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Haemocystidium spp., a species complex infecting ancient aquatic turtles of the family Podocnemididae: First report of these parasites in *Podocnemis vogli* from the Orinoquia

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ABSTRACT

The genus *Haemocystidium* was described in 1904 by Castellani and Willey. However, several studies considered it a synonym of the genera *Plasmodium* or *Haemoproteus*. Recently, molecular evidence has shown the existence of a monophyletic group that corresponds to the genus *Haemocystidium*. Here, we further explore the clade *Haemocystidium* spp. by studying parasites from Testudines. A total of 193 individuals belonging to six families of Testudines were analyzed. The samples were collected in five localities in Colombia: Casanare, Vichada, Arauca, Antioquia, and Córdoba. From each individual, a blood sample was taken for molecular analysis, and peripheral blood smears were made, which were fixed and subsequently stained with Giemsa. The prevalence of *Haemocystidium* spp. was 1.55% (n = 3/193); all infected individuals belonged to *Podocnemis vogli* (Savanna Side-necked turtle) from the department of Vichada. This is the first report of *Haemocystidium* spp. in Colombia and in this turtle species. The phylogenetic analysis of a mitochondrial *cytb* fragment revealed *Haemocystidium* spp. as a monophyletic group and as a sister taxon of *Haemoproteus catharti* and the genus *Plasmodium*. *Haemocystidium* spp. are difficult to identify by morphology only. As a result, it is possible that some of the taxa, such as *Haemocystidium* (*Simondia*) *pacayae*, represent a species complex. The parasite found in our study is morphologically indistinguishable from *Haemocystidium* (*Simondia*) *pacayae* reported in Peru. However, the new lineage found in *P. vogli* shows a genetic distance of 0.02 with *Hae. pacayae* and 0.04 with *Hae. peltocephali*. It is proposed that this divergent lineage might be a new species. Nevertheless, additional molecular markers and ecological features could support this hypothesis in the future.

1. Introduction

The phylum Apicomplexa (Levine, 1988) encompasses at least 5,000 recognized species of obligate intracellular protozoan parasites (Morrison, 2009). Among them, blood parasites belonging to the order Haemosporida (Danilewsky 1885) are classified into the families Leucocytozoidae (infecting birds), Haemoproteidae, and Garniidae (found in birds and reptiles), and Plasmodiidae (which infects birds, reptiles, and mammals). Despite significant advances in the description of species diversity of the genus *Plasmodium*, which includes the species that cause human malaria (WHO, 2018), the information for other taxonomic groups that parasitize wildlife is limited and fragmented. Such a knowledge gap has driven several phylogenetic studies within the order Haemosporida (Javanbakht et al., 2015; Maia el at., 2016; Boundenga et al., 2017; Pacheco et al., 2018b). The lack of information is particularly critical in haemosporidian parasites in reptiles.

The haemosporidians found in reptiles have been classified into several families; Haemoproteidae, genera *Haemoproteus* sp. and *Haemocystidium* sp.; Plasmodidae, genus *Plasmodium* sp.; Garnidae, genera *Garnia* sp. and *Fallisia* sp. (Levine, 1970; Adl et al., 2012, 2019;

Abbreviations:H: *Haemoproteus*, *Hae*: *Haemocystidium*

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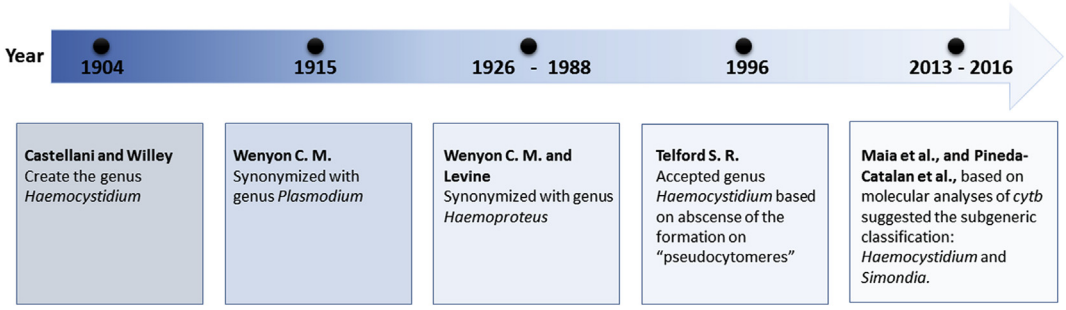


Fig. 1. Timeline of the taxonomic classification of the genus *Haemocystidium*. Relevant events in the description of this genus.

Perkins, 2014); and the family Leucocytozoidae, genus *Saurocytozoon* sp. (Lainson et al., 1974). Among those, the genus *Haemocystidium* has been one of the least studied, and its status has been controversial. Being synonymized as *Plasmodium* or *Haemoproteus*, after its description (Castellani, and Willey, 1904, Fig. 1).

Currently, the genus *Haemocystidium* has not been fully accepted by the academic community due to the limited studies available in this group, both in terms of hosts and parasite species (Javanbakht et al., 2015). New molecular lineages attributed to this genus have been reported in samples from the gecko species *Hemidactylus luqueorum* and *Ptyodactylus hasselquistii* captured in Oman, Asia (Maia et al., 2016), and in turtles, *Pelusios castaneus* and *Kinixys erosa*, from Gabon, Central Africa (Supplementary Table 1; Boundenga et al., 2017). The few reports from South America have found three *Haemocystidium* species: *Haemocystidium* (*S.*) *pacayae* infecting the freshwater turtles *Podocnemis unifilis* and *Podocnemis expansa* (Peru), *Hae. geochelonis* in the tortoise *Chelonoidis denticulata* (Brazil) and *Hae.* (*S.*) *pelthocephali* in *Peltocephalus dumerilianus*, *Podocnemis unifilis,* and *P. expansa* (Brazil and Peru), (Lainson and Naiff, 1998; Pineda-Catalan et al., 2013). However, there is an extraordinary diversity of turtles in Colombia, and there are no studies directed to assess the presence and diversity of haemosporidian infecting these hosts.

There are nine families and 36 species of turtles in Colombia, 28 are freshwater turtles, two are exclusively terrestrial, and six are sea turtles. Fifteen of these species are classified into various categories of threat (Ministerio del Medio Ambiente, Vivienda y Desarrollo Territorial, 2017). The few studies conducted on Testudines have focused on their microorganisms such as bacteria (Pachón, 2009) or pathological studies of some of their parasites such as *Toxoplasma* spp. and *Filaroides* spp. (Herrera, 2008), while the biodiversity of haemoparasites that may affect them is poorly known. This study had two objectives: (1) To analyze the presence of haemosporidian parasites in wild turtles belonging to six families distributed in diverse ecoregions in Colombia; and (2) to discuss the classification of species belonging to *Haemocystidium* genus based on morphological and molecular data.

