

Article

Haemosporidians in Non-Passerine Birds of Colombia: An Overview of the Last 20 Years of Research

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Abstract: The Neotropics are highly diverse in avian species. Neotropical countries contribute a large part of the estimated diversity of haemosporidian parasites reported for the planet's tropical zones. However, sampling is limited and biased, illustrated by only 30% of the genetic records (barcodes) from non-passerines, most of them not linked to a nominal species. This paper aimed to perform the molecular and morphological characterization of the haemosporidians that infect non-passerine birds from Colombia deposited in the biological collection named “Grupo de Estudio Relación Parásito Hospedero (GERPH)”. We analyzed 1239 samples from twelve biomes and two animal care facilities. Phylogenetic relationships using barcodes and mitochondrial genomes were estimated. In addition, the reports of haemosporidian infections in non-passerine birds from the Neotropics recorded after 1978 were summarized. We reported the presence of thirteen morphological haemosporidian species, four potential new species deposited in GERPH, a host range expansion for two *Plasmodium* species, and a barcode sequence for *Haemoproteus caprimulgi*. We confirmed the species associated with 56 molecular lineages reported in other neotropical countries at the genus level. Thus, biological collections and curated databases such as MalAvi are essential to support integrative approaches demanded in modern taxonomy.

Keywords: Neotropical birds; *Plasmodium*; *Haemoproteus*; *Leucocytozoon*; mtDNA; biological collections



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1. Introduction

Haemosporidia are a highly diverse group of parasites hosted by reptiles, mammals, and birds [1]. These protozoans have been confirmed to infect many avian families on all continents [2]. Traditionally, research on avian haemosporidians relies on species that can be captured using mist nets as a trapping method, thus skewing the information obtained towards passerine birds [3].

The Neotropical region in about ten terrestrial ecoregions [4–6] harbors more than 4000 avian species, representing almost half of the world's species [7]. In fact, the three most biodiverse countries in the world's Aves are found in this region [8]. Agreeing with this, the avian Haemosporida are highly diverse at low latitudes [9], where the Neotropical countries contribute about 40% of the unique lineages reported for tropical countries (Malavi database [10]). However, recent findings of new lineages [11–13] suggest that

higher values of parasite diversity can be expected to be found compared to the known host diversity in Central and South American countries [14].

Haemosporidian research in the Neotropics began at the dawn of the 20th century with the characterization of the *Haemoproteus columbae* life cycle ([15] cit. [16]). White et al. [17] published the first comprehensive compilation of studies on the avian haematozoa of the Neotropics, including infection reports from 31 bird orders from Mexico to Argentina. In that article, the haemosporidian genera *Plasmodium*, *Leucocytozoon*, and *Haemoproteus* were reported in 22 of the 30 non-passerine orders of birds. Then, Gabaldon et al. [18] reported the presence of a fourth parasite genus named *Fallisia* in a Columbiform species for the first time. Later, Valkiūnas [2] reported fourteen *Plasmodium*, twelve *Haemoproteus*, and three *Leucocytozoon* species in non-passerine hosts for this geographic area, most of them described before 2000 [16]. The findings reported between 1908 and 2005 were made using blood smears as a diagnostic tool (Figure 1). Currently, haemosporidian biodiversity characterization uses combined information from blood smears, mitochondrial lineages, host-parasite interactions, and ecological traits [16]. From the first studies in avian haemosporidians in the Neotropics until now, at least twenty-four species have been described in non-passerine hosts [2,16,19–23].

