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ECOSYSTEMS

Hepatozoon Miller, 1908 parasites in the Colubridae snakes *Clelia clelia* (Daudin, 1803) and *Drymarchon corais* (Boie, 1827) from the Eastern Amazonia

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Abstract: Based on the genetic, morphological, and morphometric data of blood gamonts, we identified *Hepatozoon* parasites in colubrid snakes sampled in the Eastern Amazon region. *Hepatozoon trigeminum* was detected in the mussurana snake *Clelia clelia* and exhibited wide and elongated gamonts (mean dimensions: 14.25±0.65 × 4.31±0.43 µm) with an evident parasitophorous vacuole. *Hepatozoon odwyerae* sp. nov. was described in the indigo snake *Drymarchon corais*, whose gamonts have elongated and thin bodies (mean dimensions: 13.41±0.79 × 3.72±0.35 µm) with one end more tapered than the other. Phylogenetic analyses, based on the amplification of a 441 bp fragment of the 18S rRNA gene, revealed that the novel sequences of *Hepatozoon* spp. from our study were closely related to hemogregarine lineages found in lizards and snakes from Brazil, forming a well-supported monophyletic clade with them. The present study provides the first species description of *Hepatoozon* in *D. corais* and a new record of a host species for *C. clelia* using the integrated taxonomic data. We also highlight the importance of further investigations into the diversity of *Hepatozoon* in snakes, a rich but underestimated group of parasites, especially in the Amazonian biome.

Key words: Apicomplexa, colubrid snakes, hemoparasites, integrative taxonomy, 18S rRNA.

INTRODUCTION

The phylum Apicomplexa Levine, 1970 comprises the greatest diversity of protozoan parasites, with approximately 6,000 named species (Votýpka et al. 2017). However, they remain poorly known in terms of biodiversity, and probably only 0.1% of the species in this phylum have been described (Morrison 2009, Duszynski 2021). All the organisms in this taxon are parasites (Levine 1988) and frequently infect humans and domestic and wild animals. They possess a set of structures known as the apical complex, which facilitates the entry and survival of these parasites in the cells of their host (Tardieux & Baum 2016).

Hepatozoon Miller, 1908 (Adeleorina, Hepatozoidae) is one of the most well-known genera of this phylum and can be found infecting blood and tissues from a wide range of vertebrate hosts around the world, such as amphibians, reptiles, birds, and mammals (Smith 1996, O'Donoghue 2017). As these intracellular parasites have a heteroxenous life cycle, invertebrates, including ticks, lice, fleas, dipterans and even leeches, act as definitive hosts and vectors (Smith 1996, Viana et al. 2010).

In snakes, infection occurs after the ingestion of the invertebrate host containing mature oocysts, or after feeding on an infected intermediate vertebrate host (i.e., frogs, lizards and rodents) (Sloboda et al. 2007, Tomé et al. 2012), as well as by vertical transmission (Kauffman et al. 2017).

The taxonomy and systematics of *Hepatozoon* are the subject of considerable controversy (Smith 1996, Hrazdilová et al. 2021). As highlighted by Zechmeisterová et al. (2021), the current main problem for the identification of such hemogregarines is the insufficient data used to perform species description, which often leads to synonymy. Moreover, the molecular phylogenies, mainly established using the 18S rDNA gene marker, have shown that this genus is not a monophyletic taxon (Kvičerová et al. 2014, Karadjian et al. 2015, Hrazdilová et al. 2021). Some attempts have been made to deal with this issue, such as the raising of a new genus, *'Bartazoon'* (Karadjian et al. 2015), which was not accepted by the scientific community, and a search for the sequence of the type species, *Hepatozoon muris* (Miller, 1908), which has yet to be found (Hrazdilová et al. 2021). Nonetheless, there is a strong consensus on the necessity of studies involving both morphological and molecular methods to detect and characterize *Hepatozoon* species, especially from wild hosts, to improve understanding of the diversity and phylogenetic relationships of these haemoparasites (Maia et al. 2016, Zechmeisterová et al. 2021).

Snakes are extraordinarily rich in species of *Hepatozoon*, with over 130 species described so far, at least 40 of which have been recorded in Brazil (Smith 1996, Úngari et al. 2018). Few, however, have genetic data available. Most of these records come from colubrid hosts (Pessôa et al. 1974, Smith 1996). Colubridae Oppel, 1811 is a large and diversified snake family that encompasses seven subfamilies, of which only two, Dipsadinae Bonaparte, 1838 and Colubrinae Oppel, 1811, occur in Brazil and throughout the Amazon region (Pyron et al. 2013, Fraga et al. 2013). The aims of the present study were, therefore, to investigate the occurrence of *Hepatozoon* spp. in the colubrid snakes, *Clelia clelia* (Daudin, 1803) and *Drymarchon corais* (Boie, 1827), and to characterize the specimens detected, using both morphological and molecular analyses.

