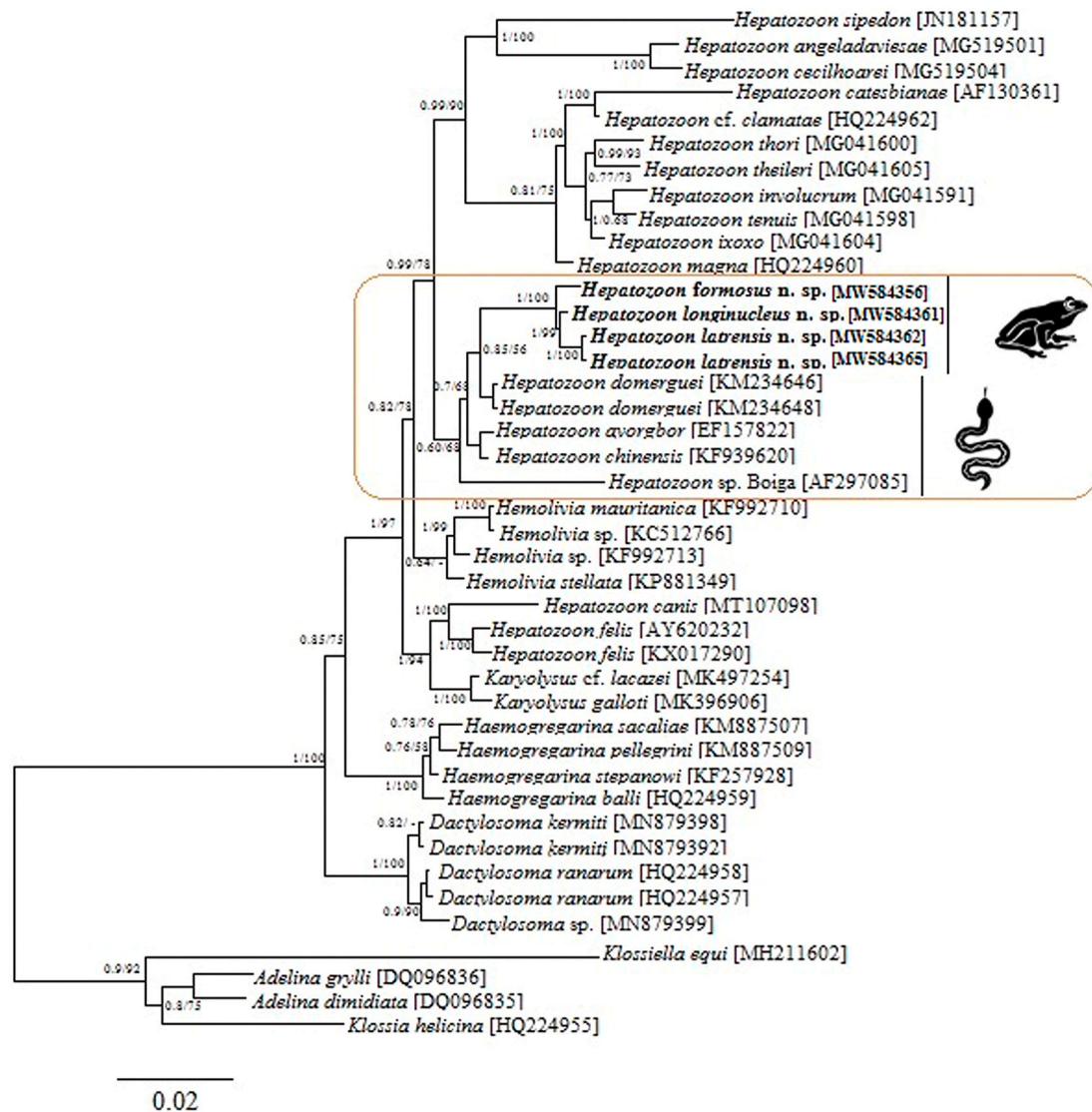
Fig. 8. Consensus phylogram of haemogregarines based on 18S rDNA sequences (1116 nt). The topology trees were inferred by Bayesian (BI) and Maximum Likelihood (ML) methods (represented by ML tree). The isolates *Adelina dimidiata* (DQ096835), *Adelina grylli* (DQ096836), *Klossia helicina* (HQ224955), and *Klossiela equi* (MH211602) were used as an out-group.

anuran hosts. *Hepatozoon latrensis* n. sp., showed 100% of gene similarity with four *Hepatozoon* sp. isolates [MK503646, MK503647, MK503645, and MK503648] reported previously reported from Brazilian anurans (Ferreira et al., 2020).