2. Materials and methods

2.1. Sampling

In total, 193 wild turtles (order Testudines) belonging to nine species representing the families Chelidae, Podocnemididae, Kinosternidae, Emydidae, Geoemydidae, and Testudinidae were sampled. These individuals were collected in the departments of Antioquia, Arauca, Casanare, Cordoba, and Vichada (Fig. 2). The numbers of individuals sampled by species and their corresponding localities are reported in Table 1. Below there is a brief description and distribution of the species included in this study. From Chelidae family, *Mesoclemmys dahli* is a Colombian trans-Andean endemic species that, except for the other species belonging to this family (genera *Chelus*, *Platemys*, *Rhinemys*, and *Phrynops*), is distributed in the tropical dry forest along the Caribbean, inhabiting shallow waters with floating vegetation (Castaño-Mora, 2002; Rueda-Almonacid et al., 2007). Specimens from three species of the genus *Podocnemis* from the Podocnemididae family were analyzed. This was a diverse group in the Late Cretaceous (at least 20 genera and 30 species) that was reduced to only three extant genera (*Podocnemis*, *Peltocephalus,* and *Erymnochelys*) and eight species (Gaffney et al., 2011) distributed in South America and Madagascar (Vargas-Ramirez et al., 2008). In this study, *Podocnemis vogli*, *P. lewyana,* and *P. unifilis* were screened for haemoparasites. *Podocnemis vogli* (Savanna Side-necked Turtle) is an omnivorous and aquatic species, which restricts its distribution to the Orinoco basin in the east of Colombia and western plains of Venezuela (Rueda-Almonacid et al., 2007). *Podocnemis unifilis* is distributed in Colombia, Venezuela, Brazil, Peru, Ecuador, Bolivia, and the Guianas, inhabiting white and black waters (Rueda-Almonacid et al., 2007). *Podocnemis lewyana*, is also a Colombian endemic species with a restricted distribution to the basins of the Magdalena and Sinú rivers; it is the only species of this family that inhabits the west of the Andes mountain range, and it has diurnal and nocturnal habits (Rueda-Almonacid et al., 2007). From the Geoemydidae family, *Rhinoclemmys melanosterna* is distributed in the Pacific, Caribbean region and lower Magdalena river (Rueda-Almonacid et al., 2007); it preferably inhabits swampy areas where heliconias are abundant. Belonging to the Testudinidae family, *Chelonoidis carbonaria* is a terrestrial tortoise with a broad distribution that includes Venezuela, Colombia, the Guyanas, Brazil, Bolivia, Paraguay, and Argentina (Castaño-Mora, 2002; Rueda-Almonacid et al., 2007). Belonging to the Kinosternidae family, *Kinosternon scorpioides* is distributed from Mexico to Argentina, inhabiting rivers, lakes, and flood plains (Rueda-Almonacid et al., 2007).

All sampled chelonians were captured by using funnel traps, fishing nets, and by hand. The blood samples were obtained from the subcarapacial sinus or the coccygeal vein and did not exceed 1% of the body weight. For each individual, three peripheral blood smears were made, which were dried immediately and fixed with absolute methanol for 5 min. At the laboratory, the blood smears were stained with Giemsa (4%, pH 7.2) for 45 min. The blood samples were preserved in absolute ethanol and stored at −20 °C for further molecular analysis. The samples were kept at room temperature in the field and then at −20 °C in the laboratory.

2.2. Ethical statement and sampling permits

Specimens were collected under the framework collection permission for wild species of biological diversity authorized by the National Environmental Licenses Authority (ANLA) to the Universidad Nacional de Colombia by resolution No. 0255 of March 14, 2014. All turtles and tortoises were released after the blood samples were collected. The samples analyzed in this study came from the collection "Banco de tejidos de la Biodiversidad Colombiana," Universidad Nacional de Colombia.

Fig. 2. Species and sampling locations for turtles analyzed in the study. Turtle species present in these localities are shown.

2.3. Blood film examination

Blood slides were examined using an Olympus CX41 microscope (Olympus Corporation, Tokyo, Japan), at 400× for 10 min, and then at 1000× for 20 min. Slides with haemoparasites were examined entirely, and digital images were obtained using an Olympus DP27 digital camera and processed with the CellSens software (Olympus Corporation, Tokyo, Japan). Morphometric analysis was performed using ImageJ software (Schneider et al., 2012), and the parasitaemia (No. of parasites/10,000 erythrocytes) was estimated from erythrocyte counts at a magnification of 1000× on areas where blood cells formed a monolayer (Staats and Schall, 1996). The taxonomic determination of haemoparasites was made comparing their morphologies and morphometry with previous reports in the literature for *Haemoproteus* and *Haemocystidium* species (Telford, 2009; Lainson, 2012; Pineda-Catalan et al., 2013; Maia et al., 2016). The comparison of morphological measurements between the *Haemocystidium* species found here and those previously reported was done using the Student's t-test.

2.4. DNA extraction and detection of haemosporidian parasites by a polymerase chain reaction

Total DNA was extracted from samples using the phenol-chloroform method (Sambrook et al., 1989) and measured by using NanoDrop Lite spectrophotometer (Thermo Scientific, Massachusetts, USA). A nested polymerase chain reaction (PCR) assay using primers targeting the parasite cytochrome b (*cytb*) gene from the mitochondria was used (Supplementary Table S2; Pacheco et al., 2018a). Reactions for primary PCR were performed in 25 μL total volume, including 2 μL of total genomic DNA template, 12.5 μL of DreamTaq Master Mix (Thermo Fisher Scientific, Germany), 8.5 μL nuclease-free water, and 1 μL of each primer. In the case of the nested PCR, reactions were performed in 50 μL total volume, with 3 μL of the primary PCR products, 1× PCR buffer, 2.5 mM $MgCl_2$, 0.3 units of Taq DNA polymerase (Thermo Scientific, Waltham, USA), 0.2 mm dNTPs (Promega, Madison, USA), and 0.4 mM of each primer. One negative control (nuclease-free water) and one positive control (*Plasmodium unalis* positive sample) were included in each PCR. Temperature profiles were the same as in the original protocol (Supplementary Table S2; Pacheco et al., 2018a). All products from the primary and nested PCR were evaluated by running 2 μL of the final products on 2% agarose gels. Amplified products were cleaned using differential precipitation with ammonium acetate protocol (Bensch et al., 2000). Fragments of DNA from all positive amplifications were sequenced in both directions using a 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA) through Macrogen (Macrogen Inc.). The sequences for the *cytb* fragments obtained in this study were identified as *Haemocystidium* species using BLAST (Altschul et al., 1997). The sequences were deposited in GenBank under the accession numbers MK976708 (GERPH: PC004), MK976709 (GERPH: PC006) and MK976710 (GERPH: PC005).

2.5. Phylogenetic analysis of the parasite cytb gene fragment

In order to link morphological characteristics with molecular lineages, only positive samples by microscopy were used in this investigation. Of those, one sample amplified in the primary PCR (GERPH: PC005) described above, and two in the nested PCR (GERPH: PC004 and GERPH: PC005), thus two different fragment sizes for the

Table 1
Turtle species analyzed in the present study, from diverse localities in Colombia. Common names, size sample, and locality are shown. *The only locality that has turtles infected with *Haemocystidium*.