MATERIALS AND METHODS

Study area and sample collection

In January 2016, one *C. clelia* specimen was captured in the Virola-Jatobá Sustainable Development Project (SDP) (-03° 9' 28.15" S; -51° 27' 51.67" W) in the municipal region of Anapu, Pará, Brazil. The SDP has an area of 38423.97 ha., with its resident human population surviving through sustainable forest management (Maestri et al. 2021). This area is formed by dense ombrophilous forest with medium and large trees (Correa et al. 2018). A specimen of *D. corais* was captured from the peri-urban region of the municipal district of Altamira (-03° 12' 01.8" S; -52°11' 29.0" W), Pará, Brazil, in May 2016. After mechanical containment of the animals, blood samples were collected by caudal vein puncture. For microscopic examination, blood smears were prepared, fixed in methanol, and stained with May-Grunwald-Giemsa solution (10%). The remaining blood samples were stored in EDTA tubes at –20 °C until DNA extraction. Snakes were euthanized with injectable anesthetic for veterinary use and fixed in 10% formalin, stored in 70% ethanol, and deposited in the collection of the Adriano Giorgi Laboratory of Zoology of the School of Biological Science, Altamira Campus, Pará, Brazil, as *C. clelia* (LZA 1416) and *D. corais* (LZA 1331). All procedures for snakes handling, sampling, and accessing genetic data were approved by the ethics committee on animal use from UNIFAP (protocol number 02/2020)

and authorized by the Ministry of Environment of Brazil (SISBIO number 32401; SISGEN number AB23235).

Microscopic analyses

The search for parasites was performed under a Leica DM4B microscope (Leica Microsystems, Heerbrugg, Switzerland) at magnifications of × 400 and × 1000. Positive slides were carefully examined, and images were captured with an attached Leica DMC4500 digital camera and processed with the LAS V4.8 software platform (Leica Microsystems Suiza Limited 2015). The length, width and area of the gamonts and nuclei were measured using this system, and parasitemia was estimated by counting the number of parasites observed in 2,000 erythrocytes (Godfrey et al. 1987).

DNA extraction, amplification, and sequencing

Genomic DNA extraction was performed following the phenol-chloroform protocol described by Sambrook et al. (1989). The DNA isolated after the procedure was suspended in 30 µl of ultra-pure sterile water, and DNA quality was verified by electrophoresis on 1% agarose gel. *Hepatozoon* spp. detection was performed by conventional Polymerase Chain Reaction (PCR) using the HepF300 (5'-GTT TCT GAC CTATCA GCT TTC GAC G-3') and Hep900 (5'-CAA ATC TAA GAA TTT CAC CTC TGA C-3') primers, which amplifies approximately 600 bp of the 18S rRNA gene (Ujvari et al. 2004). PCRs were performed in a total volume of 15 µl containing 1.5 µl of $MgCl_2$ (25 mM), 1.25 µl of 10X PCR buffer (75 mM Tris-HCl, 50 mM KCl, 20 mM (NH4)2SO4), 1.25 µl of dNTPs (10 mM), 0.3U of Taq polymerase, 1.5 µl of each primer (10 uM), 1 µl of DNA (≈ 30-50 ng/µl) and 6.7 µl of nuclease free water. The PCR conditions were as follows: 92 °C for 1 min, 35 cycles at 92 °C for 1 min, 50 °C for 50 s, 72 °C for 1.5 min, and a final extension step at 72 °C for 7 min. Amplified products were purified and sequenced in a forward direction using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and the ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The obtained sequences were edited using the BioEdit software package v7.2.5 (Hall 1999) and compared in the GenBank database using the BLASTn software (http://www.ncbi.nlm.nih.gov/BLAST). The two sequences obtained in this study were aligned with 48 sequences published in GenBank® using the MUSCLE algorithm and the Geneious v.7.1.3 software package (Biomatters; http://www.geneious.com). Phylogenetic reconstructions were based on Bayesian Inference and Maximum Likelihood methods. The jModelTest v.2.1.10 (Darriba et al. 2012) was used to select the best evolution model for phylogenetic analysis. Based on the Akaike Information Criterion (AIC), HKY + G was the best model chosen for Bayesian Inference and TPM2uf + G was chosen for maximum likelihood. Bayesian Inference was implemented using MrBayes 3.1.2 (Ronquist & Huelsenbeck 2003). Markov chain Monte Carlo (MCMC) simulations were run for 10,000,000 generations in two parallel runs, sampling one tree every 1,000 generations, with a burn-in of 25%. Maximum likelihood analysis was inferred using PhyML v.3.0 (Guindon et al. 2010), with 1000 bootstrap replicates. An estimate of the nucleotide divergence was obtained using a 441 bp alignment from the *Hepatozoon* sp. sequences obtained in this study and *Hepatozoon* spp. sequences isolated from Squamata available in the GenBank database. This analysis was performed using *p*-distance in the MEGA 6.0 software package (Tamura et al. 2013).

