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A new haemosporidian parasite from the Red-legged Seriema *Cariama cristata* (Cariamiformes, Cariamidae)

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ABSTRACT

Haemoproteids (Haemosporida, Haemoproteidae) are a diverse group of avian blood parasites that are transmitted by hematophagous dipterans. In this study, we describe *Haemoproteus pulcher* sp. nov. from a Red-legged Seriema (*Cariama cristata*) in southeast Brazil. Analysis of the mitochondrial *cytb* gene indicates this parasite is closely related to *Haemoproteus catharti* (from Turkey Vulture, *Cathartes aura*) and the unidentified haemosporidian lineages PSOOCH01 (from Pale-winged Trumpeter, *Psophia leucoptera*) and MYCAME08 (from Wood Stork, *Mycteria americana*). This group of parasites appears to represent an evolutionary lineage that is distinct from other *Haemoproteus* spp., being instead more closely related to *Haemocystidium* spp. (from reptiles), *Plasmodium* spp. (from reptiles, birds, and mammals) and other mammal-infecting haemosporidians (*Nycteria*, *Polychromophilus*, and *Hepatocystis*). Current evidence suggests that parasites of this newly discovered evolutionary lineage may be endemic to the Americas, but further studies are necessary to clarify their taxonomy, life cycle, vectors, hosts, geographic distribution and host health effects. Additionally, it should be borne in mind that some PCR protocols targeting the *cytb* gene might not reliably detect *H. pulcher* due to low primer affinity.

1. Introduction

Haemoproteids (Haemosporida, Haemoproteidae) are the most diverse group of avian blood parasites, with more than 140 species described to date (Atkinson, 2007; Valkiūnas, 2005). These parasites specialize in the infection of birds as their intermediate hosts, whereas hematophagous flies are the definitive hosts (Valkiūnas, 2005). Haemoproteid infections can cause significant health effects to their avian hosts, affecting fitness, breeding success, moulting, predation, and occasionally even causing death (Marzal et al., 2005, 2013; Ferrell et al., 2007; Møller and Nielsen, 2007; Olias et al., 2011).

Haemoproteus species are traditionally classified in two subgenera: *Haemoproteus*, which comprises six species transmitted by Hippoboscidae (louse flies), and *Parahaemoproteus* which comprises more than 130 species transmitted by Ceratopogonidae (biting midges) (Valkiūnas, 2005; Valkiūnas et al., 2013; Bukauskaitė et al., 2019; Cepeda et al., 2019). Additionally, molecular studies have shown that *Haemoproteus antigonis* and *Haemoproteus catharti* are genetically distinct from each other and from other known haemoproteids, potentially representing new genera (or subgenera) that have yet to be described, and their invertebrate hosts are not known (Bertram et al., 2017; Galen et al., 2018; Yabsley et al., 2018). Haemoproteid species are traditionally designated based on the morphology of their gametocytes in the blood of their vertebrate hosts (Valkiūnas, 2005), however the genetic diversity of these parasites is far greater than could be recognized from their morphology (Bensch et al., 2009; Outlaw and Ricklefs, 2014). More than 1800 mitochondrial cytochrome *b* gene (*cytb*) lineages of *Haemoproteus* have been deposited in the MalAvi database (http://130.235.244.92/Malavi/, database version 2.5.3; Bensch et al., 2009), and this number continues to increase. As a result, the combination of morphological

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and molecular approaches has been instrumental to clarify the taxonomy of these parasites and shed light on their ecology and evolution (Nilsson et al., 2016; Valkiūnas et al., 2019).

Cariamiformes are a group of primarily flightless terrestrial birds. The group is basal among extant Australaves, which also comprises Falconiformes (falcons and kestrels), Psittaciformes (parrots and cockatoos) and Passeriformes (songbirds) (Jarvis et al., 2014; Prum et al., 2015). Fossil and genetic evidence suggest that their divergence from other Australaves dates back to the early Paleogene period around 60–66 million years ago (Claramunt and Cracraft, 2015). Although this order was once represented by several families and included the "terror birds" (Phorusrhacidae), large flightless apex predators that dominated South America during most of the Cenozoic era, the fossil record shows that most Cariamiformes were extinct around 1.8 million years ago (MacFadden et al., 2007). At present, only two species of Cariamiformes remain, the Red-legged Seriema (*Cariama cristata*) and the Black-legged Seriema (*Chunga burmeisteri*), both of which are placed in the Cariamidae family and are endemic to South America (Winkler et al., 2020).

Although haemoproteid infection in Red-legged Seriema was briefly mentioned by Lutz and Meyer (1908), the morphology of those parasites was not characterized. Recently, Carvalho et al. (2021) reported on the detection of DNA from an unidentified *Leucocytozoon* parasite in the blood of three Red-legged Seriemas in Brazil. In this study, we describe a novel species of haemoproteid from a Red-legged Seriema in southeast Brazil.

