Haemocystidium spp., a species complex infecting ancient aquatic turtles of the family Podocemididae: First report of these parasites in Podocenemis 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 Podocenemis 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 (Danielwsky 1885) are classified into the families Leucocytozoidea (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 et al., 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.; Plasmodiidae, genus Plasmodium sp.; Garniidae, genera Garnia sp. and Fallisia sp. (Levine, 1970; Adl et al., 2012, 2019;

Abbreviations: H: Haemoproteus, Hae: Haemocystidium
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Perkins, 2014); and the family Leucocytozoidae, genus *Saurocytozoon* sp. (Lainson et al., 1974). Among those, the genus *Haemocystidium* has been one of the last 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 (Javanabhkti et al., 2015). New molecular lineages attributed to this genus have been reported in samples from the gekko species *Hemidactylus luquorum* and *Pyodactylus hassequisitt* captured in Oman, Asia (Maia et al., 2016), 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.) peltocephali* 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 haemoporidians 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 (Ministerio del Medio Ambiente, Vivienda y Desarrollo Territorial, 1998; Castellano-Mora, 2002; Rueda-Almonacid et al., 2007). The few studies conducted on Testudines have focused on their microorganisms such as bacteria (Pachón, 2009) or pathological studies (Ministerio del Medio Ambiente, Vivienda y Desarrollo Territorial, 1998; Castellano-Mora, 2002; Rueda-Almonacid et al., 2007). Belonging to the *Chelonoididae* family, *Chelonoidis carbonaria* is a terrestrial tortoise with a broad distribution that includes Venezuela, Colombia, Brazil, Bolivia, Paraguay, and Argentina (Castano-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 subcaparacal 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. 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 dahlia* is a Colombian trans-Andean endemic species that, except for the other species belonging to this family (genera *Chelus, Platymes, Rhinemys, and Phrynops*), is distributed in the tropical dry forest along the Caribbean, inhabiting shallow waters with floating vegetation (Castano-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 Guianas, Brazil, Bolivia, Paraguay, and Argentina (Castano-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).

**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.
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 MgCl₂, 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

| Name                  | Common name                  | Location                  | Sample size | NCI |
|-----------------------|------------------------------|----------------------------|-------------|-----|
| Podocnemidae          | Yellow-spotted River Turtle  | Cravo Norte (Arauca)      | 3           | 0   |
| Chelidae              | Dahl's Toad-headed Turtle    | Lorica (Cordoba)          | 3           | 1   |
| Kinosternidae         | Scorpion Mud turtle         | Lorica (Cordoba)          | 3           | 1   |
| Trachemys venusta callirostris | Colombian slider         | Savanna Side-necked Turtle | 11          | 4   |
| Rhinoclemmys melanosterna | Colombian Wood Turtle      | Yondó (Antioquia)         | 6            | 5   |
| Chelonoidis carbonaria | Red-footed Tortoise        | Yondó (Antioquia)         | 6            | 5   |
| Chelonoidis denticulata | Red-footed Tortoise        | Paz de Ariporo (Casanare) | 3            | 1   |
| Podocnemis unifilis   | Yellow-headed Side-neck Turtle | Cravo Norte (Arauca)      | 6°14′30.84″N 70°14′31.05″W |
| Podocnemis vogli      | Yellow-spotted Side-neck Turtle | Lorica (Cordoba)          | 17           | 4   |
| Kinosternum scorpioides | Scorpion Mud turtle       | Lorica (Cordoba)          | 3            | 1   |
| Trachemys venusta callirostris | Colombian slider         | Savanna Side-necked Turtle | 11          | 4   |
| Rhinoclemmys melanosterna | Colombian Wood Turtle      | Yondó (Antioquia)         | 6            | 5   |
| Chelonoidis carbonaria | Red-footed Tortoise        | Yondó (Antioquia)         | 6            | 5   |
| Chelonoidis denticulata | Red-footed Tortoise        | Paz de Ariporo (Casanare) | 3            | 1   |
| Podocnemis unifilis   | Yellow-headed Side-neck Turtle | Lorica (Cordoba)          | 6°14′30.84″N 70°14′31.05″W |

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...
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; 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, (I) coinfection with a gamont of Haemogregarina, (G) microgametocyte, and (H) macrogametocyte. Haemocystidium (S.) pacayae (GERPH: PC004-PC006) identified in P. vogli (I–L). (I) Young gametocyte, (K) microgametocyte, and (L) macrogametocyte. Bold black arrow: hemoglobin 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 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* (KFO49506, KFO49509, and KFO49513) 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 cytb 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 KFO49506, KFO49509, and KFO49513 (Pineda-Catalan et al., 2013). The lineage found in the sample GERPH: PC005 was closely related to the *Hae. pacayae* sequences identified as KFO49495, and KFO4949507 (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 (KFO49506, KFO49509, and KFO49513). 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 (KFO49506, KFO49509, and KFO49513) 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 (morphological data that can support this hypothesis. Nevertheless, a detailed description of this parasite is given.