RESULTS

Hepatozoon gamonts were detected in the blood samples from *C. clelia* and *D. corais*, with parasitemia values of 146 and 36 / 2,000 erythrocytes in the blood, respectively. Two different gamonts morphologies were found in each host (Figure 1, Table I).

In *C. clelia* (Figure 1a-c), gamonts were elongated and wide (14.25±0.65 × 4.31±0.43 µm; Table I), with both ends rounded and one pole more curved than the other, with an evident parasitophorous vacuole (PV), appearing as a 'halo', and a basophilic and granulous cytoplasm. The nucleus of this parasite was large and square-shaped (4.50±0.62 × 2.63±0.41 µm; Table I), eccentric and slightly displaced towards the curved end, with condensed dark stained chromatin filaments. This gamonts induce displacement of the host cell nucleus. No other cytopathological effect on host cells (dimensions: 19.05±1.15 × 10.94±0.79 µm) was noticed when compared with non-parasitized erythrocytes (dimensions: 18.00±1.25 × 9.58±0.94 µm) (Table I). For gamonts observed in *D. corais*, the morphological data are shown in the species description section below.

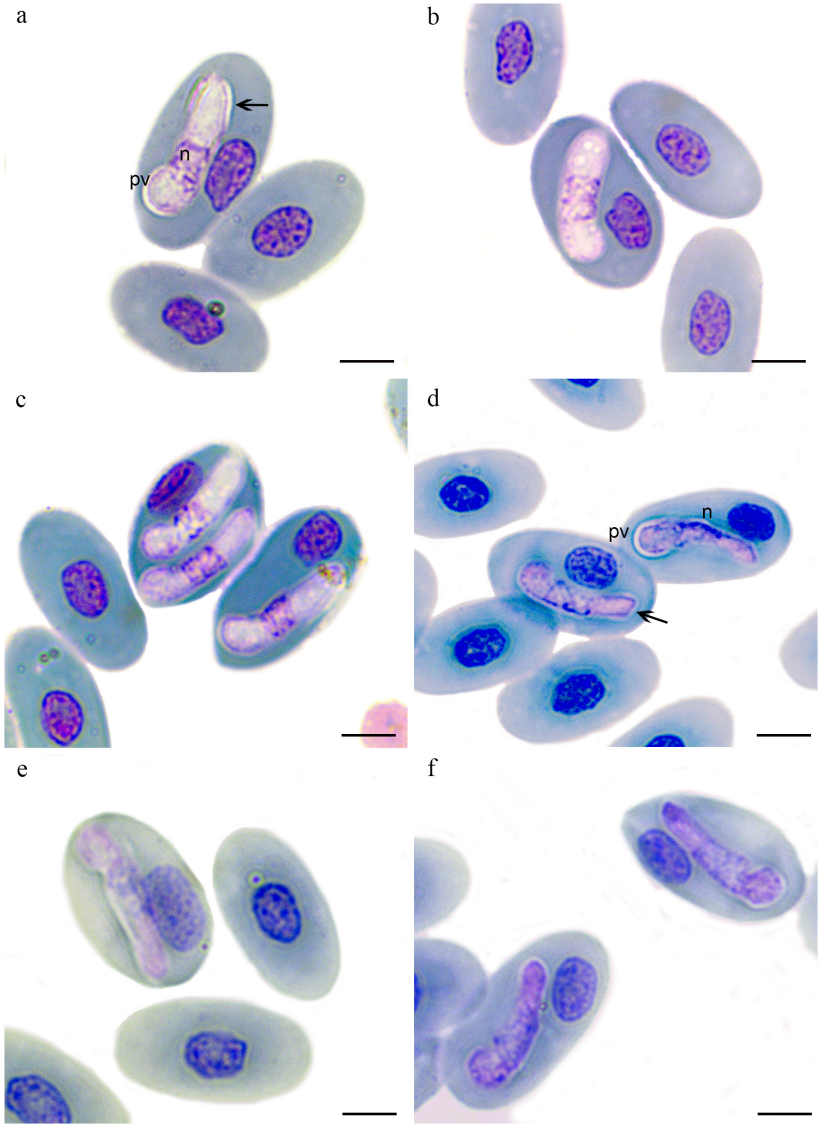

Figure 1. ***Hepatozoon*** **gamonts in the peripheral blood of snakes from the Eastern Amazon, Brazil. (a-c)** ***Hepatozoon trigeminum*** **from** ***Clelia clelia*****. (d-f)** ***Hepatozoon odwyerae*** **sp. nov., from** ***Drymarchon corais*****. Arrows indicate parasites; (n) indicates gamont nucleus; and (pv) indicates parasitophorous vacuole. Micrographs are from Giemsa-stained thin blood films. Scale bar is 10 µm.**