2. Material and methods

On 6 June 2019, an adult male Red-legged Seriema (*Cariama cristata*) was found on the shoulder of the highway ES-060, near the entrance of Paulo César Vinha State Park (20°36′02″S 40°25′34″W). It was in good body condition (2.3 kg) but presented with labored breathing, had blood in the trachea, and appeared unable to stand-up, presumably having been hit by a car. The bird was rescued by park rangers and transported to the Institute of Research and Rehabilitation of Marine Animals (IPRAM), where it was initially treated (oral glucose, oral diazepam, intramuscular tramadol, intravenous Ringer's lactate solution, oxygen mask). After 1 h, however, the bird did not show improvement of its clinical condition, with signs of severe lung hemorrhage and probable coelomic hemorrhage, presumably experiencing intense discomfort and pain. The decision was made to euthanize the bird through the intravenous administration of propofol to induce anesthesia followed by cardiorespiratory arrest.

Blood was collected from the tarsal vein before administering propofol, and was immediately used to prepare thin blood smears and to store a frozen blood sample (−20 °C). Due to logistical constraints, the carcass had to be refrigerated (2–8 °C) and was only necropsied 72 h after death; by then, moderate autolysis had occurred. Gross lesions were photographed and noted, and tissue samples were fixed in 10% buffered formalin. Formalin-fixed tissues were embedded in paraffin and 5 μm sections were obtained, stained with hematoxylin-eosin and examined under light microscopy.

Blood smears were air dried, fixed with absolute methanol and stained with eosin–methylene blue (Kyro-Quick®, Kyron Laboratories, Benrose, South Africa). Blood parasites were quantified with the assistance of digital image analysis to count 5000 erythrocytes (Gering and Atkinson, 2004) and parasites were morphologically identified using the published keys and descriptions (Valkiūnas, 2005). Gametocytes and host cell features were measured using ImageJ 1.8.0 (Schneider et al., 2012), using the morphometric parameters described by Bennett and Campbell (1972) and Valkiūnas (2005). Kruskal-Wallis tests were used to compare the measurements of uninfected erythrocytes and erythrocytes parasitized by macrogametocytes or microgametocytes. Mann-Whitney tests were used to compare the measurements of macrogametocytes and microgametocytes.

DNA was extracted with the Wizard® SV 96 Genomic DNA Purification System (Promega, Madison, WI, USA) with modifications. Briefly, 10 μL of blood were incubated with Whole Blood Lysis Buffer (400 μL) for 15 min in a shaker at 90 °C. The initial lysis was completed with Proteinase K and incubated overnight in a shaker at 37 °C. The lysates were transferred to columns and washed according to the manufacturer's instructions. DNA was eluted in 50 μL of nuclease-free water and stored at −20 °C. PCR tests targeting the mitochondrial *cytb* gene of *Haemoproteus* and *Plasmodium* were employed. Initially, a nested PCR protocol targeting a fragment of the *cytb* gene (479 bp) of *Haemoproteus* and *Plasmodium* (external primers: HaemNFI and HaemNR3; internal primers: HaemF and HaemR2) was employed as described by Hellgren et al. (2004). When this approach was not successful in spite of several attempts, a nested PCR protocol targeting the *cytb* gene of *Haemoproteus*, *Plasmodium* and *Leucocytozoon* were employed as described by Perkins and Schall (2002). This protocol employs the external primers DW2 and DW4 (amplification product = 1260 bp) followed by the internal primers DW1 and DW6 (amplification product = 1180 bp). Then, primers DW1, DW8, DW3 and DW8 were used to sequence overlapping segments of the *cytb* gene (Sanger sequencing with dye-terminator fluorescent labelling) with BigDye® Terminator v3.1 Cycle Sequencing Kit in ABI PRISM® 3500 Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA). The sequences obtained with primers DW1, DW8, DW3 and DW8 were aligned and stitched to produce a consensus sequence (S1 File).

DNA sequences were deposited in the Genbank (accession code OL906298) and MalAvi databases (lineage name CARCRI02). Phylogenetic analyses of the *cytb* gene were conducted including: (a) reference lineages of haemosporidian parasites for which *cytb* are presently available, (b) haemosporidian lineages from the Genbank and MalAvi databases with high BLAST score (Zhang et al., 2000), and (c) *Leucocytozoon* sp. lineage CARCRI01 from Red-legged Seriema (Carvalho et al., 2021). Sequences were aligned with ClustalW (Larkin et al., 2007) and trimmed to the same length as the sequence obtained in this study. MrBayes 3.2.7 (Ronquist et al., 2012) was used to produce a Bayesian tree; two Markov chains were run simultaneously for 5 million generations that were sampled every 1000 generations, and the first 1250 trees (25%) were discarded as a burn-in step. MEGA 7 (Kumar et al., 2016) was used to produce a Maximum Likelihood tree; bootstrap values were estimated from 1000 replicates. The GTR + I + G model of nucleotide evolution was used as recommended by jModelTest 2 (Darriba et al., 2012). *Leucocytozoon* was placed as an outgroup as recommended by multi-gene analyses (Borner et al., 2016; Galen et al., 2018). For comparative purposes, phylogenetic analyses were repeated using only a 479 bp segment of the *cytb* gene (as standardized in the MalAvi database; Bensch et al., 2009) which had been obtained through sequencing with primer DW1 (without consensus sequence stitching, see S1 File).