Family	Species	Common name	Sample locality (Department)	n
Chelidae	*Mesoclemmys dahli*	Dahl's Toad-headed Turtle	Lorica (Cordoba)	3
Podocnemididae	*Podocnemis unifilis*	Yellow-spotted River Turtle, Yellow-headed Side-neck, Yellow-spotted Side-neck Turtle	Cravo Norte (Arauca) 06°14′ 30.84″ N 70° 14′ 31.05″ W	1
	Podocnemis lewyana	Magdalena River Turtle	Lorica (Cordoba)	17
	Podocnemis vogli	Savanna Side-necked Turtle	Paz de Ariporo (Casanare) 5° 42′ 18.9463″ N 71° 14′ 47.5309″ W	134
			*Puerto Carreño (Vichada) 06°16′ 067″ N 67°49′ 742″ W	3*/13
Kinosternidae	*Kinosternon scorpioides*	Scorpion Mud turtle	Lorica (Cordoba)	3
Emydidae	*Trachemys venusta callirostris*	Colombian slider	Lorica (Cordoba)	11
Geoemydidae	*Rhinoclemmys melanosterna*	Colombian Wood Turtle	Yondó (Antioquia) 6° 48′ 27.0720″ N 74° 12′ 19.69″ W	4
Testudinidae	*Chelonoidis carbonaria*	Red-footed Tortoise	Yondó (Antioquia) 6° 48′ 27.0720″ N. 74° 12′ 19.69″ W	5
			Paz de Ariporo (Casanare) 5° 42′ 18.9463″ N 71° 14′ 47.5309″ W	1
	Chelonoidis denticulata	Yellow-footed Tortoise, Brazilian Giant Tortoise, Forest Tortoise, South American Tortoise, South American Yellow-footed Tortoise	Cumaribo (Vichada) 3° 22′ 26.796″ N. 69° 30′ 48.311″ W	2

cytb gene were obtained in this study (1,692 and 535 bp without the primer regions respectively). In order to compare our molecular data against all sequences that have been published for *Haemocystidium* and other haemoparasites infecting reptiles or birds (*Plasmodium, Haemoproteus* and *Leucocytozoon*), two different nucleotide alignments were performed using ClustalX v2.0.12 and Muscle as implemented in SeaView v4.3.5 (Gouy et al., 2010) with manual editing. The first alignment was constructed with 103 cytb partial sequences (233 bp excluding gaps) belonging to four genera (*Leucocytozoon*, *Haemoproteus*, *Haemocystidium*, and *Plasmodium*). Although our smallest cytb fragment for samples GERPH: PC004 and GERPH: PC006 was 535 bp in length, the region that overlapped between all sequences, including those available in the databases, was only 233 bp in length. This alignment included the sequences obtained in this study for samples GERPH: PC004, GERPH: PC005 and GERPH: PC006, as well as sequences from well-known parasite species based on morphology and haplotypes (Valkiūnas and Iezhova, 2018) that were available on the GenBank database (Benson et al., 2012) at the time of this study. The second alignment was constructed with 62 sequences corresponding to a bigger fragment of cytb (707 bp excluding gaps) also belonging to the four genera, including only the sequence obtained for GERPH: PC005 and sequences from well-known parasite species (using morphology) available on GenBank. In this case, our larger cytb fragment obtained for GERPH: PC005 had 1,692 bp, but the region that overlapped among all the sequences included was 707 bp.

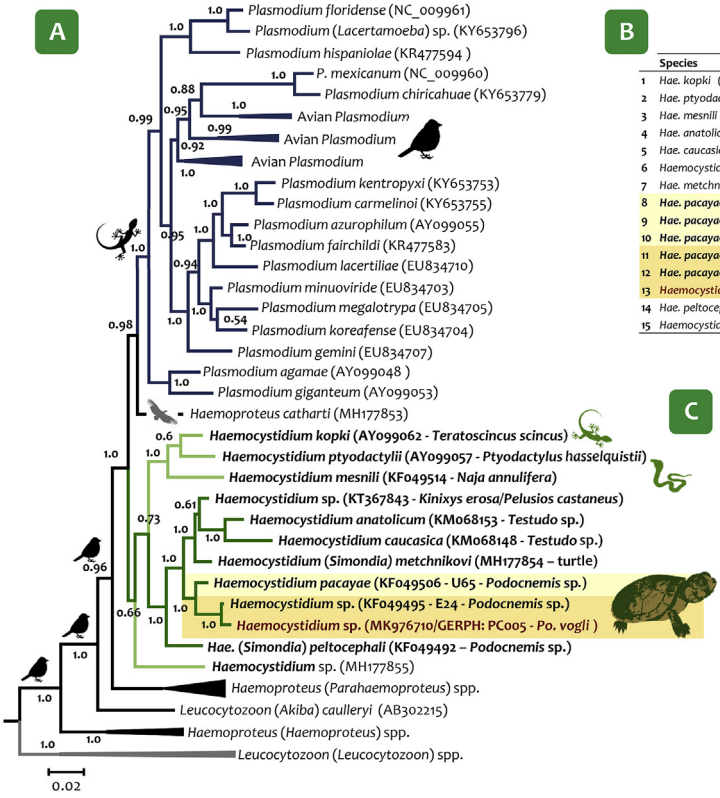
Phylogenetic trees were inferred based on the first (Supplementary Fig. S1) and second alignments (Fig. 3A), using a Bayesian method as implemented in MrBayes v3.2.6 with the default priors (Ronquist and Huelsenbeck, 2003) and the general time-reversible model with gamma-distributed substitution rates and a proportion of invariant sites (GTR +Γ + I). This model was the one with the lowest Bayesian Information Criterion (BIC) scores for both alignments as estimated by MEGA v7.0.14 (Kumar et al., 2016). Posterior probability was estimated for the nodes in MrBayes by sampling every 1,000 generations from two independent chains lasting $3X10^6$ Markov Chain Monte Carlo (MCMC) steps. The chains were assumed to have converged when the average SD of the posterior probability was < 0.01 and the value of the potential scale reduction factor (PSRF) was between 1.00 and 1.02 (Ronquist and Huelsenbeck, 2003). Then, 25% of the sample was discarded once convergence was reached as a "burn-in." For both phylogenies, *Leucocytozoon* species were used as out-group. Genbank accession numbers for all sequences used in the analyses are given in the phylogenetic trees.

Furthermore, in order to estimate the genetic distance between *Haemocystidium* species, the number of base substitutions per site and the standard error estimates between sequences of species from well-known parasites based on morphology were estimated using the Tamura-Nei model. This model was the one that better fit to the data (Tamura and Nei, 1993). In a second analysis, the number of base substitutions per site and the standard error estimates from averaging overall sequence pairs (haplotypes) within and between species were also estimated. Codon positions included were 1st + 2nd + 3rd' and all positions containing gaps and missing data were eliminated. These analyses were conducted in MEGA7 (Kumar et al., 2016) using the second alignment (707 bp excluding gaps).

