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Molecular and morphological description of the first *Hepatozoon* (Apicomplexa: Hepatozoidae) species infecting a neotropical turtle, with an approach to its phylogenetic relationships

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Research Article

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Abstract

Haemogregarines (Adeleorina) have a high prevalence in turtles. Nevertheless, there is only one *Hepatozoon* species described that infects Testudines so far; it is *Hepatozoon fitzsimonsi* which infects the African tortoise *Kinixys belliana*. Colombia harbours a great diversity of chelonians; however, most of them are threatened. It is important to identify and characterize chelonian haemoparasite infections to improve the clinical assessments, treatments and the conservation and reintroduction programs of these animals. To evaluate such infections for the Colombian wood turtle *Rhinoclemmys melanosterna*, we analysed blood from 70 individuals. By using the morphological characteristics of blood stages as well as molecular information (18S rRNA sequences), here we report a new *Hepatozoon* species that represents the first report of a hepatozoid species infecting a semi-aquatic continental turtle in the world. Although the isolated lineage clusters within the phylogenetic clades that have morphological species of parasites already determined, their low nodal support makes their position within each group inconclusive. It is important to identify new molecular markers to improve parasite species identification. In-depth research on blood parasites infecting turtles is essential for increasing knowledge that could assess this potential unknown threat, to inform the conservation of turtles and for increasing the state of knowledge on parasites.

Introduction

Among Haemogregarines, those belonging to the *Hepatozoon* genus (Miller, 1908) are the most common parasites reported infecting mammals (Wenyon, 1926; Clark, 1958; Desser, 1990), birds (Hoare, 1924; Merino *et al.*, 2014; Valkiūnas *et al.*, 2016), amphibians (da Costa *et al.*, 1973; Netherlands *et al.*, 2018) and reptiles-like snakes (Ball *et al.*, 1967; Smith *et al.*, 1994), lizards (Mackerras, 1962; Desser, 1997) and crocodiles (Carini, 1909; Soares *et al.*, 2017); but they are rarely seen in chelonians (Cook *et al.*, 2009). Haemogregarines are heteroxenous coccidians, their life cycle involves blood-sucking invertebrate vectors (fleas, ticks and mosquitoes, etc.) where sexual development occurs and various vertebrates are intermediate hosts, where the merogonic and gamontogonic cycle take place (Smith, 1996; Telford, 2008) .

Hepatozoon is a highly diverse group of parasites that has been described in almost all vertebrates around the globe (Smith, 1996; Telford, 2008) . However, the description and characterization of these organisms are not exempt from difficulties. At the morphological level, descriptions are based on the traits of the gamonts that frequently are the only visible structures in the blood films. Nevertheless, at this stage, only a few morphologic characters are available that are often poorly distinctive (Ball, 1967; Telford, 2008) ; Cook *et al.*, 2014; Dvořáková *et al.*, 2015; Hayes and Smit, 2019). Also, since for some species, there is little knowledge on the development of the parasite, it may occur that early stages that are rare in blood films might be mistakenly taken as gamonts of different species (Smith, 1996). Therefore, for species identification, some authors have recommended that when characterizing the development of the parasites throughout their life cycle, it is good to include morphological characters from other stages different from gamonts (Ball *et al.*, 1967; Smith, 1996; O'Dwyer *et al.*, 2013).

To improve our understanding of the phylogenetic relationships of Adeleorina parasites, genetic information has been included using the 18S rRNA (Barta *et al.*, 2012; Maia *et al.*, 2016). This molecular marker has a slow rate of evolution; hence it is widely used for the reconstruction of deep phylogenetic relationships at the higher taxonomic levels such as classes or orders (Hwang and Kim, 1999). Notwithstanding the above, for this particular case and using this gene, the phylogenetic relationships within the adeleorinid parasites have been mostly clarified (Barta *et al.*, 2012; Karadjian *et al.*, 2015). In fact, the analysis using these

sequences have shown that *Hepatozoon* is a paraphyletic group that includes some species of other genera such as *Karyolysus* (Karadjian *et al.*, 2015; Cook *et al.*, 2016) and it is closely related to *Hemolivia* (Kvičerová *et al.*, 2014). This suggests that the scope of the marker for this group could be slightly broader, providing information for the resolution of the genus or even species (Cook *et al.*, 2015; Borges-Nojosa *et al.*, 2017; Netherlands *et al.*, 2018). In the course of the last decades, the genetic information from the sequences of 18s rRNA has not only allowed the delimitation of some species but has played an important role in genus reassignment for at least two of the parasites described in the Testudines: *Haemogregarina parvula,* which was assigned to the *Hemolivia* (Cook *et al.*, 2015) and *Heamogregarina fitzsimonsi,* which after being placed in the *Hepatozoon* clade becomes the only species of this genus described as infecting chelonians (Cook *et al.*, 2009).

During the past decade, many studies have characterized the apicomplexan parasites that infect reptiles of tropical regions of South America (i.e. O'Dwyer *et al.*, 2013; Pineda-Catalan *et al.*, 2013; Borges-Nojosa *et al.*, 2017; Matta *et al.*, 2018; Úngari *et al.*, 2018; González *et al.*, 2019). Despite this, knowledge about Haemogregarines, particularly in Testudines, is still scarce. Here, we explore the hemoparasites associated with *Rhinoclemmys melanosterna*, a semi-aquatic turtle inhabiting forested areas in the presence of lentic water bodies (Rueda-Almonacid *et al.*, 2007). The turtle is distributed from the east coast of Panama, part of the Caribbean coast of Colombia, following the course of the Magdalena River to the Middle Magdalena region and throughout the Pacific coast region to north western Ecuador (Rueda-Almonacid *et al.*, 2007). Although the species is currently not included in any threat category either globally or in Colombia, the implications of the deep phylogeographic structure revealed for the species by Vargas-Ramirez *et al.* (2013), suggest the presence of seven evolutionary significant units (ESU) that should have conservation status.

The goals of this study were (i) to perform the morphological description and molecular characterization of the first hepatozoid species parasitizing a continental turtle in the neotropics; and (ii) to elucidate the phylogenetic relationship with other species of the genus and other adeleorinid coccidias, while discussing the suitability of the use of morphological characters and 18s rRNA sequences for the description of new species. Additionally, we discuss advances in the use of new molecular markers for species identifications of *Hepatozoon*. This study registered a new host and increases the knowledge about parasitic fauna that infect turtles in Colombia, which has been poorly studied, despite the ecological importance of its charismatic hosts. In this sense, we expect this new information to be useful for the identification of unknown threat factors that should be taken into account in the generation of conservation strategies for Testudines.