3. Results

Examination of ante-mortem blood smears revealed the presence of erythrocytic parasites in the cytoplasm of approximately 0.1% of the erythrocytes (Fig. 1 and Table 1). The following measurements showed significant differences between uninfected erythrocytes, erythrocytes infected with macrogametocytes, and erythrocytes infected with microgametocytes: host cell length ($H = 22.73$, $df = 2$, $P < 0.001$) and host cell nucleus length ($H = 20.85$, $df = 2$, $P < 0.001$); there were no such differences in host cell width ($H = 3.78$, $df = 2$, $P = 0.151$) and host cell nucleus width ($H = 3.60$, $df = 2$, $P = 0.165$). Macrogametocytes and microgametocytes showed significant differences in length ($W = 1307$, $P < 0.001$), width ($W = 1158$, $P < 0.001$), nucleus length ($W = 465$, $P < 0.001$), nucleus displacement ratio ($W = 1232$, $P < 0.001$), and number of pigment granules ($W = 1348.5$, $P < 0.001$); there was no such difference in nucleus width ($W = 1002.5$, $P = 0.198$).

Nested PCR amplification of the *cytb* gene with the Hellgren protocol was negative in four attempts, even though positive controls were amplified as expected. Nested PCR amplifications with the Perkins and

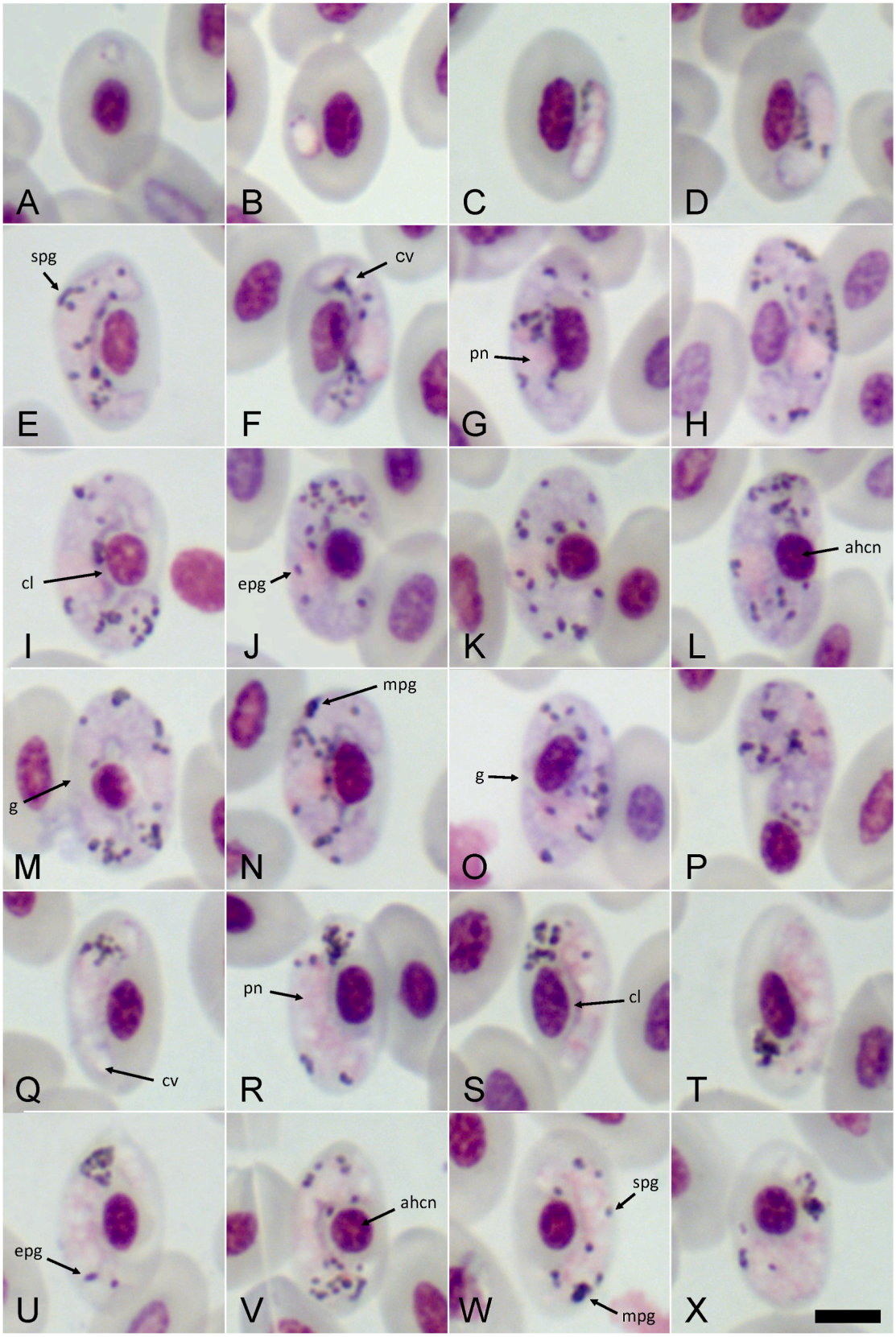

Fig. 1. Gametocytes of *Haemoproteus pulcher* sp. nov. from the blood of the Red-legged Seriema (*Cariama cristata*): A–D, young gametocytes; E–P, macrogametocytes, Q–X, microgametocytes. Legend: ahcn, atrophied host cell nucleus; cl, cleft between the parasite and the host cell nucleus; cv, cytoplasmic vacuoles; epg, elongated pigment granules; g, gap between the ends of the parasite; pn, parasite nucleus; mpg, medium-sized pigment granules; spg, small pigment granules. Eosin–methylene blue stained thin blood films. *Scale-bar*: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Morphometric parameters of gametocytes of *Haemoproteus pulcher* sp. nov. and host cells of Red-legged Seriema (*Cariama cristata*). Sample size was 30 for all measurements.