The blood samples from *C. clelia* and *D. corais* were also PCR-positive for *Hepatozoon* spp. In the BLAST search, the sequence obtained from *C. clelia* (OM032593) exhibited 99,60% of similarity with *Hepatozoon trigeminum* Úngari, Netherlands, Silva and O'Dwyer, 2022 (ON262424) from *Oxyrhopus trigeminus* Duméril, Bibron and Duméril, 1854, 99.02% with *Hepatozoon cuestensis* O'Dwyer, Moço, Paduan, Spenassatto, Silva and Ribolla, 2013 (KC342524) from *Crotalus durissus* Linneus, 1758, and 98.64% with *Hepatozoon* sp. (AY252108) from *Varanus scalaris* Mertens, 1941. *Hepatozoon* sp. obtained from *D. corais* (OM032594), meanwhile, exhibited 99.79% of identity with *Hepatozoon* sp. (MF497768) from the *Boa constrictor* Linnaeus, 1758, 99.61% with *Hepatozoon ameivae* (Carini and Rudolphi, 1912) (MN833642), and 98.45% with *Hepatozoon musa* Borges-Nojosa, Borges-Leite, Maia, Zanchi-Silva, Braga and Harris, 2017 (KX880079) from *Philondryas natterei* (Steindachner, 1870). The genetic distance between the two *Hepatozoon* species obtained in this study was 1.21%. The pairwise distance between *Hepatozoon* sp. from *C. clelia* and the *Hepatozoon* spp. sequences from other squamates ranged from 0 to 7.51% (Table II). The *p* distance between *Hepatozoon* sp. from *D. corais* and the other *Hepatozoon* species ranged from 0.24 to 7.51% (Table II). Nucleotide divergence among the named *Hepatozoon* species from snakes and lizards ranged 0 to 8.23% (Table II).

The phylogenetic tree (Figure 2) based on 441 bp partial sequences of the 18S rRNA gene revealed that the *Hepatozoon* sequences (OM032593; OM032594) generated in this study grouped in a clade composed of *Hepatozoon* spp. detected in snakes and lizards from Brazil. The sequence from *C. clelia* (OM032593) was positioned in a branch with the lineage of *H. trigeminum* (ON262424) (Fig. 2). In addition, *Hepatozoon* sp. (OM032594) obtained from *D. corais* clustered with *Hepatozoon* sp. (MF497768) from *B. constrictor* and formed a sister clade to *H. ameivae* from the *Ameiva ameiva* lizard (Linnaeus, 1758) (MN833642).

Table I. Morphometric analysis of *Hepatozoon* spp. gamonts and their host cells detected in colubrid snakes from the Eastern Amazonia, Brazil. Measurements are presented as mean ± standard deviation (SD) followed by the range (maximum and minimum values).

Species/ Characteristic	*N*	CL (µm)	CW (µm)	CA (µm²)	NL (µm)	NW (µm)	NA (µm²)
Hepatozoon trigeminum							
Gamont	30	14.25±0.65 (12.84–15.50)	4.31±0.43 (3.44–4.97)	54.84±6.06 (63.89–44.54)	4.50±0.62 (5.87–3.27)	2.63±0.41 (3.55–2.00)	9.86±1.97 (13.45–6.29)
Infected erythrocyte	30	19.05±1.15 (20.85–16.17)	10.94±0.79 (12.23–9.53)	167.49±14.78 (187.73–126.33)	6.53±0.79 (7.75–5.25)	4.35±0.37 (5.33–3.73)	24.30±3.43 (32.06–19.23)
Uninfected erythrocyte	10	18.00±1.25 (20.15–16.67)	9.58±0.94 (11.78–8.71)	146.04±21.54 (181.33–115.67)	6.17±0.64 (7.53–5.01)	4.26±0.44 (4.84–3.62)	23.75±1.78 (26.91–20.97)
Hepatozoon odwyerae sp. nov.							
Gamont	30	13.41±0.79 (14.33–10.26)	3.72±0.35 (4.50–3.04)	43.04±4.04 (52.44–32.51)	4.16±0.40 (4.81–3.25)	1.92±0.36 (2.95–1.21)	5.85±0.98 (8.91–4.29)
Infected erythrocyte	30	18.48±1.01 (20.61–15.79)	10.40±0.53 (11.17–9.54)	154.47±11.55 (175.11–130.00)	6.08±0.56 (7.54–5.17)	4.14±0.38 (4.88–3.33)	22.04±3.45 (30.85–16.96)
Uninfected erythrocyte	10	17.95±0.91 (19.96–16.80)	9.42±039 (10.01–8.86)	139.21±7.56 (156.04–130.64)	6.02±0.56 (6.86–5.40)	4.14±0.56 (4.99–3.40)	20.73±0.56 (25.64–15.93)

CL = cell length, CW = cell width, CA = cell area, NL = nucleus length, NW = nucleus width, NA = nucleus area.