3. Results

3.1. Morphological detection of haemosporidian parasites

Of the 193 captured turtles, only three individuals were positive for haemosporidian parasites using microscopy (3/193, 1.55%), all belonging to the same host species, *P. vogli.* The only haemosporidian detected in the samples analyzed were *Haemocystidium,* and its prevalence in *P. vogli* was 2.04% (n = 3/147). All three positive samples came from a single locality: Finca de las Flores in the department of



B

	Species	Genetic Distances/Standar Errors														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Hae. kopki* (AY099062)		0.007	0.009	0.012	0.014	0.010	0.009	0.009	0.009	0.009	0.009	0.009	0.010	0.010	0.012
2	*Hae. ptyodactylii* (AY099057)	0.030		0.009	0.012	0.015	0.010	0.010	0.010	0.010	0.010	0.010	0.010	0.011	0.010	0.013
3	*Hae. mesnili* (KF049514)	0.041	0.045		0.013	0.014	0.011	0.011	0.009	0.009	0.009	0.010	0.010	0.011	0.010	0.012
4	*Hae. anatolicum* (KM068153)	0.067	0.074	0.085		0.010	0.009	0.009	0.010	0.010	0.010	0.011	0.011	0.012	0.011	0.014
5	*Hae. caucasica* (KM068148)	0.093	0.100	0.097	0.041		0.011	0.012	0.011	0.011	0.011	0.012	0.012	0.013	0.012	0.016
6	*Haemocystidium* sp. (KT367843)	0.052	0.059	0.061	0.035	0.054		0.007	0.007	0.007	0.007	0.008	0.008	0.009	0.009	0.011
7	*Hae. metchnikovi* (MH177854)	0.047	0.054	0.059	0.037	0.063	0.020		0.008	0.008	0.008	0.009	0.009	0.010	0.009	0.013
8	***Hae. pacayae* (KF049506)** (1)	0.045	0.052	0.050	0.046	0.052	0.022	0.031		0.000	0.000	0.005	0.005	0.006	0.007	0.011
9	***Hae. pacayae* (KF049513)** (1)	0.045	0.052	0.050	0.046	0.052	0.022	0.031	**0.000**		0.000	0.005	0.005	0.006	0.007	0.011
10	***Hae. pacayae* (KF049509)** (1)	0.045	0.052	0.050	0.046	0.052	0.022	0.031	**0.000**	0.000		0.005	0.005	0.006	0.007	0.011
11	***Hae. pacayae* ? (KF049495)** (2)	0.052	0.059	0.056	0.052	0.059	0.029	0.037	**0.014**	**0.014**	**0.014**		0.000	0.003	0.009	0.012
12	***Hae. pacayae* ? (KF049507)** (2)	0.052	0.059	0.056	0.052	0.059	0.029	0.037	**0.014**	**0.014**	**0.014**	**0.000**		0.003	0.009	0.012
13	*Haemocystidium* sp. (MK976710/PC005)	0.054	0.066	0.063	0.059	0.066	0.035	0.044	0.020	0.020	0.020	**0.006**	**0.006**		0.010	0.012
14	*Hae. peltocephali* (KF049492)	0.048	0.054	0.054	0.056	0.067	0.037	0.035	0.022	0.022	0.022	0.037	0.037	0.044		0.013
15	*Haemocystidium* sp. (MH177855)	0.063	0.075	0.067	0.093	0.102	0.067	0.077	0.061	0.061	0.061	0.063	0.063	0.065	0.077	

C

Parasite species		Host	Genetic Distance	Standar Error
Hae. anatolicum (N=7)		*Testudo* sp.	0.004	0.002
Hae. caucasica (N=6)		*Testudo* sp.	0.002	0.001
Haemocystidium sp. (N=10)		*Kinixys erosa/Pelusios castaneus*	0.003	0.001
Hae. peltocephali (N=14)		*Podocnemis* sp.	0	0
***Hae. pacayae* (N=6)**		***Podocnemis* sp.**	**0.01**	**0.003**
***Hae. pacayae* (N=3)**	(1)	***Podocnemis* sp.**	**0.000**	**0.000**
Hae. sp. (N=3)	(2)	***Podocnemis* sp.**	**0.003**	**0.001**
1 *vs* **. 2**		*Podocnemis* sp.	0.0137	0.004

Fig. 3. (A) A Bayesian phylogenetic analysis of reptile haemosporidian parasites based on 62 partial sequences of *cytb* gene sequences corresponding to a bigger fragment of *cytb* (707 bp excluding gaps). *Leucocytozoon* genus was used as outgroup. In parenthesis are GenBank sequence accession number, isolate name, and turtle species name respectively. Branch color indicates the parasites genus: Blue, *Plasmodium* sp.; light green, *Haemocystidium* spp. infecting lizards and snakes; dark green, *Haemocystidium* sp. Infecting turtles; black, *Haemoproteus* and *Leucocytozoon* spp. **(B–C)** Estimates of evolutionary divergence between/within *Haemocystidium* spp. Genetic distances were estimated using the bigger fragment of *cytb* (707 bp excluding gaps). The number of base substitutions per site between sequences are shown in black and the standard error estimate(s) are shown above the diagonal in blue. Evolutionary divergence between/within *Haemocystidium pacayae* and *Haemocystidium* sp. (GERPH: PC005) are shown in bold and red respectively. Sequences previously identified as *Hae. pacayae* (KF049495 and KF049507) are likely *Hae.* (*Simondia*) sp. (group 2). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

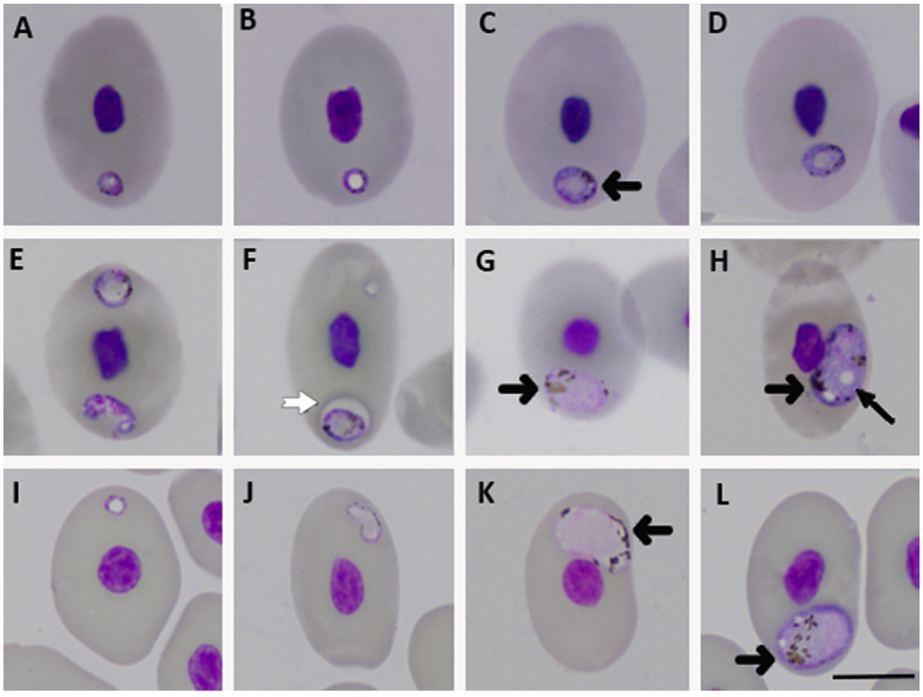


Fig. 4. *Haemocystidium* (*Simondia*) sp. (GERPH: PC005) identified in *Podocnemis vogli* (A–H). (A–F) Young gametocytes, (E) coinfection with a gamont of *Haemogregarina*, (G) microgametocyte, and (H) macrogametocyte. *Haemocystidium* (*S.*) *pacayae* (GERPH: PC004-PC006) identified in *P. vogli* (I–J). (I) Young gametocyte, (K) microgametocyte, and (L) macrogametocyte. Bold black arrow: hemozoin granules; white arrow: parasitophorous vacuole; fine black arrow: vacuole. Giemsa stain, Scale bar: 10 μm.