Feature	Mean	S.D.	Range
Uninfected erythrocyte			
Length	12.7	0.9	10.9–15.7
Width	7.3	0.4	6.3–8.0
Length of nucleus	5.0	0.6	3.6–6.3
Width of nucleus	2.7	0.2	2.4–3.1
Erythrocyte parasitized by macrogametocyte			
Length	13.5	0.7	12.4–15.4
Width	7.4	0.4	6.7–8.3
Length of nucleus	4.1	0.5	3.4–5.2
Width of nucleus	2.8	0.2	2.4–3.3
Erythrocyte parasitized by microgametocyte			
Length	13.5	0.7	12.3–15.5
Width	7.5	0.5	6.0–8.7
Length of nucleus	4.3	0.7	3.1–5.6
Width of nucleus	2.7	0.3	2.2–3.2
Macrogametocyte			
Length	13.2	0.8	11.2–14.9
Width	3.1	0.4	2.3–4.0
Length of nucleus	3.6	0.5	2.6–4.4
Width of nucleus	2.9	0.5	2.0–3.8
Nuclear displacement ratio	0.5	0.2	0.2–1.0
Number of pigment granules	21.5	3.2	17–31
Microgametocyte			
Length	11.5	0.8	10.1–13.9
Width	2.7	0.4	2.0–3.4
Length of nucleus	7.2	0.9	4.6–8.6
Width of nucleus	2.9	0.7	1.9–5.7
Nuclear displacement ratio	0.7	0.1	0.4–0.9
Number of pigment granules	14.3	2.2	11–20

Schall primers were successful, and a 1119 bp consensus sequence was obtained (approximately 92% of the complete *cytb* gene; S1 File). Bayesian tree analysis of the *cytb* gene consensus sequence (Fig. 2 and S2 File) showed that the parasite in this study was most closely related to Haemosporida sp. PSOOCH01, which was recorded in Pale-winged Trumpeter (*Psophia leucoptera*; Gruiformes: Psophiidae) in northwestern Brazil (Fecchio et al., 2018). Additionally, the parasite in this study and Haemosporida sp. PSOOCH01 also clustered with two other lineages: *Haemoproteus catharti* CATAUR01, which was recorded in Turkey Vulture (*Cathartes aura*; Cathartiformes: Cathartidae) in eastern USA (Yabsley et al., 2018), and Haemosporida sp. MYCAME08 (formerly known as MYCAMH1), which was recorded in Wood Stork (*Mycteria americana*; Ciconiiformes: Ciconiidae) in central Brazil and eastern USA (Villar et al., 2013; Fecchio et al., 2019). Together, these four lineages formed cluster that was clearly separated from other known *Haemoproteus* spp., representing instead the sister lineage to a large clade containing *Plasmodium*, *Polychromophilus*, *Nycteria*, and *Hepatocystis*. Maximum Likelihood tree (S2 File) showed differences in topology relative to the Bayesian tree, but agreed that the parasite in this study and lineages PSOOCH01, CATAUR01 and MYCAME08 formed a reasonably well-supported clade (bootstrap values = 88) that is clearly separated from other known *Haemoproteus* spp.; in the Maximum Likelihood tree, this group of parasites was placed as a sister lineage to *Haemocystidium* spp. Phylogenetic analyses considering only a 479 bp segment of the *cytb* gene (as standardized by the MalAvi database) produced similar results (S2 File). Comparison of the primers employed in the Hellgren PCR protocol to their corresponding annealing sites in the parasite detected in this study revealed a relatively poor sequence match (≤87%) for primers HaemF, HaemR2 and HaemNR3 (Fig. 3).

Necropsy revealed that the bird was in good body condition, and the gross findings were attributable to blunt-force trauma: cerebral hematoma, hemoceloma due to liver bruising, lung congestion and hemorrhage, spleen subcapsular hematoma, and kidney congestion and hemorrhage. Tissue meronts consistent with those of Haemosporida were seen in endothelial cells of venules and capillaries of the lungs (common; S3 Figure) and kidneys (rare). Megalomeronts were not seen. It is possible that tissue meronts (but very unlikely for megalomeronts) were present in other organs but were not recognizable due to autolysis. Lung endothelial meronts had average length (mean ± S.D.) of 11.1 ± 2.3 μm (range: 7.2–16.7; n = 30) and average width of 7.9 ± 1.4 μm (range: 4.7–11.2; n = 30), with a round to elongate shape (length-to-width ratio: 1.4 ± 0.4, range: 1.0–2.8; n = 30) and containing tens to hundreds of merozoites measuring 1.3 ± 0.2 μm (range: 1.1–1.9; n = 20) by 1.1 ± 0.3 μm (range 0.7–1.7; n = 20). Meronts in the lungs were occasionally accompanied by mild lymphocytic infiltration, but in most cases, there was no evident inflammatory response associated with their presence.