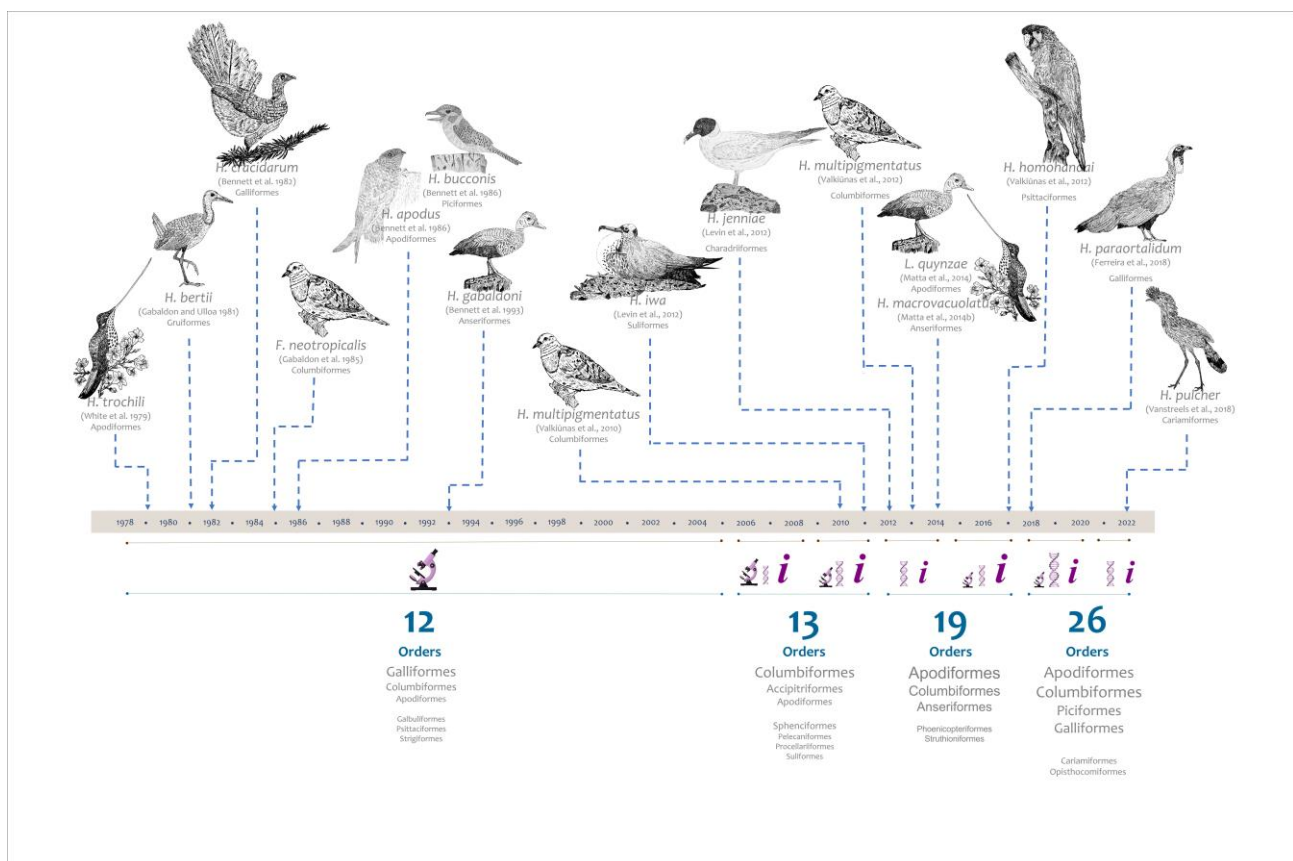


Figure 1. Schematic representation of the findings of haemosporidians in Neotropical non-passerine birds since the last comprehensive revision in 1978. Over the timeline, the parasite species are described with the host and references. Under the timeline, brown thin lines represent the time periods for the number of studies carried out using only microscopy (represented by the figure of the microscope), methods based on PCR (represented by the DNA molecule), or combined evidence (represented by the letter *i*). The size of the icons represents the number of studies carried out with each methodology. The thin blue lines represent the time spans for which the number of orders studied is counted. At the bottom, the size of the name represents the number of records associated with each order, the smallest being those with the fewest number of studies in the specified time range.

The first record of a PCR-based diagnosis of haemosporidian parasites in neotropical non-passerine birds was done by Durrant et al. [24] in 2006 (Figure 1). Given the higher detection rate of molecular methods over microscopy (e.g., [25]), the number of explored avian orders has increased (Figure 1). The first *Leucocytozoon* species reported in Apodiformes (Hummingbirds) came from Colombia, and was published in 2014 [20]. In that report, the authors emphasize a change in the distribution of this parasitic genus, commonly found only in the highlands. Then, several new *Leucocytozoon* lineages were reported in South America's highlands for non-passerines [13,21,26,27], etc. To date, according to MalAvi [10], nearly 700 lineages that remain identified at the genus level have been reported for neotropical non-passerine hosts.