Vichada, where 13 *P. vogli* were captured (06°16.067′ N. 67°49.742′ W) so the prevalence of *Haemocystidium* sp. in this area was 23.1% (Fig. 2, Table 1). Parasites were found infecting mature erythrocytes (Fig. 4). The parasitaemia was 0.35% for the individual GERPH: PC004, 0.68% for GERPH: PC005, and 0.29% for GERPH: PC006. The most frequent haemoparasites observed in these samples were haemogregarines (84.53%, n = 164/193). All individuals infected with *Haemocystidium* showed co-infection with haemogregarine gamonts (data not shown). All the slides were deposited in the biological collection of "Grupo de Estudio Relación Parásito Hospedero (GERPH)" at the Department of Biology, Universidad Nacional de Colombia, Bogotá, Colombia.

3.2. Genetic distances and phylogenetic relationships of Haemocystidium genus

A fragment of *cytb* gene was obtained for each infected turtle. The genetic distances between *Haemocystidium* species estimated as the number of base substitutions per site are shown in Fig. 3B. The *cytb* gene fragments (535 bp) of samples GERPH: PC004 and GERPH: PC006 were 100% identical to the sequence reported for *Haemocystidium pacayae* (KF049506; Pineda-Catalan et al., 2013; Supplementary Fig. S1). However, the genetic distance between *Haemocystidium pacayae* (KF049506, KF049509, and KF049513) and the parasite infecting sample GERPH: PC005 was 0.02 ± 0.006 (Fig. 3B).

Bayesian phylogenetic trees of *Haemocystidium* parasites, generated using the first (103 *cytb* partial sequences with 233 bp; Supplementary Fig. S1) and the second alignments (62 c*ytb* partial sequences with 707 bp; Fig. 3A) showed similar topologies. All the species identified as *Haemocystidium* parasites infecting reptiles (snakes, lizards, and turtles) form a monophyletic group sharing a common ancestor with *Haemoproteus catharti* and the genus *Plasmodium*. Two lineages of *Haemocystidium* parasites identified as 1 and 2 (Fig. 3C), were found infecting three individuals of *P. vogli* and were grouped with the lineages reported so far as *Hae. pacayae*. The lineage found infecting the samples GERPH: PC004 and GERPH: PC006 (group 1) was identical to the *Hae. pacayae* sequences available on the GenBank database under the accession numbers KF049506, KF049509, and KF049513 (Pineda-Catalan et al., 2013). The lineage found in the sample GERPH: PC005 was closely related to the *Hae. pacayae* sequences identified as KF049495, and KF0449507 (group 2, with a mean distance within the group of 0.003 ± 0.001; Fig. 3C, and Supplementary Fig. S1). Genetic distances of 0.02 ± 0.006 were obtained between GERPH: PC005 and sequences named as *Hae. pacayae s.l.,* group 1 (KF049506, KF049509, and KF049513). As a comparison, the genetic distance within all available sequences identified as *Hae. pacayae s.l.* is an order of magnitude greater than the ones estimated using the haplotypes for other well-known species like *Hae. kopki*, *Hae. ptyodactylii*, *Hae. mesnili*, *Hae. anatolicum* and *Hae. caucasica* (see Fig. 3C, Supplementary Fig. S1). Given that the genetic distance between GERPH: PC005 lineage and the sequences named as *Hae. pacayae s.l.,* group 1 (KF049506, KF049509, and KF049513) was 0.02 ± 0.006, the lineage GERPH: PC005 could be considered as a new species; however, there is still not enough molecular data that can support this hypothesis. Nevertheless, a detailed description of this parasite is given.

3.3. Morphological description Haemocystidium (Simondia) sp.

Parasites do not induce hypertrophy of the red blood cells (morphometrics have been provided in Table 2). The morphological description is based on GERPH: PC005.

3.3.1. Young gametocytes

The outline of gametocytes is not amoeboid (even); the shape varies from circular (33%), oval (30%) or ellipsoid (37%). The presence of a large vacuole can be observed in the youngest gametocytes (Fig. 4A–D). As the gametocyte develops, it acquires a doughnut shape (Fig. 4B–E), the vacuole in some instances is big, so the morphology of the gametocyte resembles the ring stage typical in *Plasmodium falciparum* (Fig. 4E). In at least 12.5% of the cases, it was possible to observe a structure similar to a parasitophorous vacuole (PV) described in other haemoparasites. In these cases, the parasite and PV together measured between 5.87 and 30.12 μm^2 (Fig. 4F).

3.3.2. Microgametocytes

Microgametocytes are rounded (9.39 × 5.88 μm), the pigment granules are small, and distributed both randomly and at the poles of the parasite (Fig. 4G). The parasite nucleus is diffuse, located at the periphery. Occasionally, small vacuoles can be observed in the parasite (Fig. 4G). The proportion of macrogametocytes:microgametocytes was 1:2.

3.3.3. Macrogametocytes

Cytoplasm is granular in appearance and pigment granules may form clumps (Fig. 4H). Macrogametocytes might have small vacuoles (Fig. 4H), but in advanced stages, vacuoles are conspicuous (Fig. 4H). Nucleus is compact and located laterally. We did not find significant differences ($p < 0.001$) at the morphometric level between *Haemocystidium* sp., and *Hae.* (*S.*) *pacayae*, and *Hae.* (*S.*) *peltocephali* (Table 2). However, the genetic distance of the *cytb* fragments between *Hae.* (*Simondia*) sp. (GERPH: PC005) and *Hae. pacayae* (e. g., KF049506) is 0.02, and *Hae.* (*Simondia*) sp. with *Hae.* (*S.*) *peltocephali* (KF049492) is 0.04 (Fig. 3B and C).

4. Discussion

The reports of *Haemocystidium* sp. worldwide are limited. Thus this study represents the third report of species belonging to this genus in South America and the first in Colombian Podocnemidids. It is important to highlight that the overall prevalence of *Haemocystidium* found in *P. vogli* from Colombia (2.04%, 3/147) is lower than that previously reported by Pineda-Catalan et al. (2013) in *P. expansa* of 12.5% (12/96) and *P. unifilis* of 9.5% (13/136). These differences could be explained by the use of molecular diagnostic methods used by Pineda-Catalan et al. (2013). These authors detected low parasitaemia by microscopy and a maximum of eight parasitic forms by blood smear (Pineda-Catalan et al., 2013). Low parasitemia was also detected in our samples. However, estimating prevalence through microscopy can underestimate the true rates of infection, mainly in cases of chronic (Jarvi et al., 2002) or mild infection.