4. Discussion

Currently, 7166 anuran species have been described around the world, with 1039 species reported from Brazil (Frost, 2020). In addition, anurans are known to harbour a great number of haemoparasites, with haemogregarines being the most commonly reported (Barta and Desser, 1984; Smith, 1996; Telford, 2009). In the present study anurans of Bufonidae, Hylidae, and Leptodactylidae were screened for intracellular blood parasites, however, only species from Leptodactylidae were positive for species of *Hepatozoon*. Barta and Desser (1984) evaluated the prevalence of haemoparasites in blood smears from Canadian frogs and found higher prevalence among aquatic lifestyle frogs when compared to terrestrial ones. Another study conducted in South Africa analysed the diversity of haemoparasites from anurans with different lifestyles (aquatic, semi-aquatic, semi-terrestrial, and terrestrial), and also

reported a higher prevalence of species of *Hepatozoon* among semi-aquatic and semi-terrestrial anurans (Netherlands et al., 2015). These authors suggest that the number of potential vectors may be higher surrounding aquatic habitats, as compared to terrestrial habitats (Netherlands et al., 2015). Furthermore, a recent study in Brazil by Ferreira et al. (2020) reported higher prevalence for haemogregarine parasites in the semi-aquatic nocturnal *L. latrans* (100%) as compared to terrestrial nocturnal *R. diptycha*. Therefore, the present study corroborates with those described above, since the Leptodactylidae includes species with a semi-aquatic lifestyle. Moreover, although there are only a few reports on *Hepatozoon* prevalence among Brazilian frogs, individuals of Leptodactylidae are commonly reported to be parasitised by species of *Hepatozoon* (Cunha and Muniz, 1927; Da Costa, et al. 1973; Leal et al., 2015; Ferreira et al., 2020).

Cunha and Muniz (1927) reported in detail the schizogony in the liver, lung, and intestine of a haemogregarine infecting *Leptodactylus latrans* from Brazil. Another study, by Da Costa et al. (1973) used morphological and morphometric data, to formally described *H. leptodactyli,* infecting individuals of Leptodactylidae. From 90 *Leptodactylus pentadactylus* and *L. latrans* analysed, only 17 (17.17%) frogs

were positive for *H. leptodactyli*. In this study, from 40 Leptodactylidae frogs collected, only four (10%) were positive. Moreover, using microscopy screening, Leal et al. (2015) reported on the presence of species of *Hepatozoon* in blood smears of eight (11.76%) *L. chaquensis* and two (2.60%) *L. podicipinus* from Mato Grosso do Sul State.

With regards to other Brazilian anurans, Sousa and Filho (1974) reported a 1% prevalence of species of *Haemogregarina* (this was most likely an incorrect identification of species of *Hepatozoon*) from 100 anurans screened. Intra-erythrocytic gamonts infecting the blood smears of one *Rhinella crucifer* (Wied-Neuwied, 1821) (syn. *Bufo crucifer*) from Rio de Janeiro State, Brazil, were found with a parasitaemia of 0.5%. In another study by Kattar (1986), from the 100 Brazilian anurans analysed, eight (8%) were positive for haemogregarine parasites infecting blood smears of *Rhinella diptycha* (Cope, 1862) (syn. *Bufo paracnemis*) collected at the municipality of João Pessoa, Paraíba State, Brazil. However, gametocyte morphology conforms rather to species of *Hemolivia* as compared to species of *Hepatozoon*.

In relation to Leal et al. (2009), the authors reported a 10% prevalence of haemogregarines in three Brazilian frog species, *Leptodactylus chaquensis* Cei, 1950, *L. podicipinus* Cope, 1862, and *Pithecopus hypochondrialis* (Daubin, 1800), from Mato Grosso do Sul State and São Paulo State. The parasitaemia found in this study was 1.17% for *H. formosus* n. sp. 0.2% for *H. longinucleus* n. sp., and 0.5%, and 18.21% for *H. latrensis* n. sp. These findings may be related to the physiological and immunological patterns of the host and also, the parasite pathogenicity. Lower parasitaemia can be associated with a chronic state of infection, resulting in a cyclic merogonic phase, to maintain the infection for a long time (Tomé et al., 2012). Besides, Tender et al. (2000) suggest a relationship between species of *Hepatozoon* and *Toxoplasma gondii* infections, which can persist throughout the hosts life.