In Colombia, the biological collection named Grupo de Estudio Relación Parásito Hospedero (GERPH) hosts samples from twenty non-passerine bird orders collected in at least ten different ecosystems since 1999. This study aimed to perform a molecular and morphological characterization of the Haemosporidians infecting non-passerine birds deposited in the GERPH biological collection. We also present an update on the haemosporidian infection reports in non-passerine birds from Mexico to Argentina for the last 40 years. That would provide a more accurate perception of the state of Neotropical research, allowing future studies to focus efforts on the species and ecosystems less sampled to date.

2. Materials and Methods

2.1. Samples

This study analyses samples of 1239 individuals belonging to 178 species, 34 families, and 20 orders of non-passerine birds deposited in the biological collection GERPH. These samples have been obtained mainly from wild birds collected in 23 localities distributed in 12 of the 32 continental biomes of Colombia (Table S1, Figure 2). There are also samples from animals treated and housed in animal care facilities after being found injured or seized from illegal traffickers. It is essential to highlight that these birds were sampled at the time of admission to the rescue center before receiving treatment or having contact with other specimens in the facilities. A detailed description of the sampling areas is provided in Table S1.

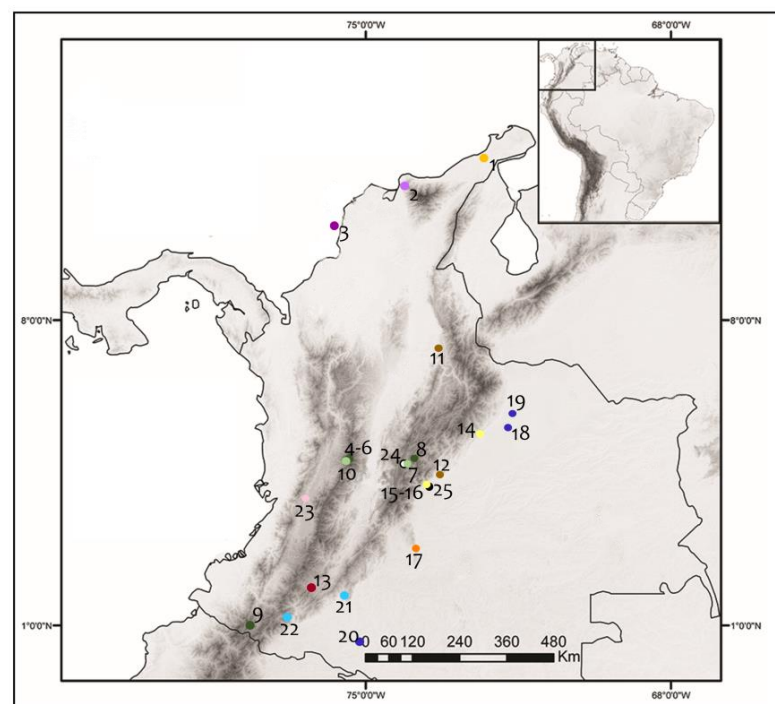


Figure 2. Geographical location of the sampling localities. Dot colors represent the biomes in each locality. Then, Manaure (1) with a dark yellow spot is placed in the Tropical desert zonobiome of the

Guajira and Santa Marta; Cienaga (2) represented by a pale purple circle is located in the Caribbean halobiome; and Barú (3) represented with a dark purple belongs to the Caribbean tropical dry zonobiome. The dark green dots indicate the places included in the High orobiome of the Andes: Los Nevados National Natural Park (NNP) (4), Chingaza National Natural Park (NNP) (5), and Aldana (6). The localities sampled in the middle orobiomes of the Andes represented by light green dots were Universidad Nacional de Colombia- Bogotá Campus (UNAL) (7), Ucumari National Regional Park (NRP) (8), El Cedral (9), and Quimbaya Flora and Fauna Sanctuary (FFS) (10); while in the low orobiomes of the Andes, brown dots indicate the location of San Gil (11) and Medina (12). The dark red dot represents San Agustín (13) in the upper Magdalena alternate hygric or subxerophytic zonobiome. Yopal (14), Villavicencio (15), and San Miguel (16) where the sampled places in the Amazon-Orinoco peiniobiome represented by light yellow dots, while La Macarena NNP (17) represented in orange corresponds to the Macarena Orobiome. Sampling places in the helobiomes of the Orinoco and the Amazon (18–20), and tropical humid Zonobiome of the Orinoco and Amazon (21–22) are represented by dark and pale blue respectively. For helobiomes of the Cauca Valley, the pink dot indicates the Sonso Lagoon (23). As for the animal care facilities, they are represented by a white dot (URRAS–24) and a black dot (Ocarros–25).