Considering the genetic divergence, phylogenetic positions and morphological features we identified the sequence isolated from *C. clelia* as *H. trigeminum*, and we proposed that the parasite found in *D. corais* is a new species of *Hepatozoon*.

Taxonomic summary

Genus *Hepatozoon* Miller, 1908

-*Hepatozoon odwyerae* sp. nov.

Type host: *Drymarchon corais* (Boie, 1827) (Colubridae: Colubrinae), Caninana, Indigo Snake.

Other hosts: Unknown.

Type locality: Peri-urban region in the municipal district of Altamira (-03° 12' 01.8" S; -52° 11' 29.0" W), Pará, Brazil.

Other locality: Unknown.

Type material: Hapantotype (two blood slides) from *Drymarchon corais* were deposited in the collection of the Adriano Giorgi Laboratory of Zoology of the School of Biological Sciences, Altamira Campus, Pará, Brazil (LZA – 1417 and 1418).

Site of infection: Blood erythrocytes.

Prevalence: one of one individual of *Drymarchon corais* was infected.

Parasitemia: The parasitemia was 36 parasites for every 2,000 erythrocytes (1.8%).

Vector: Unknown.

Gene sequence: The 18S ribosomal gene sequences were deposited in the GenBank database under accession number OM032594.

Zoobank registration: In accordance with section 8.5 of the International Code of Zoological Nomenclature (ICZN), details of the new species were submitted to Zoobank. The Life Science Identifier (LSID) for *H. odwyerae* sp. nov. is urn:lsid:zoobank.org:pub:AB980FE4-644B-427F-8B91-DFE2C1BDA5A1.

Table II. Pairwise distances among partial 18S rDNA sequences of *Hepatozoon* spp. detected from snakes and lizards.

Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1. *H. odwyerae* sp. nov. (OM032594)																		
2. *H. trigeminum* (OM032593)	0.0121																	
3. *Hepatozoon* sp. (MF497768)	0.0024	0.0145																
4. *H. ameivae* (MN833641)	0.0024	0.0097	0.0048															
5. *H. ayorgbor* (EF157822)	0.0291	0.0218	0.0315	0.0266														
6. *H. angeladoviesae* (MG519501)	0.0678	0.0678	0.0654	0.0654	0.0678													
7. *H. annulatum* (ON262426)	0.0316	0.0243	0.0340	0.0291	0.0170	0.0728												
8. *H. bashtari* (MN497412)	0.0266	0.0194	0.0291	0.0242	0.0024	0.0678	0.0194											
9. *H. cecilhoarei* (MG519504)	0.0751	0.0751	0.0775	0.0726	0.0726	0.0169	0.0801	0.0726										
10. *H. cevapii* (KC342525)	0.0363	0.0291	0.0387	0.0339	0.0194	0.0726	0.0049	0.0218	0.0799									
11. *H. chinensis* (KF939622)	0.0242	0.0169	0.0266	0.0218	0.0048	0.0654	0.0218	0.0024	0.0702	0.0242								
12. *H. cuestensis* (KC342524)	0.0169	0.0097	0.0194	0.0145	0.0266	0.0702	0.0291	0.0291	0.0775	0.0339	0.0266							
13. *H. domerguei* (KM234646)	0.0266	0.0169	0.0242	0.0242	0.0121	0.0726	0.0267	0.0097	0.0823	0.0291	0.0121	0.0291						
14. *H. massardi* (KC342526)	0.0387	0.0315	0.0412	0.0363	0.0218	0.0751	0.0730	0.0242	0.0823	0.0024	0.0266	0.0363	0.0315					
15. *H. musa* (KX880079)	0.0169	0.0097	0.0194	0.0145	0.0315	0.0678	0.0340	0.0291	0.0751	0.0387	0.0266	0.0097	0.0291	0.0412				
16. *H. pyramidumi* (MT025290)	0.0291	0.0218	0.0315	0.0266	0.0048	0.0702	0.0218	0.0024	0.0751	0.0242	0.0048	0.0315	0.0121	0.0266	0.0315			
17. *H. quagliattus* (ON237463)	0.0678	0.0605	0.0702	0.0654	0.0654	0.0678	0.0704	0.0630	0.0751	0.0751	0.0605	0.0702	0.0702	0.0775	0.0702	0.0654		
18. *H. sipedon* (JN181157)	0.0726	0.0654	0.0751	0.0702	0.0702	0.0751	0.0752	0.0678	0.0823	0.0799	0.0654	0.0751	0.0751	0.0823	0.0751	0.0702	0.0121	
19. *H. trigeminum* (ON262424)	0.0121	0.000	0.0145	0.0097	0.0218	0.0678	0.0243	0.0194	0.0751	0.0291	0.0169	0.0097	0.0194	0.0315	0.0097	0.0218	0.0605	0.0654

Etymology: The specific name, "*odwyerae*", is a tribute to the parasitologist Prof. Lucia Helena O´Dwyer, from Universidade Estadual Paulista (Unesp), São Paulo, Brazil, in order to honor her scientific contribution to the knowledge of hemogregarines from Brazilian wildlife, especially with regard to snakes.