Materials and methods

Sample collection and blood film examination

Analysed samples were obtained from individuals captured in the wild, as well as individuals held in captivity in rescue animal centres (Fig. 1, Table 1). Wild turtles were sampled from eight sites in four departments in their natural range of distribution (Fig. 1). At all localities, turtles were found near bodies of water (i.e. swamps, lagoons and marshes) inside of or near forested areas. Forty-six tissue samples from those individuals for polymerase chain reaction (PCR) identification procedures came from the Banco de ADN y Tejidos de la Biodiversidad (BTBC), of the Genetics Institute, Universidad Nacional de Colombia. Meanwhile, captive individuals were sampled from two places outside their range of distribution; the animals had been seized from illegal wildlife trafficking. The turtles sampled in the Unidad de Rescate y Rehabilitación de Animales Silvestres (URRAS) in Bogotá at 2600 meters above sea level (m.a.s.l), were kept in plastic pools inside rooms with controlled environmental conditions. The average temperature of the enclosure was 30°C with a 12/12 photoperiod. The turtles from the Estación de Biología Tropical Roberto Franco (EBTRF) in Villavicencio at 459 m.a.s.l., animals were held in artificial ponds, surrounded by vegetation; and the temperature varied from 24 to 26°C, and the relative humidity ranged between 79 and 95%.

From all individuals, about 1 mL of blood was taken from the subcarapacial venous sinus and did not exceed 1% of the body weight. Three thin blood films were made and the remaining blood sample was stored in ethanol 96% for further molecular analyses. The blood films were quickly dried using a fan, fixed with methanol for 5 min and stained with Giemsa at 4% as proposed by Rodríguez and Matta (2001).

The blood films obtained from the individuals sampled in Yondó (Antioquia) and URRAS (Bogotá) were analysed using an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan) at a magnification of 100 × and photographs were taken using the Olympus DP27 integrated camera and the CellSens software (Olympus Corporation, Tokyo, Japan). The Haemogregarines were identified to genus using morphological and morphometric characteristics (Telford, 2008); Cook *et al.*, 2015; Javanbakht *et al.*, 2015). Parasitaemia was established by the relationship between the number of infected erythrocytes in a total of 10 000 screened erythrocytes. This resulted from the close observation of 100 optical fields at 1000 magnification.

DNA extraction and 18s rRNA amplification

DNA was extracted from blood samples preserved in absolute ethanol using the DNeasy Blood and Tissue extraction kit (QIAGEN, Hilden, Germany). 18S rRNA gene amplification was performed using the primers set HepF300/HepR900 (Ujvari *et al.*, 2004) to obtain a fragment of approximately 600 base pairs (bp). The original protocol was modified by adding five cycles with an annealing temperature of 50°C for 45 s prior to the 35 cycles of amplification that were indicated. The PCR products were visualized in a 1.5% agarose gel, cleaned using differential precipitation with ammonium acetate protocol (Bensch *et al.*, 2000) and sequenced in both directions using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA).

Phylogenetic analysis

Three alignments using sequences of different lengths were generated to estimate the phylogenetic relationships of the new *Hepatozoon* species described here, as well as the lineages of *Haemogregarina* sp. reported in this study. All databases included 59 sequences of 18s rRNA of *Hepatozoon* (43 lineages), *Hemolivia* sp. (5), *Karyolysus* sp. (1), *Haemogregarina* sp. (8, including lineages here reported) and *Dactylosoma* sp. (2, used as outgroup). In the first alignment, full-size sequences up to 1800 bp were analysed while in the second and third databases, lineages of 1000 and 585 bp, respectively, were used (Table 2). Such alignments were constructed in MEGA 7 (Kumar *et al.*, 2016) and were aligned with MAFFT (Katoh *et al.*, 2002), available at https://www.ebi.ac.uk/Tools/msa/mafft/.

The phylogenetic reconstructions were estimated using both Bayesian inference (BI) as well as Maximum Likelihood (ML). The BI analyses were carried out using MrBayes version 3.1.2 (Ronquist *et al.*, 2012), and implemented in the platform CIPRES Science Gateway V 3.3 (Miller *et al.*, 2010). These analyses were performed under the general time-reversal model (GTR + I + G) suggested by jModelTest 2.1.1 (Darriba *et al.*,

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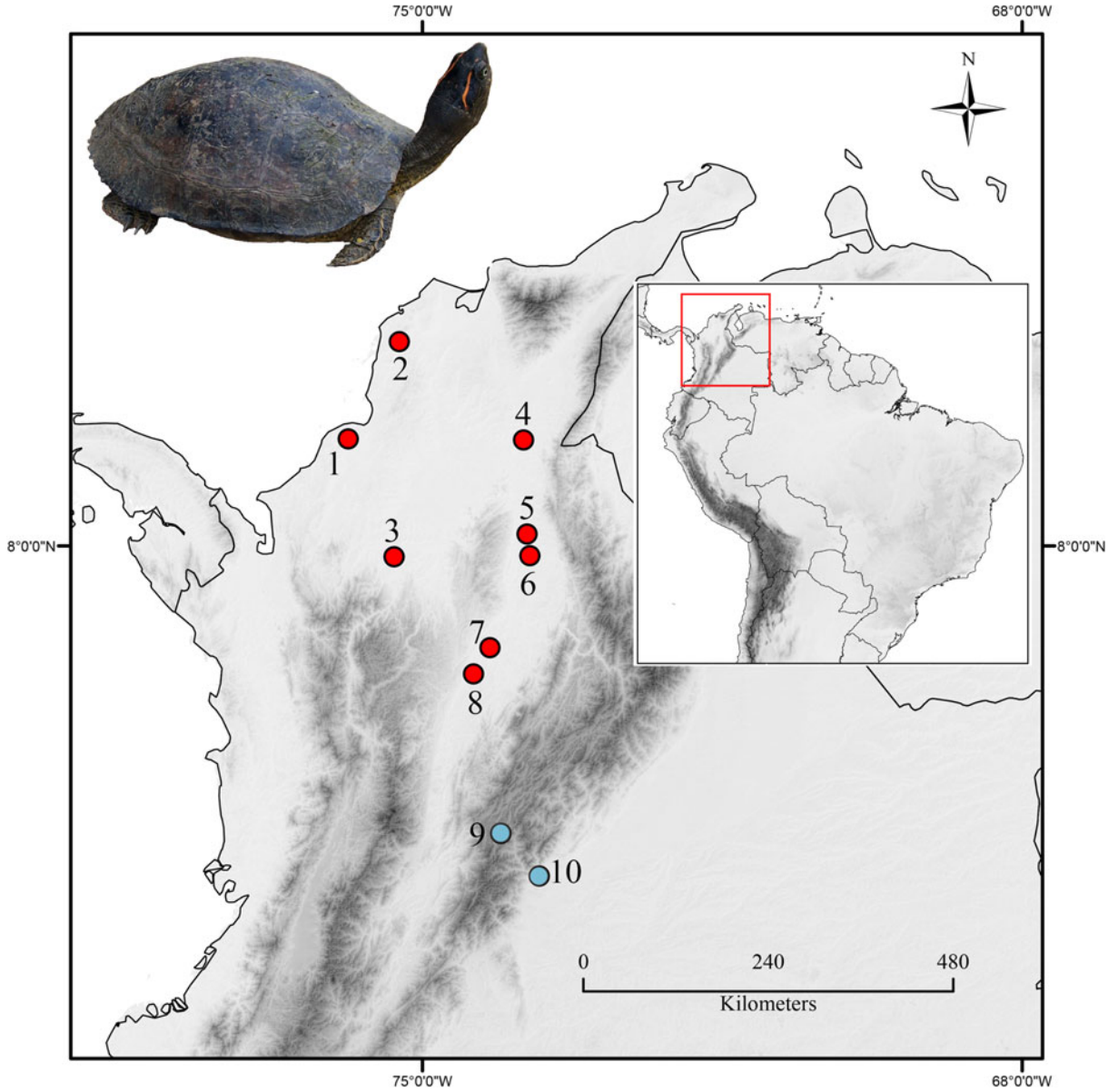

Fig. 1. The geographical location of the sampling places. Names and coordinates are provided in Table 1, according to the numbering. Red dots (dark grey in printed version) indicate the places where free-living turtles were captured and sampled and the blue dots (Light grey dots) correspond to rescue animal centres. Inset Photo: Female *Rhinoclemmys melanosterna* from Arjona, Bolivar.