There are only a few recognized species of *Hepatozoon* from anurans globally that have been characterized using morphological, morphometric, and molecular tools. Namely two species of *Hepatozoon* from North America, *Hepatozoon catesbianae* (Stebbins, 1904) and *Hepatozoon clamatae* (Stebbins, 1905). From South Africa, *Hepatozoon tenuis* Netherlands, Cook and Smit, 2017; *Hepatozoon involucrum* Netherlands, Cook and Smit, 2017; *Hepatozoon thori* Netherlands, Cook and Smit, 2017; *Hepatozoon theileri* (Laveran, 1905) and *Hepatozoon ixoxo* Netherlands, Cook and Smit, 2014. Lastly from Europe, there is only one species with morphological, morphometric, and molecular data available namely *Hepatozoon magna* (Grassi and Feletti, 1891) (see Barta et al., 2012; Netherlands et al., 2014a; Netherlands et al., 2014b; Netherlands et al., 2018). However, these species of *Hepatozoon* from anurans differ in morphology compared to *H. formosus* n. sp., *H. longinucleus* n. sp. or *H. latrensis* n. sp., and through molecular diagnosis reported interspecific divergences ranging from 8.01% to 4.97%, between isolates from this study and those mentioned above.

Furthermore, the phylogenetic analysis with small base pairs sequences, recovered species of *Hepatozoon* isolated from Brazilian anurans and snakes as monophyletic and sister to species isolated anurans and snakes isolates from South Africa, Europe, and North America. These findings are in contrast to the findings of Netherlands et al. (2018), who suggested that species of *Hepatozoon* from anurans are monophyletic, providing new insights to the phylogenetic relationships and evolution of species of *Hepatozoon* form anurans. This divergence in clades could be explained by the geographical distribution and isolation between Brazil (South America) and Africa, Europe and North America.

Moreover, four *Hepatozoon* sp. isolates from Brazilian anurans, *Rhinella diptycha* [MK503646, MK503647and MK503648] and *Leptodactylus latrans* [MK503645], reported by Ferreira et al. (2020) showed 100% of gene similarity with one species from this study, *Hepatozoon latrensis* n. sp. Although Ferreira et al. (2020) only provided molecular data, being unable to compare the developmental stages among them, the identical gene similarity observed suggest that these sequences belong to the same species, *H. latrensis* n. sp.

The comparison among the three species from the present study and

Brazilian snakes revealed a lower interspecific divergence, ranging from 0.62%–5.98%, with *H. musa* (MF497767) presenting 99.38% identity to *H. latrensis* n. sp. *Hepatozoon musa* described from the snake *Philodryas nattereri* Steindachner, 1870 from Ceará State, north-eastern Brazil, belong to a different group of animals and differ in lifestyle behaviour also, comparing with the anuran host of *H. latrensis* n. sp. The *P. nattereri* is a diurnal arboreal and terrestrial species, compared to the nocturnal semi-aquatic life-style observed in *L. latrans* (Uetanabaro et al., 2008; Pimenta et al., 2014; Harrington et al., 2018). Moreover, the morphological data revealed significant differences between both species.

The phylogenetic proximity between *Hepatozoon* from anurans and snakes has been reported in the literature (Netherlands et al., 2014a, 2014b; Netherlands et al., 2018; Ferreira et al., 2020). It is known that anurans can play an important role as intermediate host for some reptile-associated *Hepatozoon* spp. (Smith et al., 1994; Laison et al., 2003). Laison et al. (2003) observed an infection in *Caiman crocodilus* (Linnaeus, 1758) specimens that had been fed with infected *Leptodactylus fuscus* (Schneider, 1799) and *Lithobates cartesbeianus* (Schaw, 1802). In another study, Smith et al. (1994) reported a snake *Nerodia sipedon* (Linnaeus, 1758) infected with *Hepatozoon sipedon* through an ingestion of a positive *Lithobates pipiens* (Schreber, 1782) frog. Therefore, the closeness of anuran-reptile *Hepatozoon* may be related with the predation through the parasite life-cycle, jumping from one vertebrate host to another and leading to adaptation to a new host, resulting in speciation (Ferreira et al., 2020).

Although this study has provided longer sequences, most available sequence isolates of species of *Hepatozoon* from Brazilian reptiles and amphibians are short fragments (<500 bp). Therefore, future studies should include obtaining longer sequences, as well as sequence data from faster evolving markers (mitochondrial), for further comparison of the phylogenetic position of species of *Hepatozoon* from anurans.

4.1. Conclusion

The present study used morphological and molecular data to characterise and describe three new species of *Hepatozoon* parasitising the Brazilians frogs, *L. labirinthycus* and *L. latrans*, from the municipality of Nova Xavantina, Mato Grosso State, Brazil. This data shows the value of using a more holistic approach to determine the diversity of *Hepatozoon* species from anurans in this biodiversity hotspot. Although, in the present study the diversity of known species of *Hepatozoon* described from Brazilian anurans has significantly increased, there are likely still several more species to be described from this area. Future work should include screening more anuran species from Brazil across the different families.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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