Description

Gamonts (Figure 1d-f, Table I): Elongated, thin and slightly curved; with one end tapered and the other rounded and flared; within a large PV; and with a purple-stained uniform cytoplasm. Nuclear chromatin usually diffuses in the cytoplasm; when condensed is elongated and with dark staining at one end or in the middle of the parasite. Gamonts measured (n = 30):

Figure 2. Consensus phylogenetic tree from Bayesian inference and maximum likelihood methods based on the 18S rRNA gene partial sequences (441bp) of *Hepatozoon* spp. isolated from *Clelia clelia* (OM032593) and *Drymarchon corais* (OM032594), and sequences deposited in in the GenBank database. Numbers at the nodes indicate posterior probabilities/ bootstrap, represented on the Bayesian Inference tree. Posterior probabilities/bootstrap lower than 50 are not shown. Sequences obtained in this study are indicated in bold.

13.41±0.79 × 3.72±0.35µm; and area 43.04±4.04 µm^2. Nucleus size (n = 30): 4.16±0.40 × 1.92±0.36 µm; and area 5.85±0.98 µm^2.

Effects on the host cell: Gamonts cause displacement of the host cell nucleus to the lateral or superior region of the cell. No other morphological effects on host cells (dimensions: 18.48±1.01 × 10.40±0.53 µm) were noted when compared to non-parasitized erythrocytes (dimensions: 17.95±0.91 × 9.42±039 µm) (Table I).

Remarks: Sambom and Seligmann (1907) described a hemogregarine in a congeneric host *Drymarchon couperi* (Holbrook, 1842) –syn., *Coluber corais couperi* Holbr. –, kept at the London Zoo. This parasite was later named as *Hepatozoon rarefaciens* (Sambon and Seligmann, 1907) and found by Ball et al. (1967) in high prevalences (n = 19/20) in specimens from North America. *Hepatozoon rarefaciens* gamonts have large and wide (15.4 x 5.5 µm) bodies, with both ends rounded, and cause remarkable hypertrophy in infected erythrocytes (Ball et al. 1967). Thus, they differ remarkably from the small gamonts of *H. odwyerae* sp. nov. (13.41 × 3.72 µm). Compared to phylogenetically closely related hemogregarine species, *H. ameivae*, *H. cuestensis*, *H. musa*, *H. trigeminum* and *Hepatozoon* sp. (MF497768), previously detected in squamates from Brazil, *H. odwyerae* sp. nov. gamonts were morphologically and morphometrically different. *Hepatozoon ameivae* gamonts are longer and wider (14.28 × 4.50 µm) than those of *H. odwyerae* sp. nov., and, as previously mentioned, this parasite visibly interacts with the host cell nucleus, which is also not seen in the *H. odwyerae* sp. nov. parasites (Picelli et al. 2020). *Hepatozoon cuestensis* and *H. musa* (O'Dwyer et al. 2013, Borges-Nojosa et al. 2017) have longer and thinner gamonts (17.05 × 3.6 µm and 18.94 × 3.76 µm, respectively) than *H. odwyerae* sp. nov. Additionally, both ends of *H. musa* gamonts are rounded and considerable curved, with the evident nuclei located in the middle of the parasite (Borges-Nojosa et al. 2017), differing from those in *D. corais* in this study. Regarding the other species found here in *C. clelia*, *H. trigeminum* has larger gamonts (14.25 × 4.31 µm) with very visible nuclei, which is not observed in *H. odwyerae* sp. nov. Finally, gamonts of the unnamed *Hepatozoon* sp. (MF497768) from the snake *B. constrictor* possess similar dimensions (13.3 × 4.6 µm) with *H. odwyerae* sp. nov., but in contrast to the latter both ends are rounded, there is a large and condensed nucleus, and their presence does not lead to displacement of the erythrocyte nucleus (Úngari et al. 2018).

DISCUSSION

To the best of our knowledge, this is the first study to combine molecular and morphological data to identified two *Hepatozoon* species, *H. trigeminum* an *H. odwyerae* sp. nov., in *C. clelia* and *D. corais* snakes. These findings reinforce the fact that snakes are unique hosts for *Hepatozoon* parasites, and how much we have yet to learn about this diversity (Smith 1996, Poulin 2014). So far, in Brazil, there were previously only seven named species of *Hepatozoon* spp. from snakes with available sequences (*H. cevapii*, *H. cuestensis*, *Hepatozoon massardii* O'Dwyer, Moço, Paduan, Spenassatto, Silva and Ribolla, 2013, *H. musa, Hepatozoon quagliattus* Úngari, Netherlands, Silva and O´Dwyer, 2021, *Hepatozoon annulatum* and *Hepatozoon trigeminum* Úngari, Netherlands, Silva and O'Dwyer, 2022), with *H. cevapii* the only one registered in the Amazonian biome (Paula et al. 2021). Indeed, including our new sequences, this represents 20% (n = 8/40) of species morphologically described in Brazil (Smith 1996, Úngari et al. 2022), a contradictory situation considering the modern molecular technologies and the improvements in

parasitological research (Morand 2018, Selbach et al. 2019).