2012) as the best of 88 models according to the corrected information criterion of Akaike (AIC). For BI two independent Markov Chain Monte Carlo (MCMC) simulations were run simultaneously; using 5×10^6 generations sampled every 500 generations. Convergence was reached when the average standard deviation of the posterior probability was less than 0.01 and was also assessed using the software Tracer v1.6 (Rambaut *et al.*, 2013). After discarding 25% of the trees as burn-in, 37 500 trees were used to build the majority rule consensus tree, which was visualized and edited using FigTree version 1.3.1 (Rambaut and Drummond, 2010).

The ML analyses were performed using the software PhyML 3.0 (Guindon *et al.*, 2009) using the same model mentioned above, leaving the 'estimated' option for the proportion of invariable sites and the gamma shape parameter. In this phylogenetic analysis nodal supports were calculated using 1000 bootstrap replicates.

Genetic distances between taxa were calculated for both alignments, whereas between and within genera were estimated only for the first alignment using the Kimura two-parameter substitution model implemented in the software MEGA 7 (Kumar *et al.*, 2016).

Results

Sample collection and blood film examination

Samples of 70 turtles *R. melanosterna* were screened for blood parasites. Although PCR tested all individuals, only 24 had a blood smear available for microscopic analysis. The other 46 samples came from the BTBC, which has a different purpose and does not collect blood films (Fig. 1, Table 1). Eight samples were positive (overall prevalence: 11.42%) four of them were screened by microscopy, but all the samples were positive by PCR. Seven infected with *Haemogregarina* sp. (prevalence: 10%) and one that corresponds to a single infection of the new *Hepatozoon* species (prevalence: 1.42%).

Taxonomic summary

Suborder: Adeleorina Léger, 1911
Family: Hepatozoidae Wenyon, 1926
Genus: *Hepatozoon* Miller, 1908
Hepatozoon (*simidi* sp. nov

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Table 1. Localities and report of infection of the studied individuals of the Colombian wood turtle *Rhinoclemmys melanosterna*

Locality	Coordinates		$n(n_{mic})$	*Hepatozoon* sp.	*Haemogregarina* sp.
	N	*W*			
Antioquia					
3. Caucasia[a]	7.87128	−75.327	10 (0)	0	0
8. Puerto Berrio[a]	6.5002	−74.399	1 (0)	0	0
7. Yondó[a]	6.8057	−74.206	4 (4)	1*	3
Bolívar					
2. Arjona[a]	10.267	75.336	12 (0)	0	0
Cesar					
4. Chimichagua[a]	7.87128	−75.327	1 (0)	0	0
6. Terraplen[a]	7.88283	−73.744	7 (0)	0	0
5. Loma Corredor[a]	8.1358	−73.775	2 (0)	0	0
Cordoba					
1. Lorica[a]	9.2442	−75.864	10 (0)	0	4
Cundinamarca					
9. Bogotá Unidad de Rescate y Rehabilitación de Animales Silvestres (URRAS)[b]	4.6397	−74.083	13 (13)	0	0
Meta					
10. Villavicencio Estación de Biología Tropical Roberto Franco (EBTRF)[b]	4.14009	−73.634	10 (7)	0	0
Total			70 (24)	1	7
Prevalence by genus				1.42	10
Overall prevalence of infection	11.42				

N, total number of samples; n_{mic}, number of samples examined by microscopy; *, individual infected with *H. simidi* sp. nov.
aLocalities where the turtles were captured from the wild.
bAnimal rescue centres where animals were held in captivity.

Type host: *Rhinoclemmys melanosterna* Gray, 1861 (Geoemydidae) Colombian wood turtle.

Type locality: free-living environment in 'El Silencio' natural reserve (6.8057N, −74.206W), Middle Magdalena river valley rain forest, municipality of Yondó, Antioquia, Colombia.

Type material: Hapantotype, three blood smears from *R. melanosterna* were deposited at the biological collection 'Grupo de Estudio Relación Parásito Hospedero' (GERPH), at the Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia.

Site of infection: mature erythrocytes

Prevalence: One individual was positive (1.42%) for *Hepatozoon simidi* sp. nov.

Parasitemia: the parasitemia for *Hepatozoon simidi* sp. nov, was 0.68%.

Distribution: This species was found only in the type locality.

Vector: Unknown.

DNA sequences: the 18S rRNA lineage RM4 (585 bp) obtained from type host *R melanosterna* was deposited in GenBank under accession N°MT754271.

Etymology: The species name refers to the word 'simidi', which is used by the 'Embera' native group to name 'turtle.' These native people live in a part of the geographical area of Colombia, where *R. melanosterna* is found.

Description of blood stages

The morphology found reflects different stages of development of the parasite. *Immature gamonts* (Fig. 2A–H) are cylindrical with a straight central axis and rounded ends, or slightly curved 'bean-like' (Fig. 2E–H). Interestingly, it should be noted that 100% of the gamonts cause the host cell nucleus to be pushed aside. A capsule may surround the parasite (Fig. 2B, E, H); the pale-blue cytoplasm has a granular appearance and sometimes possesses fine vacuoles and granules of different sizes (Fig. 2C, D, G, H). A round vacuole is often seen at one end of the parasite (Fig. 2A and D). Uncondensed chromatin is observed at the central (Fig. 2A) or subcentral position (Fig. 2B).

Mature gamonts, this stage shows larger parasites (Fig. 2I–L; Table 2), which causes a great deformation of infected cells. The chromatin is condensed, and the nucleus of RBC is displaced from a central position to a lateral position (Fig. 2J) or polar position (Fig. 2L) or even expelled from the host cell (Fig. 2I). The parasite shows an intense blue-stained cytoplasm, irregular in appearance. Multiple pigment granules with variable affinities for the dye from pink to purple (Fig. 2J and L) are mainly distributed around the parasite nucleus (Fig. 2K) but can also be found dispersed throughout the entire body of the gamont (Fig. 2I and L). In at least 70% of the mature parasites, clear space between the parasite and RBC's cytoplasm is observed; it could be a capsule or parasitophorous vacuole (Fig. 2K, L).