The prevalence reported here is low, even when compared to similar works that use microscopy as the only detection method such as in the case of *Pe. dumerilianus*, where a 50% prevalence was reported (Lainson and Naiff, 1998). In other organisms such as geckos, the reported prevalence is comparable to this study's findings, being 6.7% in *Lygodactylus capensis grotei* (Telford, 2005). The parasitaemia observed in the current study is low compared to those reported for Omani geckos (0.3%, Maia et al., 2016). Despite this, our results are considerably lower when compared to the parasitaemias reported in *Hae. lygodactyli* in acute (56.4%) and chronic infection (20.2%) (Telford, 2005) or in *Elseya latisternum* (between 1 and 50%) (Jakes et al., 2001). Low parasitaemia could prevent the uptake of the vector of the two sexual forms of gametocytes during feeding, thus affecting the transmission of the parasite. Although the tabanid fly *Chrysops callidus* (Diptera: Tabanidae) was shown as a vector for *Hae. metchnikovi* in the turtle *Chrysemys picta* in North America (DeGiusti et al., 1973), the vectors of these blood parasites in South America are unknown. Studies aimed at identifying the vector could help to decipher the host-parasites relationship between *Haemocystidium* and *Podocnemis*.

Of the eight extant species of the Podocnemididae (Rhodin et al., 2017), *Podocnemis vogli* has a relatively narrow distribution; nevertheless, it is sympatric with *Podocnemis expansa*, *Podocnemis unifilis* and *Peltocephalus dumerilianus* in several regions of the Amazon and

Table 2
Morphometrical features of *Haemocystidium* (*Simondia*) complex species described in South America and their hosts. Measurements of infected and non-infected erythrocytes are shown.

Characteristic	*Hae.* (S) PC004 and PC006	*Hae.* (*S*) sp. PC005	*Hae.* (*S.*) *pacayae* Pineda-Catalan et al. (2013)	*Hae.* (*S.*) *peltocephali* Pineda-Catalan et al. (2013)	*H. peltocephali* (Lainson and Naiff, 1998)
Host	*Podocnemis vogli*		*P. expansa*	*P. unifilis*	*Peltocephalus dumerilianu*
	This study				
Non-infected erythrocytes	*n = 15*	*n = 15*	–	–	–
Length	22.5 ± 1.4	20.51 ± 2.92	–	–	–
	(18.9–24.6)	(18.97–26.02)	–	–	–
Wide	14.1 ± 1.2	14.86 ± 1.33	–	–	–
	(11.4–16.0)	(11.79–17.46)	–	–	–
Area	251.3 ± 28.1	258.86 ± 21.14	–	–	–
	(214.5–310.6)	(219.63–292.91)	–	–	–
Nucleus					
Length	5.9 ± 0.7	5.66 ± 0.45	–	–	–
	(5.0–7.2)	(5.05–6.12)	–	–	–
Wide	4.2 ± 0.5	4.03 ± 0.39	–	–	–
	(3.7–5.3)	(3.71–4.52)	–	–	–
Area	20.8 ± 2.7	18.30 ± 4.05	–	–	–
	(15.9–26.4)	(12.86–27.66)	–	–	–
Microgametocyte	*n = 1*	*n = 4*	*n = 12*	*n = 6*	
Length	10.44	8.44 ± 1.29	10.86 ± 2.30	10.11 ± 0.97	6.7–12.5
	–	(7.56–10.30)	(5.91–14.13)	(9.01–11.54)	
Wide	7.14	4.84 ± 0.44	6.87 ± 0.94	8.81 ± 1.04	6.0–9.0
	–	(4.46–5.47)	(5.36–8.31)	(7.01–9.91)	–
Area	67.54	41.09 ± 6.61	64.64 ± 19.77	74.73 ± 17.32	–
	–	(36.24–50.29)	(26.67–92.74)	(62.16–98)	–
No Pigment granules	> 10	> 10 Aggregated	–	–	12–30
	n = 5	*n = 3*	–	–	Average 21
	0.42 ± 0.08	0.31 ± 0.11	–	–	n = 17
	(0.30–0.53)	(0.16–0.54)	–	–	
Number of vacuoles			0.58 ± 1	0	
Nucleus					
Length	5.42	–	–	–	–
	–	–	–	–	–
Wide	0.65	–	–	–	–
	–	–	–	–	–
Area	0.35	–	–	–	–
	–	–	–	–	–
Macrogametocytes	*n* = 2	*n = 4*	*n = 19*	*n = 19*	
Length	9.45 ± 0.05	10.63 ± 1.23	11.03 ± 3.16	10.07 ± 1.77	7.4–12.6
	(9.41–9.48)	(9.38–11.55)	(5.22–18.85)	(6.15–14.07)	
Wide	6.12 ± 0.19	5.94 ± 0.95	7.68 ± 2.25	8.95 ± 1.41	6.2–11.1
	(5.99–6.26)	(5.26–7.23)	(4.70–12.76)	(5.69–11.11)	
Area	47.56 ± 2.88	52.01 ± 11.61	73.31 ± 32.29	76.84 ± 24.34	–
	(45.52–49.59)	(40.47–65.89)	(22.92–134.83)	(28.11–130.92)	
Nº. Maximum of pigment granules	> 10	> 10 Aggregated	–	–	15–20
	n = 6	*n = 8*	–	–	
	0.28 ± 0.03	0.27 ± 0.05	–	–	
	(0.20–0.31)	(0.21–0.38)	–	–	
Number of vacuoles		1.25 ± 0.5 (1–2)	–	–	3
Nucleus	*n* = 2	*n = 3*	–	–	–
Length	5.78 ± 2.19	2.79 ± 0.76	–	–	–
	(4.23–7.34)	(2.25–3.33)	–	–	–
Wide	0.67 ± 0.32	1.26 ± 0.16	–	–	–
	(0.44–0.89)	(1.14–1.37)	–	–	–
Area	3.12 ± 0.03	3.46 ± 1.38	–	–	–
	(3.10–3.14)	(2.18–5.27)	–	–	–
Infected erythrocytes	*n = 3*	*n = 8*	–	–	–
Length	23.40 ± 2.16	22.01 ± 2.12	–	–	–
	(20.43–26.38)	(19.67–25.53)	–	–	–
Wide	14.07 ± 0.61	14.71 ± 1.41	–	–	–
	(13.32–14.74)	(11.81–15.98)	–	–	–
Area	261.32 ± 21.00	262.88 ± 33.21	–	–	–
	(224.95–325)	(219.18–325.06)	–	–	–
Nucleus					
Length	5.63 ± 0.23	5.61 ± 0.82	–	–	–
	(5.30–5.90)	(4.53–6.74)	–	–	–
Wide	4.24 ± 0.29	4.04 ± 0.41	–	–	–
	(3.95–4.67)	(3.63–4.60)	–	–	–
Area	19.41 ± 0.76	19.54 ± 2.24	–	–	–
	(18.51–20.33)	(17.41–24.45)	–	–	–