2.2. Microscopic Examination

Microscopic examination was performed at low 400× and high magnification 1000×. Positive smears were photographed using the CellSens software (Olympus Corporation). We used the taxonomic keys provided in Valkiūnas [2], Valkiūnas and Iezhova [28] and the original descriptions for parasite taxonomic identification. In addition, graphic material from parasitic species deposited in the collection of Dr. G. Valkiūnas at the Institute of Ecology (CDVA) and specimens from the International Reference Centre for Avian Haematozoa (IRCAH) were revised to compare the parasite species morphologies observed in these blood smears to corroborate species determinations.

Overall and individual parasite genus frequencies were calculated as the positive individuals over the total sampled, expressed as a percentage.

2.3. DNA Extraction, Amplification of Cytochrome b (Cytb) Barcodes and mtDNA Genomes

DNA was extracted using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) when collected samples were stored in EDTA, or the standard phenol chloroform protocol [29] for samples collected in ethanol or SET buffer. Molecular diagnosis was made only for samples whose smears were positive (n = 108). It is important to mention that for some of these positive samples, there was no blood available for molecular tests (n = 20). Once DNA was obtained, amplification of cytochrome b (*cytb*) fragments was performed using the primers and protocols suggested by Hellgren et al. [30] and Pacheco et al. [31]. To check for non-patent coinfections with other haemosporidians, independent amplifications were conducted using the primers of Pacheco et al. [31]. Amplicons were cleaned with ammonium acetate [32] and sequenced in both senses on an ABI 3730xl System (Applied Biosystems, Foster City, CA, USA). A total of 17 partial *cytb* gene sequences were reported in this study and submitted to GenBank under accession numbers OL689174, OL689176, OL689177, and OP087637-OP087649.

Second, for those positive samples with good DNA quality and parasitemia partial parasite mtDNA genomes (≈6 kb) were amplified by a PCR protocol with Takara LA Taq™ Polymerase (TaKaRa Mirus Bio, Madison, WI, USA) following Pacheco et al. (2018b) using the oligos forward AE170-5'-GAGGATTCTCTCCACACTTCAATTCGTA CTTC-3' and reverse AE171-5'-CAGGAAAATWATAGACCGAACCTTGGACTC-3' [33]. PCRs were performed in 50 µL using 2 µL of total genomic DNA. PCR conditions were a partial denaturation at 94 °C for 1 min and 30 cycles with 30 s at 94 °C and 7 min at 67 °C, followed by a final extension of 10 min at 72 °C. PCR products (50 µL for each sample) were visualized in agarose gels (1%). Excised bands (bands of ≈6 kb) were purified using a QIAquick® Gel extraction kit (Qiagen, GmbH, Hilden, Germany). Finally, these products were cloned using pGEM®-T Easy Vector systems (Promega, Madison, WI, USA) following

the manufacturer's directions. A minimum of three to four clones per sample (both strands) were sequenced at GENEWIZ from Azenta Life Sciences (Middlesex County, NJ, USA). A total of five new mtDNA genomes for morphospecies were reported in this study and submitted to GenBank under accession numbers OP701681-OP701685.

2.4. Phylogenetic Analysis

For *cytb* gene phylogenetic analysis, the partial sequences obtained from non-passerine birds were manually edited in MEGA v7.0. [34]. Three alignments were constructed, one for each of the three haemosporidian genera. Those alignments included lineages obtained from the positive specimens deposited in the biological collection GERPH, along with parasite sequences isolated from passerine and non-passerine birds deposited in GenBank and MalAvi [10]. Thus, for *Haemoproteus*, an alignment of 465 bp composed of 15 sequences from GERPH specimens, 83 sequences from public databases, and 3 *Leucocytozoon* species as outgroup was performed. In this alignment, 76 lineages were associated with morphospecies.

For *Plasmodium*, 59 partial *cytb* gene sequences were used, 8 from GERPH specimens, and 24 linked with morphospecies. Three *Leucocytozoon* lineages were added as an outgroup. The total length for this alignment was 448 bp.