Remarks

To date, *Hepatozoon fitzsimonsi* is the only parasite that has been found parasitizing chelonian hosts (Cook *et al.*, 2009). The species described here is the second *Hepatozoon* species reported in a cryptodiran turtle species and the first species reported in a freshwater turtle species of the family Geoemydidae. Despite the phylogenetic proximity with *H. fitzsimonsi* hosts, gamonts of *H.*

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Table 2. Morphometric measurements of gamonts and host cells of *Hepatozoon simidi* sp. nov. Measurements of *H. fitzsimonsi*, *H. colubri* and *H. rarefaciens* are provided for comparison

	Hepatozoon simidi sp. nov *Rhinoclemmys melanosterna* (Geoemydidae)	*Hepatozoon fitzsimonsi Kinixys belliana* (Testudinidae)[a]	*Hepatozoon colubrid Python reticulatus Erythrolamprus aesculapii* (Squamata, Pythonidae, Colubridae)[b,c].	*Hepatozoon rarefaciens Drymarchon corais* (Squamata, Colubridae)[d]
Uninfected erythrocytes				
Cell area	145.96–190.2 (158.49 ± 14.16)			
Cell length	15.99–18.14 (17.216 ± 0.774)			17
Cell width	10.33–12.80 (11.045 ± 0.789)			10
Nucleus area	15.64–26.99 (20.674 ± 3.875)			
Nucleus length	3.94–5.88 (5.046 ± 0.620)			
Nucleus width	4.38–5.63 (4.985 ± 0.457)			
Cell area	194.11–244.82 (226.7 ± 7.19)			
Cell length	21.16–23.24 (22.32 ± 0.76)			
Cell width	11.17–12.79 (12.04 ± 0.58)			
Nucleus area	22.23–25.13 (23.732 ± 1.23)			
Nucleus length	3.62–6.98 (4.456 ± 1.42)			
Nucleus width	3.67–7.33 (6.031 ± 1.39)			
Immature gamonts	$n = 26$	$n = 12$		
Host cell-parasite complex				
Area	194.8–291.0 (229.72 ± 10.82)			
Length	19.59–24.97 (21.77 ± 1.57)			
Width	10.70–16.27 (12.43 ± 1.81)			
Parasite				
Parasite area	79.08–126.08 (104.02 ± 11.63)			
Parasite length	15.17–19.20 (16.97 ± 1.08)	14.3–19.6 (17.8 ± 1.2)	5–6[b]	11–22 (15.4)
Parasite width	6.27–8.75 (7.11 ± 0.51)	1.6–3.0 (2.3 ± 0.4)		3–10 (5.5)
Parasite nucleus length	3.61–6.99 (5.88 ± 0.75)	1.4–2.8 (2.0 ± 0.4)		
Parasite nucleus width	3.61–6.51 (4.82 ± 0.84)	0.7–1.0 (0.9 ± 0.1)		
Mature gamonts	$n = 34$	$n = 36$		
Host cell-parasite complex				
Area	207.35–314.43 (262.30 ± 12.85)			
Length	21.13–25.75 (23.39 ± 0.56)			
Width	11.73–14.48 (13.28 ± 0.63)			
Parasite				
Parasite area	90.83–129.29 (110.13 ± 7.50)			
Parasite length	15.85–18.70 (17.42 ± 0.59)	17.1–17.7 (17.5 ± 0.3)	9–9.5[b]/15–17[c]	
Parasite width	6.64–8.05 (7.25 ± 0.36)	3.3–4.3 (3.9 ± 0.5)	3.8–4.7[c]	
Parasite nucleus length	3.05–5.12 (3.98 ± 0.42)	4.5–5.0 (4.8 ± 0.3)		
Parasite nucleus width	4.99–7.51 (6.04 ± 0.49)	2.4–3.2 (2.9 ± 0.4)		

Measurements are given in μm or μm^2. Minimum and maximum values and mean ± S.D. are provided.
[a]According to Cook *et al.* (2009).
[b]According to Börner (1901).
[c]According to Han *et al.* (2015).
[d]According to Ball *et al.* (1967).

simidi sp. nov are similar to *Hepatozoon rarefaciens*, a parasite that has been found infecting colubrid snakes in Canada. However, *H. rarefaciens* gamonts are slightly shorter and slender than *H. simidi* sp. nov (Table 3). Unfortunately, there are no genetic lineages from *H. rarefaciens* available for comparison.

Hepatozoon simidi sp. nov can distinguish from *H. fitzsimonsi* and other *Hepatozoon* species by combining their morphological features with the nuclear molecular marker's information. For this species, it is noteworthy that the mature gamonts' width is almost twice the width of most of the genus species already described;

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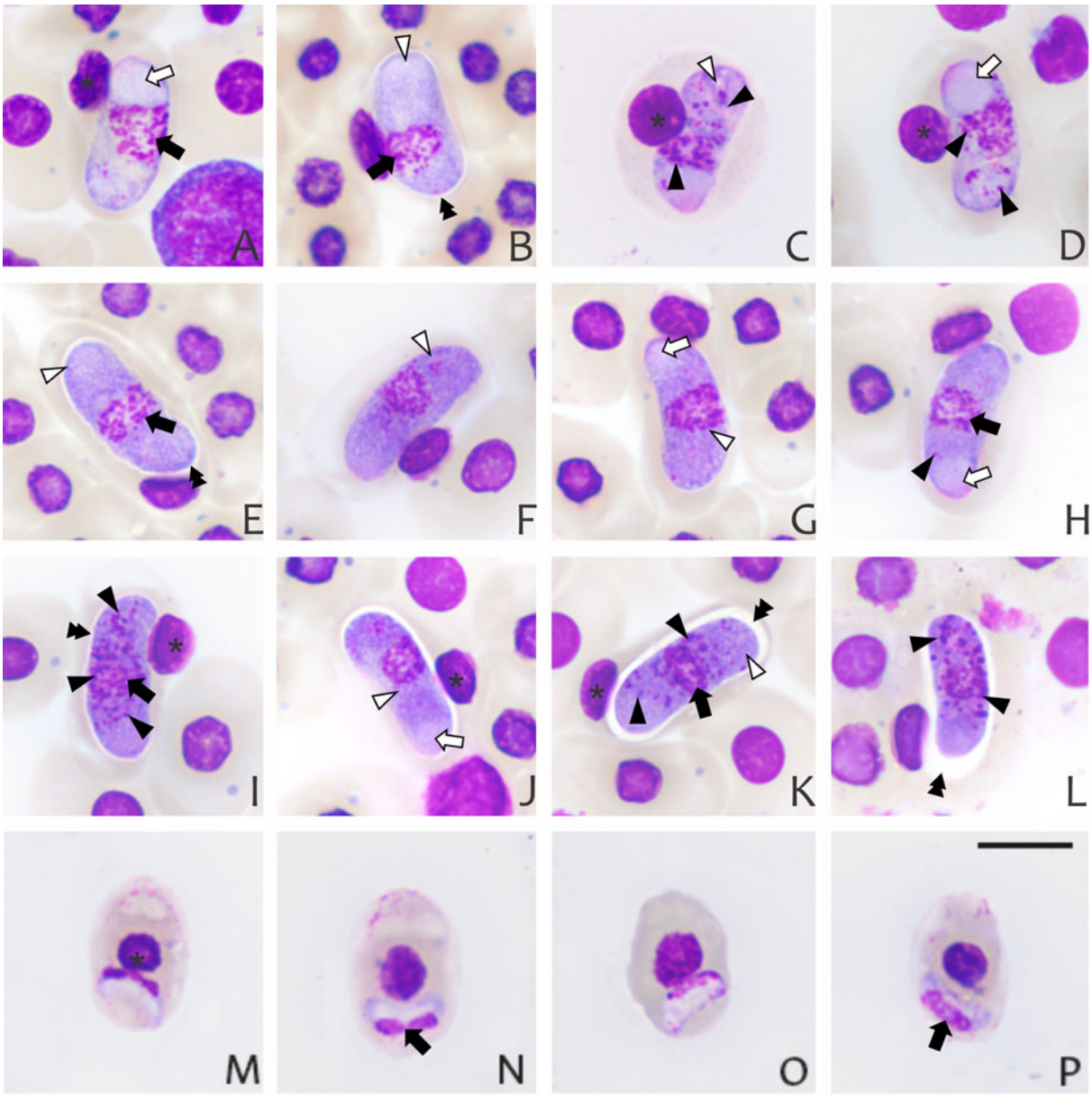