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 μm² (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.) peltoccephali* (Table 2). However, the genetic distance of the cytb fragments between *Hae. (Simondia)* sp. (GERPH: PC005) and *Hae. pacayae* (e. g., KFO49506) is 0.02, and *Hae. (Simondia)* sp. with *Hae. (S.) peltoccephali* (KFO49492) 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 Podocnemids. It is important to highlight that the overall prevalence of *Haemocystidium* found in *P. vogli* from Colombia (2.64%, 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 parasitaemia was also detected in our samples. However, estimating prevalence through microscopy can under-estimate 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* (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 *Peltoccephalus 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) parvus 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 dumerilii                         |
| **This study**|                           |                      |                                             |                                                 |                                          |
| **Non-infected erythrocytes** |                           |                      |                                             |                                                 |                                          |
| n = 15        | 22.5 ± 1.4                | 20.51 ± 2.92         | (18.9–24.6)                                 | (18.97–26.02)                                  |                                          |
| Length        | (11.4–16.0)               | (11.79–17.46)        |                                             |                                                 |                                          |
| Wide          | 251.3 ± 28.1              | 258.86 ± 21.14       |                                             |                                                 |                                          |
| Area          | (214.5–310.6)             | (219.63–292.91)      |                                             |                                                 |                                          |
| **Nucleus**   |                           |                      |                                             |                                                 |                                          |
| Length        | 5.9 ± 0.7                 | 5.66 ± 0.45          |                                             |                                                 |                                          |
| Wide          | 4.2 ± 0.5                 | 4.03 ± 0.39          |                                             |                                                 |                                          |
| Area          | 20.8 ± 2.7                | 18.30 ± 4.05         |                                             |                                                 |                                          |
| **Microgametocyte** |                           |                      |                                             |                                                 |                                          |
| n = 1         | 10.44                     | 8.44 ± 1.29          | 10.86 ± 2.30                                | 10.11 ± 0.97                                   | 6.7–12.5                                 |
| Length        | (7.56–10.30)              | (5.91–14.13)         | (9.01–11.54)                                | (7.01–9.91)                                    |                                          |
| Wide          | 67.54                     | 41.09 ± 6.61         | 64.64 ± 19.77                               | 74.73 ± 17.32                                  |                                          |
| Area          | (36.24–50.29)             | (26.67–92.74)        | (62.16–98)                                  |                                                 |                                          |
| **No Pigment granules** |                           |                      |                                             |                                                 |                                          |
| > 10          | > 10                      | Aggregated           |                                             |                                                 | 12–30                                    |
| n = 5         |                           |                      |                                             |                                                 |                                          |
| 0.42 ± 0.08   |                           | 0.31 ± 0.11          |                                             |                                                 |                                          |
| (0.30–0.53)   |                           | (0.16–0.54)          |                                             |                                                 |                                          |
| **Number of vacuoles** |                           |                      |                                             |                                                 |                                          |
| 0.58 ± 1      |                           | 0                   |                                             |                                                 |                                          |
| **Nucleus**   |                           |                      |                                             |                                                 |                                          |
| Length        | 5.42                      |                      |                                             |                                                 |                                          |
| Wide          | 0.65                      |                      |                                             |                                                 |                                          |
| Area          | 0.25                      |                      |                                             |                                                 |                                          |
| **Macrogametocytes** |                           |                      |                                             |                                                 |                                          |
| n = 2         | 9.45 ± 0.05               | 10.63 ± 1.23         | 10.07 ± 1.77                                | 10.74 ± 1.29                                   | 7.4–12.6                                 |
| Length        | (9.41–9.48)               | (9.38–11.55)         | (6.15–14.07)                                | (5.99–11.11)                                   |                                          |
| Wide          | 4.12 ± 0.19               | 5.94 ± 0.95          | 7.68 ± 2.25                                 | 8.95 ± 1.41                                    | 6.2–11.1                                 |
| 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)             | (22.92–130.92)       | (28.11–130.92)                               |                                                 |                                          |