Leucocytozoon phylogenetic reconstruction included 48 lineages of this parasite's genus, along with 2 sequences of *Plasmodium* and 2 of *Haemoproteus*. Seven lineages were isolated from specimens deposited in the GERPH collection, while the remaining forty-one were obtained from public databases. Also, 23 lineages were associated with identified morphospecies. The total length for this analysis was 456 bp. All sequences were aligned with MAFFT (Ref. [35], available at <https://www.ebi.ac.uk/Tools/msa/mafft/>, accessed on 13 September 2022).

For the phylogenetic analysis, the general time-reversible model was used with gamma-distributed substitution rates; a proportion of invariant sites (GTR + G + I) was selected in Jmodeltest [36] as the best fit model of nucleotide substitution, according to the Akaike information criterion. The phylogenetic relationships were estimated using maximum-likelihood (ML) implemented in W-IQ-TREE (Ref. [37], available at <http://iqtree.cibiv.univie.ac.at/>, accessed on 13 September 2022), performing a bootstrap of 1000 replicates. In addition, a Bayesian inference analysis was conducted with MrBayes v 3.2 [38] using two MCMC simulations 5×10^6 generations, with samplings every 100 generations. The convergence was checked by the average standard deviation of split frequencies inferior to 0.01. A majority consensus rule was applied to obtain a phylogenetic hypothesis of over 75,000 trees.

In addition, a fourth alignment was performed using 70 partial avian parasite mtDNA genomes (5234 bp excluding gaps). The alignment included sequences from the Genbank (N = 65) of the three genera (*Leucocytozoon*, *Haemoproteus*, and *Plasmodium*) and the new mtDNA genome sequences (N = 5) obtained in this study for some well-identified morphospecies using morphology. This alignment was done using MUSCLE, as implemented in SeaView v4.3.5 [39], with manual editing. Then, using both the Bayesian (MrBayes) and maximum likelihood (W-IQ-TREE) methods, phylogenetic relationships were inferred using six partitions [33]. These partitions corresponded to the three non-protein coding regions between the ORFs (fragmented SSU rRNA and LSU rRNA) and the three protein-coding genes, keeping their order in the mtDNA genome. Also, a general time-reversible model was used with gamma-distributed substitution rates and a proportion of invariant sites (GTR + G + I) for each partition of the alignments. This model had the lowest Bayesian information criterion (BIC) scores estimated by jModelTest [36]. The Bayesian support for the nodes was inferred by sampling every 1000 generations from two independent chains lasting 10^4 Markov chain Monte Carlo steps. Convergence between chains was assumed once the average standard deviation of the posterior probability was <0.01, and the value of the potential scale reduction factor was between 1.00 and 1.02 [38]. Then, as a 'burn-in', 25% of the sample was discarded once convergence was reached.

For each of the four alignments constructed in this investigation, genetic distances were calculated using the Kimura 2 parameters (K2P) model of nucleotide substitution implemented in MEGA v.7.0 [34].

3. Results

A total of 108 specimens from 11 avian orders were found infected with haemosporidian parasites. Such infected individuals were distributed in nine of the twelve biomes studied and from the two rescue centers. For *Haemoproteus*, there were 82 positive birds (6.62%), while for *Plasmodium* and *Leucocytozoon* there were 15 and 11 (1.21% and 0.89%), respectively.

Haemoproteus parasites were distributed in all the biomes except for those located in the Caribbean region (tropical desert zonobiome–I; Caribbean halobiome–II; and Caribbean tropical dry zonobiome–III). Meanwhile, *Plasmodium* was found only for middle orobiomes of the Andes (V), the helobiomes of the Orinoco and the Amazon (X), and the tropical humid zonobiome of the Orinoco and Amazon (XI). *Leucocytozoon* was restricted to places whose altitudes exceeded 2100 m asl; then, the genus was found only in the Andean mountains in the high altitude (IV) middle altitude (V) orobiomes and in the Upper Magdalena alternate hygric or subxerophytic zonobiome (VII). For the rescue centers, only the infections caused by *Plasmodium* and *Haemoproteus* are registered (Table 1).