3.1. *Haemoproteus pulcher sp. nov*

Type-host: Red-legged Seriema *Cariama cristata*. Male, adult bird caught on 6 June 2019.

Type-locality: Paulo César Vinha State Park, a coastal area of tropical semideciduous forest ("non-flooded forest formation" *sensu* Assis et al., 2004) within the Atlantic Forest biome, in the Guarapari municipality, Espírito Santo state, Brazil (20°36′02″S 40°25′34″W; 3 m above sea level).

Site of infection: Mature erythrocytes; endothelial cells (lungs, kidneys).

Prevalence: One of one bird.

Type-specimens: Hapantotype (accession number G466233, ex *Cariama cristata*; parasitemia intensity approximately 0.1%, 6 June 2019, collected by L. Egert) is deposited at the International Reference Centre for Avian Haematozoa (IRCAH) of the Queensland Museum (Brisbane, Australia). Parahapantotype deposited at the Coleção de Protozoários, Instituto Oswaldo Cruz (Rio de Janeiro, Brazil; accession code COLPROT-927).

Distribution: Only known from type-locality.

Representative DNA sequence: Mitochondrial cytochrome *b* gene, lineage CARCRI02 (1119 bp, GenBank accession code OL906298).

Etymology: From Latin pulcher = beautiful; the species name should be treated as a Latin adjective. The name is a reference to the statement by Lutz and Meyer (1908) that they had seen "a beautiful species of halterids" (in Portuguese: "uma bonita espécie de halterídeos") in the blood of Red-legged Seriema.

Description (Fig. 1 and Table 1)

Young gametocytes (Fig. 1A–D). Young gametocytes may develop at any position, with median-to-subpolar positioning being most frequent (Fig. 1B–D). Occasionally, growing gametocytes can slightly displace the host cell nucleus laterally (Fig. 1B) or slightly rotate it (Fig. 1D). Younger gametocytes usually have a smooth membrane and do not touch the host cell nucleus (Fig. 1A and B).

Macrogametocytes (Fig. 1E–P). Macrogametocytes are relatively pale staining, but still show sufficient staining contrast to be differentiated from microgametocytes. Growing macrogametocytes have a relatively smooth membrane (Fig. 1E), but the membrane facing towards the host cell nucleus can become undulated as the parasites grow larger (Fig. 1F and G), occasionally developing folds (Fig. 1H, I, 1M, and 1N). Growing gametocytes frequently touch the host cell nucleus on the sides but not on the poles (Fig. 1G, Q, and 1S), although in some cases they apparently do not touch the host cell nucleus and an evident cleft can be seen between the parasite and the host cell nucleus (Fig. 1I and N). Fully grown macrogametocytes are usually appressed to the host cell outer membrane and markedly displace the host cell nucleus laterally (Fig. 1E–J), or occasionally towards the pole (Fig. 1P). The host cell is slightly elongated when parasitized by a macrogametocyte (on average, the length of parasitized erythrocytes is 0.8 μm greater; Table 1), and the host cell nucleus is often atrophied into a round or ovoid shape (on average, the nuclear length of parasitized erythrocytes is 0.9 μm smaller; Table 1), with a darker staining of its chromatin (Fig. 1J–M and 11P). A slight rotation (up to 45°) of host cell nucleus occurs occasionally