Fig. 2. *Hepatozoon simidi* sp. nov. (A–L) and *Haemogregarina* sp. (M–P) found in the Colombian Wood Turtle (*Rhinoclemmys melanosterna*). Young gamonts (A–H), and mature gamonts (I–L) of *Hepatozoon simidi* sp. nov. from the bloodstream of the type host. Black arrows indicate the parasite nucleus, whereas the white arrows show the vacuole-like patches at the tip of parasite structures. Black arrowheads indicate the granules and white arrowheads, the tiny vacuoles in the cytoplasm. Asterisks are located over the host cell nucleus. Giemsa-stained blood films. Scale bar = 10 μm.

and gamonts possessed equally wide ends that give a characteristic appearance of a slightly curved cylinder (Table 3).

Phylogenetic analysis

Using sequences with different lengths, three phylogenetic hypotheses were generated and different rearrangements of clades and taxa were observed. Overall, both tree-building methods for phylogenetic reconstruction showed almost the same topology. *Hepatozoon* parasites appear into four different clades (Fig. 3, clades A–E), and *Karyolysus* was included in one of them (Fig. 3, clade E). In the phylogenetic reconstruction using full-length sequences (Fig. 3A), *Haemogregarina* (clade II) parasites diverge from a clade that includes *Hepatozoon, Karyolysus* and *Hemolivia*, depicted in clade I. In such clade I, *Hemolivia* lies basal to *Hepatozoon* clades from amphibia (Fig. 3, clade A) and reptile (clades B and C).

Hepatozoon simidi sp. nov. was located in a small clade (Fig. 3, clade C) along with *H. colubri* in all hypotheses performed (Fig. 3 and Fig. S1) with a low nodal support. This small clade was placed in a polytomy that included lineages from other reptiles, and the clade of amphibian *Hepatozoon* (Fig. 3, clades A and B), whose genetic divergences ranged between 0.03 (clade B *vs* clade C) and 0.05 (clade A *vs* clade C- Table S1). Furthermore, the new *Hepatozoon* species was separated by its sister taxa *H. colubri* by a genetic distance of 0.019; and from the second most closely related *H. fitzsimonsi* by a divergence of 0.03 (Table 4).

Haemogregarina lineages amplified in this study were placed basal of two distinct clades including parasite species reported infecting old-world Testudines (Fig. 3 clades G, H and I). The genetic distance between neotropical and old-world parasites of this genus ranged between 0.49 (clade G *vs* clade H) and 0.082 using 585 bp sequences (Fig. S1 B, clade G *vs* clade I), or 0.076 using sequences of 1000 bp (Fig. S1 A, clade G *vs* clade I;

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Table 3. 18S rRNA sequences aligned to construct the phylogenetic hypothesis. Sequence length used in each phylogenetic hypothesis of Fig. 3 and Fig. S1 are provided

				Sequence lengths (bp)		
Clade	Host	Parasite	GenBank No	Fig. 3A	Fig. S1 A	Fig.S1 B
Dactylosomatidae (Outgroup)	*Pelophylax lessonae* (*syn. esculentus*)	*Dactylosoma ranarum*	HQ224957	1808	1122	580
	Ptychadena anchietae	*Dactylosoma kermiti*	MN879398	1737	1122	580
Haemogregarina	*Platysternon megacephalum*	*Haemogregarina pellegrini*	KM887509	1412	1125	583
	Mauremys caspica	*Haemogregarina stepanowi*	KF992697	1421	1124	582
	Sacalia quadriocellata	*Haemogregarina sacaliae*	KM887507	1418	1124	582
	Chelydra serpentina	*Haemogregarina balli*	HQ224959	1817	1126	584
	Podocnemis unifilis	*Haemogregarina* sp.	MW246122	1423	1125	583
	Rhinoclemmys melanosterna	*Haemogregarina* sp. RM1	MT754268	585	585	584
	Rhinoclemmys melanosterna	*Haemogregarina* sp. H14	MT754269	583	583	583
	Rhinoclemmys melanosterna	*Haemogregarina* sp. H10	MT754270	582	582	582
Hepatozoon	*Lamprophis fuliginosus Boie*	*Hepatozoon ayorgbor*	EF157822	1773	1127	585
	Boiga irregularis	*Hepatozoon boiga*	AF297085	1996	1127	589
	Canis lupus familiaris	*Hepatozoon canis*	MH615006	1816	1124	582
	Elaphe carinata	*Hepatozoon* sp.	KF939620	1470	1088	585
	Martes martes	*Hepatozoon marten*	EF222257	1757	1124	582
	Abrothrix olivaceus	*Hepatozoon* sp.	FJ719817	1738	1127	585
	Podarcis bocagei	*Hepatozoon* sp.	JX531954	1365	1041	582
	Caiman crocodilus	*Hepatozoon* sp.	MW246123	1394	1127	585
	Cerdocyon thous	*Hepatozoon* sp.	KC127679	1028	798	600
	Panthera tigris tigris	*Hepatozoon felis*	HQ829446	1094	831	582
	Hemidactylus mabouia	*Hepatozoon* sp.	KM234615	1356	1029	585
	Felis silvestris silvestris	*Hepatozoon silvestris*	KX757032	1669	1135	593
	Sus scrofa leucomystax	*Hepatozoon apri*	LC314791	1007	930	582
	Hepatozoon procyonis	*Nasua nasua*	MF685409	1060	1015	583
	Hyperolius marmoratus	*Hepatozoon thori*	MG041603	1640	1127	585
	Amietia delalandii	*Hepatozoon theileri*	MG041605	1673	1127	585
	Afrixalus fornasini	*Hepatozoon tenuis*	MG041596	1701	1127	585
	Hyperolius marmoratus	*Hepatozoon involucrum*	MG041591	1658	1127	585
	Ctenosaura pectinata	*Hepatozoon* sp.	MG456821	1409	1089	585
	Sauromalus sp.	*Hepatozoon* sp.	MG456822	1411	1089	586
	Heloderma horridum	*Hepatozoon* sp.	MG456823	1378	1089	585
	Haemaphysalis bancrofti	*Hepatozoon ewingi*	MG593275	1680	1027	585
	Gallotia galloti	*Hepatozoon* sp.	MG787248	1696	1024	582
	Tarentola delalandii	*Hepatozoon* sp.	MG787251	1698	1125	582
	Spalerosophis diadema	*Hepatozoon aegypti*	MH198742	1315	948	469
	Caiman crocodilus	*Hepatozoon caimani*	MF435048	1429	1065	585
	Algyroides marchi	*Hepatozoon* sp.	JX531944	1368	1041	582
	Philodryas nattereri	*Hepatozoon musa*	KX880079	1384	1021	542
	Lithobates (ex. Rana) clamitans	*Hepatozoon clamatae*	HQ224963	1655	1127	585
	Lithobates catesbeianus	*Hepatozoon catesbianae*	AF130361	1824	1133	586
	Amblyomma maculatum	*Hepatozoon americanum*	AF176836	1413	1140	597
	Sclerophrys pusilla	*Hepatozoon ixoxo*	MG041604	1631	1127	585