Table 1. Non-passerine bird samples deposited in the “Grupo de estudio relación parásito hospedero” biological collection (GERPH). The number of tested specimens is indicated as n_T . Localities and biomes are as in Figure 2 and Table S1. *P*: *Plasmodium*, *H*: *Haemoproteus*, *L*: *Leucocytozoon*. Lineages obtained are provided. ^a: *cytb* fragments (478 pb), ^b: Mitochondrial complete genomes, *: New lineages reported in this study. The remaining negative birds are registered at the end of the table, along with the biome (super index), and number of sampled specimens in parenthesis.

Species	n_T	Haemosporidian			Biome	Locality	Parasite Species	Lineage
		<i>P</i>	<i>H</i>	<i>L</i>				
Accipitriformes								
Accipitridae								
<i>Rupornis magnirostris</i>	4		1		V, VI, X, IX	6, 12, 17, 18	<i>Haemoproteus</i> sp.	-
<i>Buteo platypterus</i>	1		1			24	<i>Haemoproteus nisi</i>	-
Anseriformes								
Anatidae								
<i>Anas discors</i>	13		2		II, XII	2, 23	<i>Haemoproteus</i> sp.	-
<i>Cairina moschata</i>	18		2		V, VI	11,12	<i>H. gabaldoni</i>	OL456193 ^a /OP701681 ^b -OP701682 ^b -CAIMOS01 KJ592828 ^a /KJ499987 ^b -DENAUT01
<i>Dendrocygna autumnalis</i>	33		14		X	19	<i>H. macrovacuolatus</i>	
Apodiformes								
Trochilidae								
<i>Adelomyia melanogenys</i>	24			1	V, VII	5, 8, 10, 13	<i>L. qyunzae</i>	KF309188 ^a -HEAME01
<i>Boissonneaua flavescens</i>	3		1		IV, V	4, 5, 10	<i>H. witti</i>	
<i>Chalybura buffonii</i>	2		1		VI, VII, VIII	1, 7, 15	<i>Haemoproteus</i> sp.	-
<i>Coeligena helianthea</i>	16			1	V, IV	2, 7, 13	<i>L. qyunzae</i>	KF309189 ^a -COHEL01
<i>Ensifera ensifera</i>	9		2		IV	4, 5, 7	<i>H. witti</i>	KY653794 ^a -TROAED20

Table 1. Cont.

Species	n_T	Haemosporidian			Biome	Locality	Parasite Species	Lineage
		<i>P</i>	<i>H</i>	<i>L</i>				
<i>Eriocnemis cupreovertris</i>	14		1		IV	5, 7, 8	<i>H. witti</i>	KY653794 a-TROAED20
<i>Eriocnemis derbyi</i>	51		1		IV, V	4, 10	<i>H. witti</i>	KY653794 a-TROAED20
<i>Eriocnemis vestita</i>	33		3		IV	7	<i>H. witti</i>	KY653794 a-TROAED20
<i>Glaucis hirsutus</i>	17	1	1		VIII, X, XI	16, 20, 21	<i>Plasmodium nucleophilum</i> ,	OP087644 a-DENPET03
<i>Helianthus amethysticollis</i>	6			1	IV, V	5, 7, 8, 9	<i>L. quynzae</i>	KF309188 a/KF479480 b-HEAME01
<i>Helianthus exortis</i>	37		1		IV, V	4, 5, 7	<i>Haemoproteus</i> sp.	-
				1			<i>L. quynzae</i>	KF309188 a-HEAME01
<i>Lafresnaya lafresnaya</i>	19		1		IV, V	4, 5, 7	<i>H. witti</i>	-
<i>Metallura tyrianthina</i>	56			6	IV, V	4, 5, 7, 10	<i>L. quynzae</i>	KF309188 a-HEAME01
<i>Opisthoprora euryptera</i>	2			1	IV	5, 7	<i>L. quynzae</i>	-
Caprimulgiformes Caprimulgidae								
<i>Chordeiles acutipennis</i>	3		1		X	19	<i>H. caprimulgi</i>	OP087649 a/OP701683 b-CHOACU01 *
<i>Nyctidromus albicollis</i>	3		2		VIII, XI	14, 21	<i>H. caprimulgi</i> ,	OL689174 a-NYCTALB02 *
		1					<i>Plasmodium</i> sp.	OP087647 a-NYCTALB03 *
<i>Nyctipolus nigrescens</i>	1		1		IX	17	<i>H. caprimulgi</i>	OL689174 a-NYCTALB02 *
Charadriiformes Scolopacidae								
<i>Numenius phaeopus</i>	13		1		II, III, XII	2, 3, 23	<i>H. contortus</i>	-
Columbiformes Columbidae								
<i>Claravis pretiosa</i>	6		3		VIII, IX	14, 17	<i>Haemoproteus</i> sp.	OL689177 a-COSQU01
<i>Columba livia</i>	40		16		IV, V, VI	7, 8, 12	<i>H. columbae</i>	KY653761 a-HECOL01
<i>Columbina minuta</i>	4		1		IX, X	17, 18	<i>Haemoproteus</i> sp.	-
<i>Columbina passerina</i>	3		2		VIII	16	<i>Haemoproteus</i> sp.	-
<i>Columbina squammata</i>	6		2		VIII, X	14, 16, 18	<i>Haemoproteus</i> sp.	
								OL689176 a/OP701685 b-COLPAS03 OP087648 a-COPIC01
<i>Columbina talpacoti</i>	13		6		I, VIII, X	14, 17, 18, 19	<i>H. paramultipigmentatus</i>	
<i>Leptotila rufaxilla</i>	12		1		VIII, IX, X	14, 15, 17, 18	<i>Haemoproteus</i> sp.	-
<i>Leptotila verreauxi</i>	11		2		V, VIII, X	10, 14, 16, 20	<i>Haemoproteus</i> sp.	-
<i>Patagioenas cayennensis</i>	1		1		VIII	14	<i>Haemoproteus</i> sp.	-
<i>Zenaidura macroura</i>	30		4		IV, V, VIII, IX, XI	8, 10, 16, 17, 22	<i>Haemoproteus</i> sp.	-
<i>Zenaidura macroura</i>	1		1		VII	13	<i>Haemoproteus</i> sp.	-