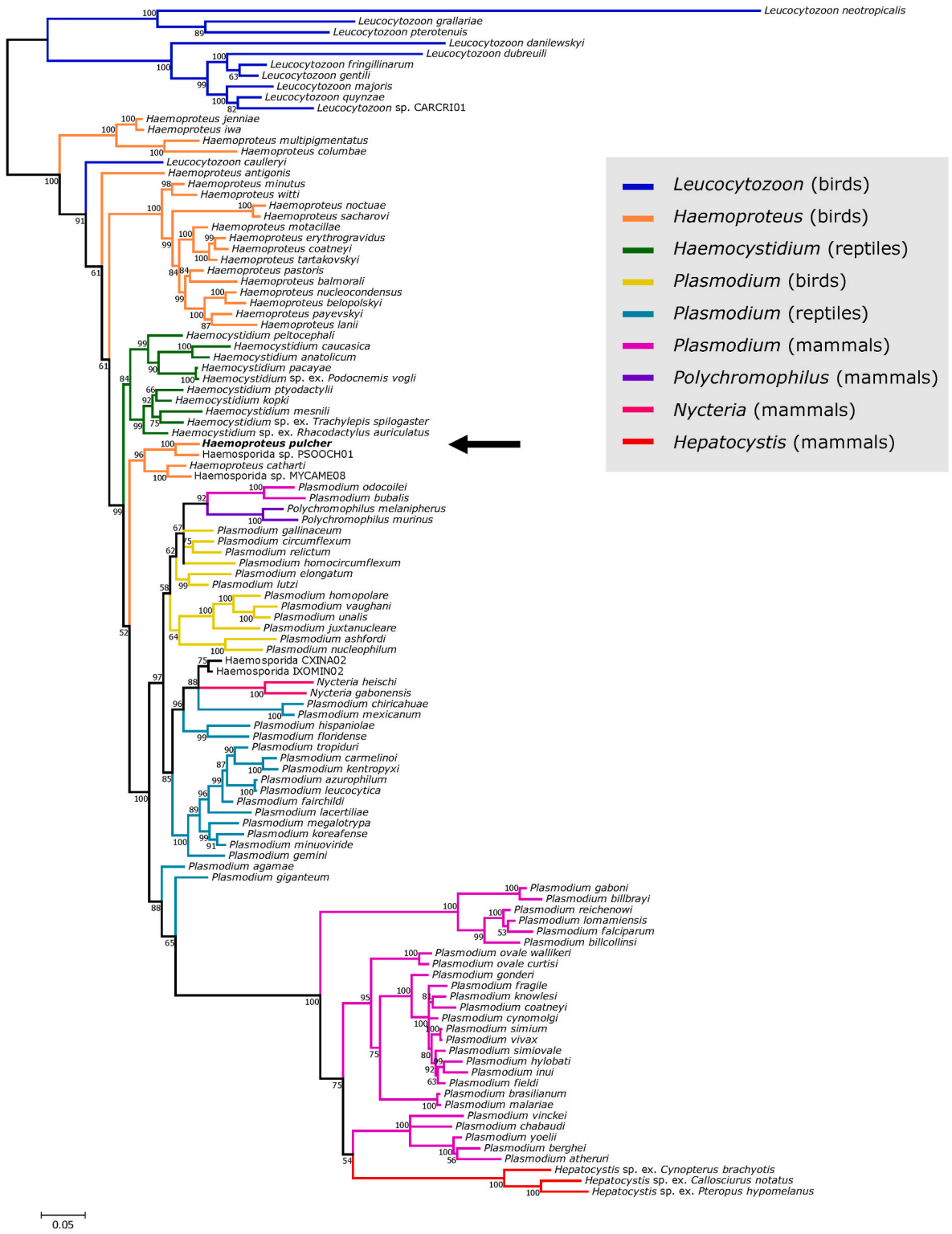

Fig. 2. Bayesian phylogenetic hypothesis of the relationship between *Haemoproteus pulcher* sp. nov. and other Haemosporida based on the mitochondrial *cytb* gene. Branch lengths are drawn proportionally to the extent of changes (scale-bar is shown). Values adjacent to nodes represent posterior probabilities.

(Fig. 1H and O). Macrogametocytes tend to enclose the host cell nucleus, but the ends of the parasite never encircle the host cell nucleus completely, leaving a small gap where the host cell cytoplasm is visible (Fig. 1M and O). The nucleus of macrogametocytes is usually median or subpolar, but never polar, and it is not usually appressed to the host cell nucleus. Small pigment granules (<0.5 μm) are abundant and randomly scattered in the cytoplasm. One or two medium-sized pigment granules (0.5–1.0 μm) are occasionally present (Fig. 1N). Large pigment granules (>1.0 μm) are absent. The number of pigment granules ranges between 17 and 31 (Table 1). Dumbbell-shaped and discoid macrogametocytes are absent. Macrogametocytes with highly amoeboid outline or finger-like projections are absent, and the parasites do not enucleate the host cell. Rod-like pigment granules are absent, even though small pigment granules may occasionally appear to be slightly elongated (Fig. 1J).

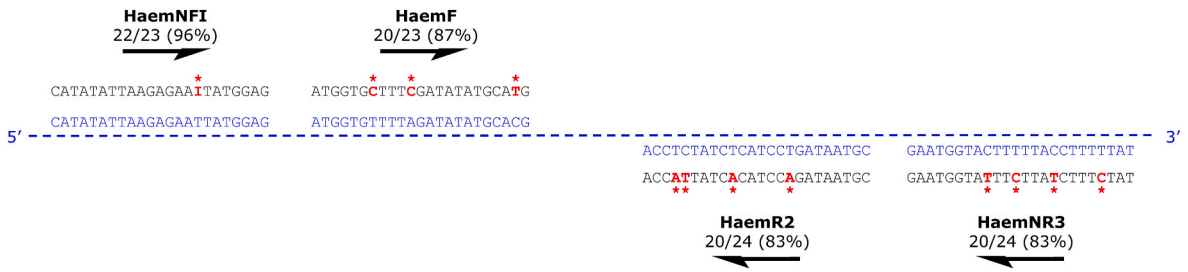

Fig. 3. Comparison of the sequence of outer primers (HaemNFI and HaemNR3) and inner primers (HaemF and HaemR2) from a frequently-used nested PCR protocol to detect avian *Haemoproteus* and *Plasmodium* based on the amplification of a 479 bp segment of the mitochondrial *cytb* gene (Hellgren et al., 2004) to the corresponding segments of the cyt-*b* gene of *Haemoproteus pulcher* obtained in this study (blue). Asterisks highlight mismatches between the sequences and the primers. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Large vacuoles are occasionally present (Fig. 1F). There are no volutin granules.