Other studies had already detected the presence of *Hepatozoon* parasites in both host species, but only using light microscopy techniques. Hemogregarines were reported in *C. clelia* in Costa Rica (Moreno & Bolanos 1977) and in French Guiana (Thoisy et al. 2000), with prevalences of 29% (n = 2/7) and 100% (n = 3/3), respectively. However, in the Costa Rica study, gamont description was carried out based on morphological group without specifying which morphology was observed in *C. clelia* (Moreno & Bolanos 1977). In the survey carried out in French Guiana, however, no information related to the morphology of the parasite was provided (Thoisy et al. 2000). Lutz (1901), meanwhile, was the first to mention a hemogregarine parasitizing *D. corais* and other ophidians in Brazil, at the time he named *Drepanidium serpentium* Lutz, 1901, and which was later revised by Smith (1996) to *Hepatozoon serpentium* ([Lutz, 1901] Sambon, 1907). Nevertheless, when carefully analyzing Lutz's study (1901), it is difficult to be certain about the validity of this species, or compare it with *H. odwyerae* sp. nov., as despite the morphological data presented, the author considered different gamont morphologies, obtained from several host species, as a single species, and did not mention which of the gamonts was seen in the blood from *D. corais*. Thus, in these studies, there are unfortunately not enough data to allow comparisons with *H. trigeminum* and *H. odwyerae* sp. nov.

Here we provide the first record of *H. trigeminum* in a new host species, *C. clelia*, at relatively high parasitemia level (7.3%). This hemogregarine species was recently described by Úngari et al. (2022) infecting, with a parasitemia of 1.4%, a single specimen of the *O. trigeminus* snake from municipality of Cocalinho, State of Mato Grosso, in Brazilian Midwest. *Hepatozoon* parasites are widely recognized for their low specificity to vertebrate hosts (Paula et al. 2021, Úngari et al 2022), so it is not unexpected to find *H. trigeminium* in two different host species. *Clelia clelia* and *O. trigeminus* are Dipsadinae snakes belonging to the Pseudoboini tribe and are phylogenetically closely related (Pyron et al. 2013), in addition their geographic distribution in Brazil overlaps in some localities, as in the region where the two studies were carried out (Nogueira et al. 2019, Costa et al. 2022). This may make it easier for different species to share potential vectors and potential prey (paratenic hosts), which could aid *H. trigeminum* in switching hosts successfully.

According to the morphological description made by Úngari et al. (2022), the *H. trigeminum* forms observed in the blood of *C. clelia* resemble immature gamonts, although in our study the parasites are slightly smaller in their average dimensions (this study: body 14.25 × 4.31 µm, and nucleus 4.50 × 2.63 µm; Úngari et al. (2022): body 14.53 × 4.71 µm, and nucleus 4.63 × 4.57 µm). Furthermore, the authors describe the nucleus of this parasitic form as oval and here, according to our perception, it appears to be more square-shaped. However, these variations are very subtle and may be related to the number of parasites measured (this study: 30; Úngari et al. (2022): 25), the tools used in both studies, the subjective effect of the observer performing the description, or due gamont plasticity in different host species (Perkins et al. 2011, Paula et al. 2021, Úngari et al. 2022).

Regarding the natural history of *C. clelia* and *D. corais*, both species are widely distributed through South America. In Brazil, however, *C. clelia* occurs mainly in the Amazonian region, while *D. corais* was recorded in all ecoregions, except the Araucaria Forest and Pampas Grasslands (Nogueira et al. 2019). The mussurana *C. clelia* is a large Dipsadinae snake, reaching up

to 2.3 m in length, and is primarily terrestrial and nocturnal, inhabiting forested areas (Gaiarsa et al. 2013). Although it is considered a generalist species (Alencar et al. 2013), it is well known for its preference for feeding on other snakes (ophiophagy), including highly venomous species (i.e., *Bothrops* spp.) (Delia 2009, Fraga et al. 2013). The indigo snake *D. corais*, meanwhile, is a diurnal Colubrinae species with semi-arboreal habits, found in forests and open areas, with few studies suggesting that it has a generalist diet, preying mainly on anurans and eventually other snakes (Bernarde & Abe 2010, Prudente et al. 2014, Pelegrini et al. 2019). Despite other items in the diet of these snakes (frogs, lizards, and small mammals) (Gaiarsa et al. 2013, Prudente et al. 2014), which are the most indicated paratenic hosts of *Hepatozoon* (Lainson et al. 2003, Paperna & Lainson 2004, Perles et al. 2019), ophiophagy reveals other possible routes of infection, with snakes also potentially acting in the trophic transmission of the parasite. This hypothesis requires testing, but may be plausible, considering the high diversity and low specificity of hemogregarines (Smith 1996, Paula et al. 2021), the lack of elucidated life cycles of these parasites, and also due to the various ophiophagus vertebrate species (Pelegrini et al. 2019), including other snakes recorded as being parasitized by *Hepatozoon* in Brazil (Pessôa et al. 1974, Borges-Nojosa et al. 2017), such as *Erythrolamprus aesculapii* (Linnaeus, 1758), *Hydrodynastes gigas* (Dúmeril, Bibron and Dúmeril, 1854), *O. trigeminus* and *Philodryas nattereri* (Steindachner, 1870) (Marques & Puorto 1994, López & Giraudo 2004, Coelho-Lima et al. 2020, Sales et al. 2020).