Table 1. Cont.

Species	<i>n</i> _T	Haemosporidian			Biome	Locality	Parasite Species	Lineage
		<i>P</i>	<i>H</i>	<i>L</i>				
Galbuliformes Galbulidae								
<i>Galbula ruficauda</i>	8	1			VIII, X	14, 18	<i>P. tejera</i>	OL689171 a-/OP701684 b-SPMAG04
Bucconidae								
<i>Monasa nigrifrons</i>	2		1		XI	21	<i>Haemoproteus</i> sp.	OP087643 a-MONNIG02
Gruiformes Rallidae								
<i>Porphyrio martinica</i>	30	9			V, -	7, 8, 23, 24	<i>Plasmodium elongatum</i> , <i>Plasmodium</i> sp.	OP087639 a-GRW06 OP087637 a-CYCYA01
Pelecaniformes Ardeidae								
<i>Butorides striata</i>	5	1			V, XII,	8, 23, 24	<i>Plasmodium</i> sp.	OP087640 a-BUSTR03 *
Strigiformes Strigidae								
<i>Megascops choliba</i>	1		1			24	<i>Haemoproteus syrnii</i> -like	OP087641 a-PSDIS01
<i>Asio clamator</i>	4		4			25	<i>Haemoproteus syrnii</i> -like	OP087645 a-ASICLA01 *
Trogoniformes Trogonidae								
<i>Trogon personatus</i>	4	1			V	4, 6, 7, 9	<i>Plasmodium</i> sp.	KF537290 a-TROPER01 KY653806 b-TROPER02
Total infected		15	82	11				
Non infected			1131					