Microgametocytes (Fig. 1Q–X). Microgametocytes have the same general characteristics as macrogametocytes with the usual sexual dimorphic characters. Microgametocytes tend to be less voluminous (on average, 1.7 μm shorter and 0.4 μm narrower; Table 1) than macrogametocytes. Microgametocytes usually do not touch the host cell nucleus as frequently as macrogametocytes, leaving clefts between the parasite and the host cell nucleus that are more frequent and prominent than those seen in macrogametocytes. Microgametocytes do not encircle the host cell nucleus to the same extent as macrogametocytes. Pigment granules are less abundant than in macrogametocytes (Table 1), and tend to aggregate near the poles more frequently than in macrogametocytes.

3.2. Remarks

This species is provisionally placed in the genus *Haemoproteus* because: (a) it parasitizes avian erythrocytes; (b) erythrocytic stages present hemozoin pigment granules; and (c) erythrocytic meronts were not seen. However, analysis of the mitochondrial cytochrome *b* gene suggests this parasite is a representative of an evolutionary branch that is separate from most known *Haemoproteus* species. Further studies are therefore necessary to clarify the taxonomy of this parasite.

If *Haemoproteus* species that infect other avian species within Australaves are considered, *H. pulcher* is morphologically most similar to *Haemoproteus elani* from Falconiformes from the Holarctic, Ethiopian and Oriental regions (Valkiūnas, 2005). Unlike *H. elani*, however, *H. pulcher* shows a greater tendency to enclose and nearly encircle the host cell nucleus, its inner membrane is more undulated and occasionally forms folds, and it lacks volutin granules or small vacuoles. When avian species other than Australaves are considered, arguably the *Haemoproteus* species with the greatest morphological similarity to *H. pulcher* is *H. rotator*, which thus far has only been recorded on the Pin-tailed snipe (*Gallinago stenura*; Charadriiformes: Scolopacidae) from the Phillipine Islands (Valkiūnas, 2005). However, the gametocytes of *H. pulcher* do not occupy the cytoplasm of the host cell completely, they cause nuclear atrophy more frequently than *H. rotator*, and they do not cause the rotation of the host cell nucleus as frequently as *H. rotator*.

4. Discussion

Lutz and Meyer (1908) were the first to record haemosporidian infection in Red-legged Seriemas, with the following notes (translated from Portuguese): "*Cariama cristata*: A beautiful species of halterids was found in the blood of two seriemas, and it seems these parasites must not be rare in this species. Its shape seems a bit different." Since this brief report, the only other record of haemosporidian infection in Cariamiformes was the molecular detection of *Leucocytozoon* sp. lineage CARCRI01 from three Red-legged Seriemas from Distrito Federal, central Brazil (Carvalho et al., 2021).

The parasite documented in this study is morphologically consistent with *Haemoproteus*, which is why we provisionally place it in this genus. However, phylogenetic analysis of the *cytb* gene suggests that this parasite (*Haemoproteus pulcher* sp. nov.), together with *Haemoproteus catharti* (from Turkey Vulture) and Haemosporida sp. lineages PSOOCH01 (from Pale-winged Trumpeter) and MYCAME08 (from Wood Stork), represent a clade that is more closely related to *Haemocystidium* spp. and *Plasmodium* spp. than to other known *Haemoproteus* spp. It seems likely that this group of parasites (*H. pulcher*, *H. catharti*, PSOOCH01, and MYCAME08) represents a genus or subgenus that has yet to be described. There are several open questions and uncertainties about the taxonomy of Haemosporida (e.g. whether *Plasmodium* should be split into several genera, whether the subgenera *Haemoproteus* and *Parahaemoproteus* should be raised to genus level, whether *Haemoproteus antigonis* and *Leucocytozoon caulleryi* should be assigned to new genera, etc.) (Omori et al., 2008; Perkins, 2014; Bertram et al., 2017; Galen et al., 2018). For this reason, we feel it is more judicious to wait until further information about the natural history and genomes of Haemosporida (especially from birds and reptiles) is available before the taxonomy of this order can be comprehensively re-assessed and the appropriate taxonomic placement of *H. catharti* and *H. pulcher* can be determined.

The placement of *H. catharti* and *H. pulcher* in the genus *Haemoproteus* relies on the understanding that these parasites do not undergo merogony within erythrocytes; if this were the case, these parasites would be assigned to the genus *Plasmodium* instead (see Valkiūnas, 2005). It should be noted however that in the original description of *H. catharti,* Greiner et al. (2011) reported finding rare "unusually thick, elongate immature schizonts" in the erythrocytes of some turkey vultures with *H. catharti*, which they interpreted as evidence of concurrent infection by an unidentified *Plasmodium* sp. In light of the molecular evidence put forth since then, the question arises on whether the erythrocytic meronts (schizonts) documented by Greiner et al. (2011) could have been produced by *H. catharti*. In this study we did not find erythrocytic meronts, but this does not fully dismiss the possibility that *H. pulcher* could develop these stages under different circumstances (e.g. if erythrocytic merogony only occurs during acute infections or follows well-defined circadian cycles). Further studies to fully characterize the life cycle of *H. catharti, H. pulcher* and other lineages of this phylogenetic group would therefore be valuable to clarify whether erythrocytic merogony does occur or not, which will have important taxonomic implications.