| **Nº. Maximum of pigment granules** |                           |                      |                                             |                                                 |                                          |
| > 10          | > 10                      | Aggregated           |                                             |                                                 | 15–20                                    |
| n = 6         |                           |                      |                                             |                                                 |                                          |
| 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) |                        |                      |                                             |                                                 |                                          |
| **Nucleus**   |                           |                      |                                             |                                                 |                                          |
| Length        | 5.78 ± 2.19               | 2.79 ± 0.76          |                                             |                                                 |                                          |
| Wide          | 0.67 ± 0.32               | 1.26 ± 0.16          |                                             |                                                 |                                          |
| Area          | 3.12 ± 0.03               | 3.46 ± 1.38          |                                             |                                                 |                                          |
| (3.10–3.14)   | (2.18–5.27)               |                      |                                             |                                                 |                                          |
| **Infected erythrocytes** |                           |                      |                                             |                                                 |                                          |
| n = 3         | 23.40 ± 2.16              | 22.01 ± 2.12         |                                             |                                                 |                                          |
| Length        | (20.43–26.38)             | (19.67–25.53)        |                                             |                                                 |                                          |
| Wide          | 14.07 ± 0.61              | 14.71 ± 1.41         |                                             |                                                 |                                          |
| 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          |                                             |                                                 |                                          |
| Wide          | 4.24 ± 0.29               | 4.04 ± 0.41          |                                             |                                                 |                                          |
| 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 lewuya (n = 17) and Trachemys venusta californiensis (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 Ha. geochelonis in a single individual Ch. denticulata. More testudinids from both sides of the Andean corridors 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 (Mattá 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; Ungari 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 cyt b 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. (MH1777855), 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. 3). Regarding the clade of species that infect lizards and snakes, Telford (1996), analyzed tissues infected with Ha. kopiti and Ha. ptyodactylti, 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 Mysteria 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 0.066 (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 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 ameboïd 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 Podocnemids 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 Haemamaeba testudinis (Laveran, 1905) and H. chelodinae (Mackerras, 1961; Jakes et al., 2001); in snakes: H. mesnili (Telford, 2007) and in lizard: Hae. papernae, Hae. quetaensis (Telford, 1996), and H. bryodactylti (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 (Martienssen 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 cyt b lineages with a genetic distance > 5% are associated with morphologically differentiable species; however, the same authors report that the species H. minitus 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 | Species 1: Gallus gallus<br>Species 2: Passer domesticus | 0.034 | No |
| Perkins (2000) | cytb | 540 bp | Plasmodium azurophilum red blood cell | Plasmodium azurophilum white blood cell | Anolis gundlachi | 0.031 | Yes |
| Palinauskas et al. (2015) | cytb | 479 bp | Plasmodium homocircumflexum | Plasmodium circumflexum | Lanius collurio<br>Species 2: Accipiter nisus<br>Species 2: Buteo jamaicensis | 0.055 | Yes |
| Valkiūnas et al. (2010) | cytb | 706 bp | Leucocytozoon mathisi | Leucocytozoon buteoni | Species 1: Accipiter nisus<br>Species 2: Buteo jamaicensis | 0.109 | Yes |
| Pacheco et al. (2013) | Complete mtDNA | Species 1: 5949 bp<br>Species 2: 5966 bp | Plasmodium falciparum<br>Plasmodium vivax | Plasmodium reichenowi<br>Plasmodium cynomolgi | Homo sapiens<br>Pan troglodytes | 0.025 | No |
| Mantilla et al. (2013) | Complete mtDNA | Species 1: 5889 bp<br>Species 2: 6003 bp | Plasmodium falciparum<br>Plasmodium vivax<br>Plasmodium knowlesi | Plasmodium reichenowi<br>Plasmodium cynomolgi<br>Plasmodium knowlesi | Homo sapiens<br>Zenaida macroura<br>Atele diadema | 0.012 | No |
| Walther et al. (2014) | cytb | 478 bp<br>Species 2: 468 bp | Plasmodium homopolar<br>Plasmodium petterhageni | Plasmodium reichenowi<br>Plasmodium cynomolgi | Homo sapiens<br>Atele diadema | 0.036 | No |
| Muehlenbein et al. (2015) | Complete mtDNA | Species 1: 5957 bp<br>Species 2: 5976 bp | Plasmodium. knowlesi<br>Haemoproteus species<br>Haemoproteus species<br>Haemoproteus<br>Haemoproteus<br>Haemoproteus | Plasmodium reichenowi<br>Plasmodium cynomolgi<br>Plasmodium cynomolgi<br>Plasmodium reichenowi<br>Plasmodium reichenowi<br>Plasmodium reichenowi | Homo sapiens<br>Musca domestica<br>Musca domestica<br>Musca domestica<br>Musca domestica<br>Musca domestica | 0.032 | No |
| Maia et al. (2016) | cytb | 607 bp | Haemoproteus species<br>Plasmodium. sp. | Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium | Homo sapiens<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus | 0.074 | No |
| van As et al. (2016) | cytb | 500 pb | Plasmodium. sp. | Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium<br>Plasmodium | Homo sapiens<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus<br>Hemidactylus | 0.034 | No |
| Matta et al. (2018) | cytb | 1039 bp | Plasmodium cabreroi | Plasmodium kentropyxi | Anolis gundlachi<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus<br>Acrocephalus scirpaceus

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 plastid 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 lutzii initially reported in Aramides cauanes and later isolated in Turdus fluscater (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 minuta 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 Podocnemididae 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...