Orinoquia (Rueda-Almonacid et al., 2007), which could explain, at least in part, the presence of *Haemocystidium* spp. in this family of turtles. It is important to emphasize that the species belonging to the Podocnemididae share habitat with species of other families of semi-aquatic turtles such as *Kinosternon scorpioides* (Kinosternidae) and also with tortoises such as *Chelonoidis carbonaria* and *Ch. denticulata* (Testudinidae), which were negative for microscopic infection by this parasite, however, the sampling of these species was limited. Based on the data analyzed to date, it would appear that *Haemocystidium* shows specificity for *Podocnemididae* species with the cis-Andean distribution. The absence of detectable *Haemocystidium* infections in *Podocnemis lewyana* ($n = 17$) and *Trachemys venusta callirostris* ($n = 11$), which are distributed along the basins of the Magdalena and Sinú rivers in Colombia, and in trans-Andean individuals of *Chelonoidis carbonaria* ($n = 6$) and *Kinosternon scorpioides* ($n = 3$), could be associated with the distribution of these turtle species and the absence of the possible vector. Lainson and Naiff (1998) reported the presence of *Hae. geochelonis* in a single individual *Ch. denticulata*. More testudinids from both sides of the Andean cordilleras should be screened to test this hypothesis that *Haemocystidium* shows specificity for *Podocnemididae* species with the cis-Andean distribution. In birds, for example, the *Leucocytozoon* distribution in Colombia is restricted to the highlands (Matta et al., 2014; Lotta et al., 2016), probably also associated with the abundance and distribution of the vectors.

The low prevalence of haemosporidian (1.55%) reported in this study contrasts with the high prevalence of haemogregarines (84.53%) found in the same group of samples analyzed. Yet, for the latter, the vector is also unknown; however, leeches as vectors have been reported before (Telford, 2009). Our results are consistent with other studies conducted in testudines, where the most commonly reported parasite is *Haemogregarina* sp. (Rossow et al., 2013; Soares et al., 2014; Úngari et al., 2018).

Concerning haemoproteids (*Haemocystidium* and previously named *Haemoproteus*), 16 species have been reported to date in reptiles (Supplementary Table 1). The genus *Haemocystidium* is characterized by the presence of malarial pigment (a trait shared with species of the genera *Plasmodium* and *Haemoproteus*) and the absence of peripheral blood erythrocytic meronts (a trait that it shares with the genus *Haemoproteus*, but that set it apart from species of the genus *Plasmodium*). Regardless of these differences, species of *Haemocystidium* in reptiles have been described as *Haemoproteus* spp. in the past (Lainson and Naiff, 1998; Jakes et al., 2001; Javanbakht et al., 2015). However, the phylogenetic analysis of the c*ytb* gene sequences previously reported (Pineda-Catalan et al., 2013; Maia et al., 2016) and those obtained in this study (Fig. 3), showed that *Haemoproteus* species infecting birds and parasites of the genus *Haemocystidium* are two distinct monophyletic groups. However, limited sampling, that results in the lack of taxa from reptilian hosts, does not allow for the elucidation of whether these are two monophyletic groups that support the subdivision of the genus *Haemocystidium* into two subgenera: *Simondia* infecting chelonians and *Haemocystidium* isolated from the Squamata as suggested by Pineda-Catalan et al. (2013) and Maia et al. (2016). Although we found that the turtle parasites could form a monophyletic group (*Simondia*), the host information of the *Haemocystidium* sp. (MH177855), included in this study's phylogenetic analyses, is not available, so it is not possible to explore this issue with the available data.

Nevertheless, our results suggest that species of *Haemocystidium* described in turtles appear to be part of four differentiated clades according to their geographical distribution: a clade formed by the species reported in Iran (*Testudo* sp.), another by the African species (*Kinixys erosa* and *Pelusios castaneus*), and two clades with the species identified in South America (*Podocnemis* sp.) (Fig. 3A, Supplementary Fig. S1). Regarding the clade of species that infect lizards and snakes, Telford (1996), analyzed tissues infected with *Hae. kopki* and *Hae. ptyodactylii*, supporting the status of the genus *Haemocystidium*. This was based on the meronts of these species not forming pseudocytomers, which is unlike species of *Haemoproteus* parasitizing birds or *Haemoproteus mesnili* (described in cobras). However, as shown in Fig. 3A, the three species mentioned above are part of the same clade, suggesting that, despite the difference in merogony, these species seem to belong to the proposed subgenus *Haemocystidium*.

The results obtained in this study contrast with those published by Boundenga et al. (2016, 2017), where the authors reported the presence of haemosporidian in *K. erosa* and *Pel. castaneus* in Gabon, Africa. In these studies, *Haemocystidium* appears as a sister clade of the genera *Leucocytozoon* and *Haemoproteus* (*Parahaemoproteus*). Likewise, new species of *Haemoproteus* have been described in birds, such as *Haemoproteus catharti* (from the Turkey vulture) and *Haemoproteus* sp. from *Mycteria americana* (Wood Stork), which constitutes a separate clade from the other *Haemoproteus* spp. of birds. These two *Haemoproteus* species appear closely related to the genus *Plasmodium* in this study. This indicates the need to extend the sampling to new taxa of both birds and reptiles in order to improve the resolution of the phylogenetic relationships of haemosporidian parasites.

It is important to highlight that the sequences identified in the individuals GERPH: PC004 and GERPH: PC006 are identical to *Hae. pacayae* (KF049506) reported by Pineda-Catalan et al. (2013), extending the geographic and host range for this species. *Haemocystidium* (*Simondia*) sp. (GERPH: PC005) is morphologically indistinguishable from *Haemocystidium peltocephali* (KF049492) and *Hae. pacayae* (KF049506) (Pineda-Catalan et al., 2013); however, the genetic distance of GERPH: PC005 with these latter species, was 0.044 and 0.02, respectively (Fig. 3B). Pineda-Catalan et al. (2013) reported several lineages for *Hae. pacayae,* among them the lineages KF049495 and KF049507; however, with these latter lineages, the genetic distance with GERPH: PC005 is only 0.006 (Fig. 3B). For that reason, although the lineages KF049495 and KF049507 were previously assigned as *Hae. pacayae*, our results suggested that both of these lineages and GERPH: PC005 could be another *Haemocystidium* (*Simondia*) species; however, more molecular markers are needed in order to support this hypothesis.

There are only reports of *Haemocystidium* sp. for *Pe. dumerilianus* and *Podocnemis* spp. (distributed in the North of South America) among the three genera belonging to the family Podocnemididae. Characteristics such as the presence/absence of vacuoles, nucleophile growth of the parasite, presence of a parasitophorous vacuole (PV), the distribution of granules in the cytoplasm of macro- and microgametocytes and the presence of amoeboid gametocytes, can be used as features that support the diagnosis in this genus (e.g., to gametocytes *Hae. peltocephali* in peripheral blood). Nevertheless, species differentiation and identification are difficult, as many characters overlap. Species described in other reptiles may be differentiated from those described in Podocnemidids due to their size, where macro and microgametocytes surround the nucleus of the host cell in Testudines: *H. metchnikovi* (Simond, 1901), *H. testudinalis* (Cook et al., 2010) synonyms *Haemamoeba testudinis* (Laveran, 1905) and *H. chelodinae* (Mackerras, 1961; Jakes et al., 2001); in snakes: *H. mesnili* (Telford, 2007) and in lizard: *Hae. papernae*, *Hae. quettaensis* (Telford, 1996), and *H. lygodactyli* (Telford, 2005).