(*Continued*)

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Table 3. (*Continued.*)

Clade	Host	Parasite	GenBank No	Sequence lengths (bp)		
				Fig. 3A	Fig. S1 A	Fig.S1 B
	Nerodia sipedon sipedon	*Hepatozoon sipedon*	JN181157	1807	1125	585
	Grandisonia alternans	*Hepatozoon_seychellensis*	KF246565	590	590	585
	Sciurus vulgaris	*Hepatozoon_sciuri*	MN104640	1492	1127	585
	Philodryas patagoniensis	*Hepatozoon* sp.	MN003368	1329	944	465
		Hepatozoon ophisauri	MN723845	1721	1127	585
	Ursus thibetanus japonicus	*Hepatozoon ursi*	EU041718	1207	1124	582
	Panthera pardus pardus	*Hepatozoon_luiperdjie*	MN793004	1002	998	582
	Zamenis longissimus	*Hepatozoon colubri*	MN723844	1609	1127	585
	Rhinoclemmys melanosterna	*Hepatozoon simidi* sp. nov	MT754271	584	585	585
Hemolivia	*Rhinella marina*	*Hemolivia stellata*	KP881349	1816	1125	583
	Kinixys zombensis	*Hemolivia parvula*	KR069083	1052	1052	582
	Oligoryzomys flavescens	*Hemolivia* sp.	KU667309	1051	1007	585
	Kinixys belliana	*Hepatozoon fitzsimonsi*[a,b]	KR069084	1034	1032	585
	Testudo graeca	*Hemoliva mauritanica*	KF992710	1418	1129	583
	Egernia stokesii	*Hemolivia mariae*	KF992712	1373	1124	582
	Rhinoclemmys pulcherrima	*Hemolivia* sp.	KF992714	1421	1124	582
Karyolysus	*Ixodes ricinus*	*Karyolysus lacazei*	MK497254	1442	1124	582

Table S1). Indeed, divergences within *Haemogregarina* parasites may reach values of 0.096 when comparing lineage (MT754270 with *Haemogregarina sacaliae,* Table 4, Fig. 3 and Fig. S1).

Discussion

Sample collection and blood film examination

This is the first report of an *Hepatozoon* parasite infecting a neotropical continental turtle, *R. melanosterna*, distributed in northwestern South America. In the Neotropics other species of *Rhinoclemmys* have been previously reported infected with Hemoregarines: in Costa Rica, the black river turtle (*Rhinoclemmys funerea*) was found infected with *Haemogregarina* sp. and probably *Hepatozoon* sp., (Rossow *et al.*, 2013) and in Nicaragua, the Central American wood turtle (*Rhinoclemmys pulcherrima*) infected with *Hemolivia* sp. (Kvičerová *et al.*, 2014). Genetic distances with the latest were for *H. simidi* sp. nov. of 0.04 (Table 4), which is between *Haemogregarina* sp. RM1 and *Hemolivia* sp. from *R. pulcherrima*; while for *Haemogregarina* lineages H10 and H14 were 0.09 and 0.05, respectively (Table 4).

At the genetic level, the closest taxon to *H. simidi* is *H. colubri* (Börner, 1901), a parasite isolated from *Zamenis longissimus* (Zechmeisterová, unpublished results) and other Colubridae (Pessoa, 1967), and also from Phytonidae (Börner, 1901). The next closest is *H. fitzsimonsi*. There are few morphological details on *H. colubri*; however, according to the original description, the parasites seem to be shorter and slender than *H. simidi* sp. nov. (Table 4).

Hepatozoon parasites can be transmitted by many blood-sucking arthropods. To the successful transmission of a heteroxenous parasite, there should be a spatiotemporal coincidence of the parasite, the host and the vector (Eldridge, 2004). Besides, some heteroxenous parasites may be transmitted horizontally or even vertically by facultative vias without the participation of true vectors (Kauffman *et al.*, 2017). *Rhinoclemmys melanosterna* is a semi-aquatic turtle that prefers swampy environments and is rarely found far away from such water bodies, so the habitat preference shown by this turtle may make transmission difficult if the vectors are ticks [as it is supposed for *H. fitzsimonsi*; (Cook *et al.*, 2009)], or blood-sucking dipterans (Smith, 1996). An alternative pathway for the transmission of *Hepatozoon* in reptiles is the ingestion of infective stages through predation (Ball, 1967; Landau *et al.*, 1972). Although *R. melanosterna* is mainly herbivorous, occasionally eats small fishes, frogs or tadpoles (Rueda-Almonacid *et al.*, 2007); thereby the infection by ingestion of an infected animal, as well as the possibility of this host species being an intermediate in a more complex life cycle, cannot be ruled out.

To date, only *H. fitzsimonsi* has been described in a Testudine host using molecular and morphological data. Although there are few distinctive characters in the gamonts that can be used in the description of the *Hepatozoon* species (Ball *et al.*, 1967), such parasite structures of *H. simidi* sp. nov. found in *R. melanosterna* were compared to those present in *H. fitzsimonsi*, revealing many distinctive features that the species in this description possesses. This parasite is even larger than others belonging to the Haemogregarine's group (Table 3, Fig. 2), that cause marked hypertrophy of the host cell from early stages. Also, the presence of large granules dispersed throughout the parasite is distinctive. The nature of these granules is still unknown; however, similar granules have been reported in haemosporidians as volutine granules (Valkiūnas, 2005; Lotta *et al.*, 2019). Electronic micrography studies are desirable for characterizing the morphological features, as well as to define more microscopic details that eventually can be used as diagnostic morphological characters.