The vectors involved in the transmission of *H. catharti* and *H. pulcher* are unknown. It is safe to presume that hematophagous dipterans must be involved, as is the case for all Haemosporida species for which the vector is known (Perkins, 2014). However, considering the unusual position of *H. catharti* and *H. pulcher* in the phylogenetic tree, with a closer relationship to *Haemocystidium* spp. and *Plasmodium* spp., it would be premature to assume that they are transmitted by the same vectors as

other *Haemoproteus* spp. (i.e. louse flies or biting midges). The vectors of *Haemocystidium* spp. are largely unknown, but tabanid flies (Tabaniidae) have been implicated in the transmission of species that infect turtles (Pineda-Catalan et al., 2013; Perkins, 2014). *Plasmodium* spp. are vectored by mosquitoes (Culicidae) with the exception of species from the subgenus *Paraplasmodium* (parasites of lizards), which are vectored by phlebotomid flies (Psychodidae: Phlebotominae) (Ayala, 1971; Perkins, 2014). Unfortunately, very little is known about dipterans that feed on Red-legged Seriemas, with the exception of a record of the louse fly *Ornithoctona erythrocephala* (Hippoboscidae: Ornithomyiinae) parasitizing this host in Brazil (Silva et al., 2021); it seems reasonable to assume that Red-legged Seriemas are also routinely parasitized by mosquitoes, which are abundant in the biomes occupied by this species (including at the type locality of *H. pulcher*, subj. obs.).

To date, representatives of the phylogenetic group comprising *H. catharti*, *H. pulcher* and closely-related lineages were only detected in the Neotropical region. Additionally, all four known/presumed hosts of these parasites (Red-legged Seriema, Turkey Vulture, Pale-winged Trumpeter, and Wood Stork) are endemic to the Americas, lending credence to the hypothesis that this group of parasites is restricted to this region. On the other hand, the fact that each representative of this phylogenetic group was recorded in a bird from a different order (Cariamiformes, Cathartiformes, Gruiformes, and Ciconiiformes) suggests these parasites occur in a broad diversity of avian hosts. It is unknown whether these parasites are host specific at family level, as is often the case for *Haemoproteus* spp., or if they are host generalists, as is frequent in *Plasmodium* spp. (Valkiūnas, 2005).

Unfortunately, the bird in this study could not be promptly necropsied and, as a result, histopathological analysis was compromised by tissue autolysis. In spite of this limitation, we were able to find meronts in endothelial cells of the lungs and, to a lesser extent, kidneys. The distribution and morphology of these meronts was consistent with previous descriptions of the exo-erythrocytic development of *Haemoproteus* spp. and *Plasmodium* spp. in birds. We did not find megalomeronts, which are known to occur in some *Haemoproteus* spp. (Valkiūnas and Iezhova, 2017; Duc et al., 2020). The bird in this study died for reasons unrelated to the haemosporidian infection, and further studies are necessary to better understand the exo-erythrocytic development and host health effects of *H. pulcher*.

The PCR protocol developed by Hellgren et al. (2004) is widely used as the standard method to detect and characterize avian *Haemoproteus* and *Plasmodium* (Bensch et al., 2009), yet we failed to amplify a segment of the *cytb* gene of *H. pulcher* using this protocol. This is not unheard of, and previous studies showed that traditional PCR protocols targeting the *cytb* gene may fail to detect some avian hemosporidian parasites such as *Plasmodium polymorphum* and *Haemoproteus iconiae* (Zehtindjiev et al., 2012; Valkiūnas et al., 2016). Fortunately, we were successful in amplifying the complete *cytb* gene using a different primer set and PCR protocol (developed by Perkins and Schall 2002). Inspection of the *cytb* sequence from *H. pulcher* revealed a relatively poor sequence match for three primers (out of four) used in the Hellgren protocol. The failure to detect *H. pulcher* using that protocol might therefore be related to low primer affinity. As discussed by Valkiūnas et al. (2016), most PCR protocols to detect avian *Plasmodium* or *Haemoproteus* were originally developed using DNA sequences from parasites that infect inhabiting passerine birds and, as such, they might be insufficiently sensitive in the amplification of DNA from distantly-related Haemosporida developing in non-passerine birds. A similar problem has also been reported when PCR primers designed using sequences from *Leucocytozoon* spp. of the Holarctic region are employed to study *Leucocytozoon* spp. of the Neotropical region (Lotta et al., 2019).

In conclusion, our results suggest that *H. pulcher* and *H. catharti* are representatives of a group of parasites that morphologically resembles *Haemoproteus* spp. but for which genetic data suggests a distinct evolutionary history. This group is likely to include other avian-infecting species that have yet to be described or which are presently assigned to *Haemoproteus* but for which genetic data is not yet available. Current evidence suggests this group of parasites may be endemic to the Americas, but further studies are necessary to clarify their taxonomy, life cycle, vectors, host and geographic distribution and host health effects.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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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.2022.02.009.

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