The species concept in haemosporidian is still a matter of debate. The concomitant use of morphological, morphometric, and genetic tools is ideal at present for addressing this issue (Martinsen et al., 2006; Hellgren et al., 2007; Perkins et al., 2011; Pacheco et al., 2013; Outlaw and Ricklefs, 2014). To date, the delimitation of species continues to be controversial, e.g. for the genus *Haemoproteus* (in birds), Hellgren et al. (2007) suggested that *cytb* lineages with a genetic distance > 5% are associated with morphologically differentiable species; however, the same authors report that the species *H. minutus* and *H. pallidus* (morphologically differentiable) differ by 0.7%, while lineages associated with morphospecies *H. balmorali* show a difference of 2.7%. Recently, Galen et al. (2018) through an integrative approach, found cryptic species of *Leucocytozoon* with a minimum difference in a single base pair in mDNA (approx. 0.2% divergence), and genetic distances for the nuclear markers between 1.07 and 5.19%.

Table 3
Genetic distances reported in haemosporidian species, using *cytb* gene or mtDNA. Presence/absence of cryptic species is indicated by yes or no.

Authors	Genetic Markers	Size fragment	Species 1	Species 2	Host	Distance	Cryptic species
Escalante et al. (1998)	*cytb*	1035 bp	*Plasmodium gallinaceum*	*Plasmodium elongatum*	Specie 1: *Gallus gallus* Specie 2: *Passer domesticus*	0.034	No
Perkins (2000)	*cytb*	540 bp	*Plasmodium azurophilum* red blood cell	*Plasmodium azurophilum* while blood cell	*Anolis gundlachi*	0.031	Yes
Palinauskas et al. (2015)	*cytb*	479 bp	*Plasmodium homocircumflexum*	*Plasmodium circumflexum*	Species 1: *Lanius collurio* Species 2: *Acrocephalus scirpaceus*	0.055	Yes
Valkiūnas et al. (2010)	*cytb*	706 bp	*Leucocytozoon mathisi*	*Leucocytozoon buteonis*	Species 1: *Accipiter nisus* Species 2: *Buteo jamaicensis*	0.109	Yes
Pacheco et al. (2013)	Complete mtDNA	Species 1: 5949 bp Species 2: 5966 bp	*Plasmodium falciparum*	*Plasmodium reichenowi*	Species 1: *Homo sapiens* Species 2: *Pan troglodytes*	0.025	No
	Complete mtDNA	Species 1: 5990 bp Species 2: 5991 bp	*Plasmodium vivax*	*Plasmodium cynomolgi*	Species 1: *Homo sapiens* Species 2: *M. radiata, Presbytis entrellus,*	0.012	No
Mantilla et al. (2013)	Complete mtDNA	Species 1: 5889 bp Species 2: 6003 bp	*Plasmodium lutzi*	*Plasmodium relictum*	Species 1: *Turdus fuscater* Species 2: *Zenaida macroura*	0.018	No
Walther et al. (2014)	*cytb*	Species 1: 478 bp Species 2: 468 bp	*Plasmodium homopolare*	*Plasmodium parahexamerium*	Species 1: *Melospiza melodia* Species 2: *Alethe diademata*	0.036	No
Muehlenbein et al. (2015)	Complete mtDNA	Species 1: 5957 bp Species 2: 5976 bp	*Plasmodium. knowlesi*	*Plasmodium coatneyi*	Species 1: *Macaca fascicularis* Species 2: Macaca mulatta	0.032	No
Maia et al. (2016)	*cytb*	Species 1: Species 2: 607 bp	*Hae.* sp. (S7155)	*Hae.* sp. (EU254531)	Species 1: *Hemidactylus luqueorum* Species 2: *Ergenia stokesii*	0.074	No
van As et al. (2016)	*cytb*	500 pb	*Plasmodium. zonuriae*	*Plasmodium intabazwe*	Species 1: *Cordylus vittifer* Species 2: *Pseudocordylus melanotus*	0.034	No
Matta et al. (2018)	*cytb*	1039 bp	*Plasmodium carmelinoi*	*Plasmodium kentropyxi*	Species 1: *Ameiva ameiva* Species 2: *Cnemidophorus cf. gramivagus*	0.02	No

Descriptions of parasite species based on morphology, host, or life-history traits have been shown to be ambiguous and inconsistent from the early days of molecular phylogenetics in Haemosporida (e.g., Escalante et al., 1998). Nowadays, an integrative taxonomic approach using morphometry, nuclear and plastids molecular markers, as well as ecological features, are essential tools in the description of these parasite species; in birds, for example, parasite morphometrical characteristics have been shown to vary according to the host (e.g. *Plasmodium lutzi* initially reported in *Aramides cajaneus* and later isolated in *Turdus fuscater* (Mantilla et al., 2013); both quite different in morphometrical measurements). The analyses of molecular markers have allowed elucidation of a large number of cryptic species (Table 3), however, those studies that use only molecular tools, usually lose valuable information such as the detection of co-infections and abortive infections (false positives), as well as morphological characters (Valkiūnas et al., 2008).

Currently, an association has been sought between the results of morphological, morphometric and genetic information analyses; this is how Hellgren et al. (2007) and Valkiūnas et al. (2009) suggested that species belonging to the genus *Haemoproteus* with a genetic distance of 0.05 in the *cytb* gene correspond to different species. Nevertheless, species such as *Haemoproteus minutus* and *Haemoproteus pallidus* (morphologically distinguishable) have only a genetic distance of 0.01. For the genus *Plasmodium* in lizards, species with a genetic distance of 0.03 may be indicative of different species, despite their morphological similarity (Perkins, 2000). Even in *Plasmodium* species, which are morphologically differentiable, there are reports of genetic distances, which vary from 0.012 to 0.034 (Escalante et al., 1998; Pacheco et al., 2013). Table 3 shows the published genetic differences for cryptic and non-cryptic species, showing high variability in their distances. Based on the above, we suggest that the genus *Haemocystidium* infecting Podocnemidids could be a complex of cryptic species.

Considering the above, the genus *Haemocystidium* is perhaps one of the most representative examples of the importance of morphological and molecular characterization, especially when considering the difficulties in the differentiation of species based solely on their morphological characters. Also, we invite to extend studies on the characterization of life cycles of haemoparasites in reptiles, and the generation of new knowledge that contributes to the elucidation of the phylogenetic relationships of haemosporidian parasites and their vectors.

5. Conclusions

The molecular lineages linked to morphological characteristics and the phylogenetic analyses reported here support that *Haemocystidium* genus (Castellani, and Willey, 1904) is a monophyletic group separated

from other haemosporidian groups. Our study extends the geographical and host range for the genus in South America. Future studies are necessary to identify the effects of the infection, its prevalence in the vertebrate host, and the vectors associated with its transmission. To date, the species of *Haemocystidium*, (at least in Podocnemidids) cannot be differentiated using solely morphological characters. Therefore, additional genetic and ecological information is needed to assess the diversity of *Haemocystidium* and to support species delimitation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2019.10.003.

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