Phylogenetic analysis

In agreement with previous studies, our phylogenetic reconstructions revealed *Karyolysus* lineages within *Hepatozoon*, making it a

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Table 4. Genetic distance calculated using K2P model of substitutions, between 18SrRNA lineages of Adeleorina parasites for the three different alignments in Fig. 3 and Fig. S1

	Genetic distance (d ± S.D.)		
Species	Full-length sequences (Fig. 3)	1000 bp (Fig. S1A)	585 pb (Fig. S1B)
Hepatozoon species from amphibians (Clade A)			
Hepatozoon tenuis vs Hepatozoon simidi sp. nov.	0.059 ± 0.010	0.059 ± 0.010	0.059 ± 0.010
Hepatozoon theileri vs Hepatozoon simidi sp. nov.	0.055 ± 0.010	0.055 ± 0.010	0.055 ± 0.010
Hepatozoon catesbianae vs Hepatozoon simidi sp. nov.	0.061 ± 0.010	0.061 ± 0.010	0.059 ± 0.010
Hepatozoon ixoxo vs Hepatozoon simidi sp. nov.	0.063 ± 0.010	0.063 ± 0.010	0.063 ± 0.011
Hepatozoon clamatae vs Hepatozoon simidi sp. nov.	0.065 ± 0.010	0.065 ± 0.010	0.065 ± 0.011
Hepatozoon involucrum vs Hepatozoon simidi sp. nov.	0.065 ± 0.010	0.065 ± 0.010	0.065 ± 0.011
Hepatozoon thori vs Hepatozoon simidi sp. nov.	0.066 ± 0.010	0.066 ± 0.010	0.066 ± 0.011
Hepatozoon species from reptiles (Clade B)			
Hepatozoon boiga vs Hepatozoon simidi sp. Nov	0.074 ± 0.012	0.076 ± 0.011	0.07 ± 0.011
Hepatozoon sciuri vs Hepatozoon simidi sp. Nov	0.044 ± 0.009	0.044 ± 0.009	0.044 ± 0.009
Hepatozoon ayorgbor vs Hepatozoon simidi sp. Nov	0.031 ± 0.007	0.031 ± 0.007	0.031 ± 0.007
Hepatozoon seychellensis vs Hepatozoon simidi sp. Nov	0.044 ± 0.009	0.044 ± 0.009	0.044 ± 0.009
Hepatozoon ophisauri vs Hepatozoon simidi sp. Nov	0.031 ± 0.007	0.031 ± 0.007	0.031 ± 0.007
Hepatozoon caimani vs Hepatozoon simidi sp. Nov	0.037 ± 0.008	0.037 ± 0.008	0.037 ± 0.008
Hepatozoon species from reptiles (Clade C)			
Hepatozoon colubri vs Hepatozoon simidi sp. Nov	0.019 ± 0.006	0.019 ± 0.006	0.019 ± 0.006
Hepatozoon species from other Testudines			
Hepatozoon fitzsimonsi vs Hepatozoon simidi sp. Nov	0.030 ± 0.007	0.030 ± 0.008	0.030 ± 0.007
Hepatozoon species from mammals (Clade D)			
Hepatozoon felis vs Hepatozoon simidi sp. Nov	0.033 ± 0.007	0.033 ± 0.008	0.033 ± 0.008
Hepatozoon apri vs Hepatozoon simidi sp. Nov	0.046 ± 0.010	0.046 ± 0.009	0.046 ± 0.009
Hepatozoon procyonis vs Hepatozoon simidi sp. Nov	0.050 ± 0.010	0.050 ± 0.009	0.050 ± 0.010
Hepatozoon ursi vs Hepatozoon simidi sp. Nov	0.052 ± 0.009	0.052 ± 0.009	0.052 ± 0.009
Hepatozoon canis vs Hepatozoon simidi sp. Nov	0.052 ± 0.010	0.059 ± 0.010	0.059 ± 0.011
Hepatozoon species from reptiles (Clade E)			
Karyolysus_lacazei vs Hepatozoon simidi sp. Nov	0.041 ± 0.008	0.041 ± 0.008	0.041 ± 0.008
Hepatozoon_sp (JX531944) *vs Hepatozoon simidi* sp. Nov	0.048 ± 0.009	0.048 ± 0.009	0.048 ± 0.009
Hepatozoon_sp (JX787251) *vs Hepatozoon simidi* sp. Nov	0.071 ± 0.012	0.071 ± 0.012	0.071 ± 0.011
Hemolivia (clade F)			
Hemolivia stellata vs Hepatozoon simidi sp. Nov	0.033 ± 0.008	0.033 ± 0.007	0.033 ± 0.007
Hemolivia párvula vs Hepatozoon simidi sp. Nov	0.035 ± 0.008	0.035 ± 0.007	0.035 ± 0.008
Hemolivia mariae vs Hepatozoon simidi sp. Nov	0.037 ± 0.008	0.037 ± 0.008	0.037 ± 0.008
Hemolivia sp in R. pulcherrima vs Hepatozoon simidi sp. Nov	0.037 ± 0.008	0.037 ± 0.008	0.037 ± 0.008
Hemolivia mauritanica vs Hepatozoon simidi sp. Nov	0.035 ± 0.007	0.035 ± 0.007	0.035 ± 0.007
Haemogregarina sp. (clades G, H, I)			
Haemogregarina sp. RM1 *vs Hepatozoon simidi* sp. Nov	0.052 ± 0.009	0.052 ± 0.010	0.052 ± 0.010
Haemogregarina balli vs Hepatozoon simidi sp. Nov	0.067 ± 0.011	0.067 ± 0.011	0.067 ± 0.011
Haemogregarina sp (MW246122) *vs Hepatozoon simidi* sp. Nov	0.072 ± 0.011	0.072 ± 0.012	0.070 ± 0.011
Haemogregarina sp. H10 *vs Hepatozoon simidi* sp. Nov	0.097 ± 0.013	0.097 ± 0.013	0.097 ± 0.013
Haemogregarina sp. H10 *vs Haemogregarina* (MW246122)	0.059 ± 0.010	0.059 ± 0.011	0.059 ± 0.011
Haemogregarina sp. H10 *vs Haemogregarina sacaliae*	0.096 ± 0.013	0.096 ± 0.014	0.096 ± 0.013
Haemogregarina sp. H10 *vs Haemogregarina* sp. RM1	0.086 ± 0.013	0.086 ± 0.013	0.086 ± 0.013
Haemogregarina sp. RM1 *vs Haemogregarina* (MW246122)	0.057 ± 0.010	0.057 ± 0.011	0.057 ± 0.009

(*Continued*)

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Table 4. (*Continued.*)

Species	Genetic distance (d ± S.D.)		
	Full-length sequences (Fig. 3)	1000 bp (Fig. S1A)	585 pb (Fig. S1B)
Haemogregarina sp. RM1 *vs Haemogregarina sacaliae*	0.050 ± 0.010	0.050 ± 0.009	0.050 ± 0.009
Dactylosoma (outgroup)			
Dactylosoma ranarum vs Hepatozoon simidi sp. Nov	0.069 ± 0.011	0.069 ± 0.010	0.071 ± 0.010