The phylogenetic assessment in the present study found that sequences of *H. odwyerae* sp. nov. and *H. trigeminum* were clustered into the "non-carnivorous" major clade along with other *Hepatozoon* lineages from herpetofauna (Zechmeisterová et al. 2021). These new species were positioned in a well-supported monophyletic group composed exclusively of lineages isolated from Squamata sampled in different Brazilian biomes. In our study this clade maintained a similar topology to previous analyzes (Paula et al. 2021, Úngari et al. 2022) with the formation of three subclades: (i) a small cluster formed by two lineages, the sequences of *H. cuestensis* in *C. durissus* from the Cerrado (O'Dwyer et al. 2013, Úngari et al. 2018), plus *H. musa* in *C. durissus* and *P. nattereri* from the Cerrado and Caatinga, respectively (Borges-Nojosa et al. 2017, Úngari et al. 2018); (ii) a subclade consisting of *Hepatozoon* sp. in *B. constrictor* from the Cerrado (Úngari et al. 2018) plus *H. odwyerae* sp. nov. in *D. corais* from the Amazonia (the present study), and, closely related to them, the lineage of *H. ameivae* in *A. ameiva* from the Amazonia (Picelli et al. 2020); and (iii) the small clade containing the sequences of *H. trigeminum* in *C. clelia* and *O. trigeminus* from the Amazonia (the present study) and Cerrado (Úngari et al. 2022), respectively, which comprises a sister taxon to all other lineages within this group. This may be the result of a possible biogeographic pattern (Harris et al. 2015, Perles et al. 2019); however, a larger sampling of hemogregarines is needed to test such a hypothesis. Furthermore, phylogenetic relationships among reptilian hemogregarines remain poorly understood.

With respect to second subclade, in addition to being phylogenetically close, *H. odwyerae* sp. nov. and *Hepatozoon* sp. (MF497767) exhibited low nucleotide divergence (0.24%), which indicates that this new sequence may be a haplotype of the lineage detected by Úngari et al. (2018). These authors suggest that *Hepatozoon* sp. (MF497767) from *B. constrictor* may be considered a new putative species, although despite presenting morphological and genetic

data, they did not describe it taxonomically. However, this small *p* distance is similar to the divergence observed between *H. cevapii* and *H. massardii,* which are closely related and considered two distinct species (O'Dwyer et al. 2013, Úngari et al. 2018, 2021, Paula et al. 2021). In addition to the morphological differences between *H. odwyerae* sp. nov. and *Hepatozoon* sp. (MF497767), highlighted in the remarks section, there are recognized limitations to the use of the 18S rRNA genetic marker to distinguish different species, especially when there is low molecular divergence (Gutiérrez-Liberato et al. 2021, Hrazdilová et al. 2021, Léveillé et al. 2021). At the present time, then, we cannot confirm that the parasite found by Úngari et al. (2018) belongs to the same species as that described in the present study.

In conclusion, the diversity of hemogregarines from snake hosts in Brazil is still greatly underestimated, especially in the Amazon region. The present study provides the first molecular detection of *Hepatozoon* spp. from *C. clelia* and *D. corais*, and the combination of this data with morphological traits allowed us to describe a new species and register a new host species from a new geographic location. Further studies with *H. trigeminum* and *H. odwyerae* sp. nov. should include other molecular markers to disentangle the puzzle of their phylogenetic relationships with other hemogregarines, especially in relation to the closeness between *H. odwyerae* sp. nov. and *Hepatozoon* sp. from *B. constrictor.* It is also important to try to identify vectors and explore hypotheses about transmission routes in ophiophagus snakes.

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AMP and LAV conceived and designed the study. EAO and EJHR performed the fieldwork. JKCC, GRP and FRP processed the data and performed the microscopic analysis. MRLS performed the molecular analysis. AMP interpreted the results and worked on the manuscript. LAV contributed to critical reading of the manuscript and supervised the findings of this work. All authors took part on the preparation, revised, and approved the final version of the manuscript.