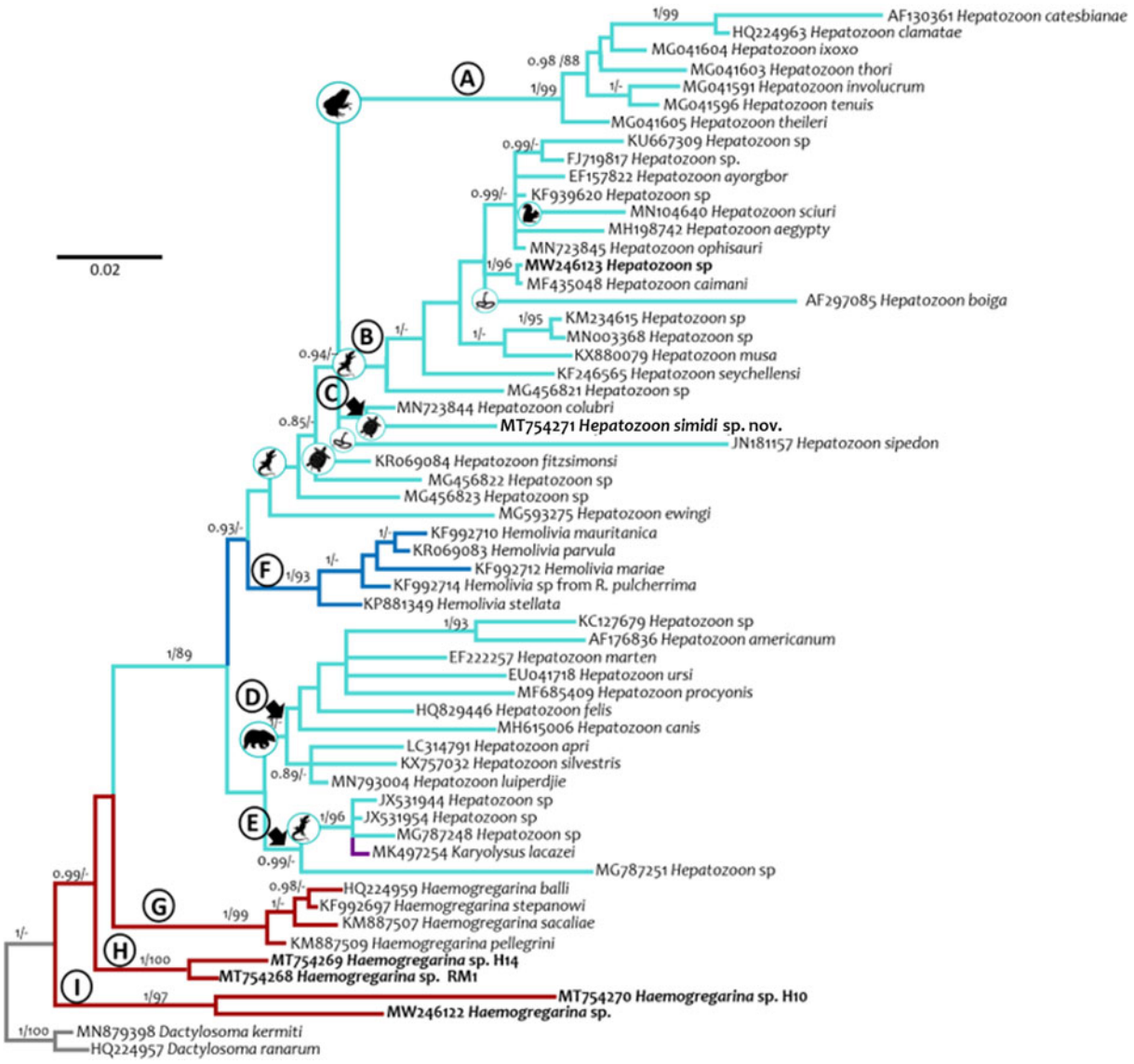

Fig. 3. Phylogenetic hypothesis obtained using Bayesian inference and maximum likelihood constructed from 18S rRNA sequences of 1800 bp. The lineages obtained in this study are highlighted in bold font. Branches colour indicates the parasite genus as follows: green (light grey in printed version) for *Hepatozoon* sp., blue (medium grey) for *Hemolivia* sp., purple (white) for *Karyolysus* sp., red (black) for *Haemogregarina* and grey (Dark grey) for the outgroup (*Dactylosoma* sp.). The silhouettes located near the clade nodes indicate the host from which the parasites were isolated: a frog for amphibians, a lizard for reptiles, a turtle for turtles and tortoises, and a bear for mammals. Bootstrap values and posterior probabilities are shown above the nodes. Nodal supports below 80/0.8 are not shown. The branch lengths are proportional to the amount of change. Scale bar indicating substitutions per site is provided.

paraphyletic genus (Barta *et al.*, 2012; Karadjian *et al.*, 2015; Cook *et al.*, 2016). Furthermore, *H. simidi* sp. nov. was consistently placed as part of a polytomy, including some other reptile and anuran parasite species, with low nodal support, most probably due to the size of the sequence analysed.

Using sequences of 18S rRNA, several authors have proposed that an interspecific genetic distance of above 1% could be enough to differentiate species, bearing in mind the low evolutionary rate mentioned (Cook *et al.*, 2015; Borges-Nojosa *et al.*, 2017; Netherlands *et al.*, 2018). Based on the large genetic distance found between the lineage MT754271 (*H. simidi* sp. nov.) with the closest lineages belonging to the genus *Hepatozoon* isolated from reptiles (2% with *H. colubri* and 3% with *H. fitzsimonsi* from the tortoise *Kinixys belliana*), we might conclude that this parasite lineage represents an undescribed parasite species.

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It is important to mention that the lineage of *H. simidi* sp. nov. fall in the clade identified as *Bartazoon* genus proposed by Karadjian *et al.* (2015). However, the *Bartazoon* genus has specific features in the sporogonic development in the invertebrate vector, described widely in Karadjian *et al.* (2015). Unfortunately, we have no information about sporogonic development or even a possible vector that allows us to give bases to designate this new species to the *Bartazoon* genus.

As for *Haemogregarina* parasites, high genetic distances found between the different lineages analysed, either from the old world and neotropical hosts, might be revealing a high diversity within this parasite genus that remains to unveil. In turn, it can also be indicative of the low number of taxa of this genus used to build the phylogenetic hypothesis.

Here we described a new parasite species belonging to the genus *Hepatozoon*. The description of *H. simidi* sp. nov. was based on both morphological and molecular approaches, and this is the first report of Adeleorinid hemoparasite infections in *R. melanosterna* from Colombia. To a more accurate description of new parasite species belonging to this group, it would be ideal to have information about the vector's development stages and tissue stages in the vertebrate (Ball *et al.*, 1967; Smith, 1996). Besides, new molecular markers would improve phylogenetic relationships. The mitochondrial genome has been seen as a good candidate given the evolution rate of the genes encoded there (Escalante *et al.*, 1998; Pacheco *et al.*, 2017) as well as their widespread use in other apicomplexa groups of parasites (Bensch *et al.*, 2000; Martinsen *et al.*, 2008; Perkins, 2008; Ogedengbe *et al.*, 2011; Witsenburg *et al.*, 2012; Borner *et al.*, 2016; González *et al.*, 2019 among others). In this regard, recent advances have been achieved for the mitochondrial genome sequencing of *Hepatozoon catesbianae* and *Hepatozoon griseisciuri* (Léveillé *et al.*, 2014, 2020), from which high genetic divergences have been found within the nominal taxa.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0031182021000184.

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Conflicts of interest. The authors declare that there are no conflicts of interest.

Ethical standards. Specimens were collected under the collection permit 255 of 2014 issued by the National Environmental Licenses Authority (ANLA) to the Universidad Nacional de Colombia by resolution. All specimens captured were released after the blood sample collection. Sampling methods were approved by the 'Institutional Bioethics committee of the Fundación Universitaria-Unitrópico' on May 22 of 2017 and the Bioethics Committee of the science Faculty of the Universidad Nacional de Colombia, by act 03-2019.

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