Negative birds: Accipitriformes: Accipitridae: *Accipiter striatus* ^{IV} (1), Anseriformes: Anatidae: *Amazonetta brasiliensis* ^{VIII} (2), *Anas andium* ^{IV} (2), *Anas georgica* ^{XI,XII} (7), *Anas platyrhynchos* ^V (4), *Anser anser* ^V (1), *Cairina moschata* ^{V,VI} (16), *Dendrocygna bicolor* ^{XII} (1), *Oressochen jubatus* ^X (1), Apodiformes: Trochilidae: *Aglaeactis cupripennis* ^{IV} (3), *Aglaiocercus kingii* ^V (2), *Amazilia* sp. ^{IX} (2), *Amazilia fimbriata* ^{VI,VIII,X} (5), *Amazilia franciae* ^V (12), *Amazilia versicolor* ^{VI,VIII} (4), *Chaetocercus mulsant* ^V (1), *Chalcostigma herrani* ^{IV} (1), *Chalcostigma heteropogon* ^V (8), *Chionomesa fimbriata* ^X (8), *Chlorestes notata* ^X (2), *Chlorostilbon mellisugus* ^{IV,VIII,IX,X} (6), *Coeligena bonapartei* ^{IV} (1), *Coeligena coeligena* ^V (13), *Coeligena lutetiae* ^{IV} (13), *Colibri thalassinus* ^V (3), *Coeligena torquata* ^{IV,V} (12), *Colibri coruscans* ^{IV,V} (67), *Discosura conversii* ^{IX} (1), *Doryfera ludovicae* ^V (2), *Eriocnemis derbyi* (50), *Eriocnemis mosquera* ^{IV} (1), *Glaucis* sp. ^X (1), *Haplophaedia aureliae* ^V (3), *Helianagel exortis* ^{IV,V} (35), *Heliodoxa leadbeateri* ^{IV} (1), *Heliodoxa rubinoides* ^V (2), *Helimaster longirostris* ^{IX} (3), *Heliothryx auritus* ^X (1), *Metallura williamsi* ^{IV} (9), *Ocreatus underwoodii* ^V (1), *Oxyopogon guerini* ^{IV} (4), *Phaethornis augusti* ^{VI,IX} (5), *Phaethornis anthophilus* ^{VIII,X} (8), *Phaethornis bourcieri* ^X (1), *Phaethornis hispidus* ^{VI,VIII,X} (8), *Phaethornis guy* ^V (7), *Phaethornis malaris* ^X (4), *Phaethornis syrmatorphus* ^V (7), *Polytmus guainumbi* ^{VIII} (6), *Pterophanes* ^{IV} (2), *Pterophanes cyanopterus* ^{IV} (2), *Ramphomicron microrhynchum* ^{IV} (6), *Schistes geoffroyi* ^V (1), *Thalurania furcata* ^X (2), *Threnetes leucurus* ^X (1), *Threnetes ruckeri* ^{IX} (1); Caprimulgiformes: Caprimulgidae: *Chordeiles* sp. ^X (3), *Systellura longirostris* ^{IV} (2), Charadriiformes: Burhinidae: *Burhinus bistriatus* ^{I,-} (2), Charadriidae: *Charadrius semipalmatus* ^{III} (6), *Pluvialis squatarola* ^{XII} (3), *Vanellus cayanus* ^{VIII} (1), *Vanellus chilensis* ^{III,V,VIII,X,XI,XII} (63), *Vanellus splendens* ^{IV} (2), Jacanidae: *Jacana jacana* ^{VIII,X,XII} (22), Laridae: *Gelochelidon nilotica* ^{II} (4), *Leucophaeus atricilla* ^{II} (3), *Sterna hirundo* ^{II} (2), *Thalasseus sandvicensis* ^{II} (3), Recurvirostridae: *Himantopus mexicanus* ^{I,II,III,XII} (15), Scolopacidae: *Actitis macularia* ^{I,XII} (4), *Calidris mauri* ^{III} (6), *Calidris melanotos* ^{III} (3), *Calidris minutilla* ^{I,III,XII} (42), *Calidris pusilla* ^I (2), *Gallinago nobilis* ^{IV} (1), *Gallinago delicata* ^{XII} (1), *Limnodromus griseus* ^{I,II} (5), *Tringa flavipes* ^{I,XII} (14), *Tringa melanoleuca* ^{I,XII} (5), *Tringa solitaria* ^{III,XII} (7), Columbiformes: Columbidae: *Geotrygon montana* ^{XI} (1), Coraciiformes: Alcedinidae: *Chloroceryle aenea* ^{VIII,X} (3), *Chloroceryle amazona* ^{VIII,X} (3), *Chloroceryle americana* ^{III,VIII} (6), *Chloroceryle inda* ^{VIII} (8), *Megaceryle torquata* ^{VIII,XII} (2), Momotidae: *Momotus momota* ^{V,IX} (5), Cuculiformes: Cuculidae: *Coccyzus minima* ^{VIII,X} (2), *Coccyzus americanus* ^{VIII} (10), *Coccyzus melacoryphus* ^V (1), *Crotophaga ani* ^{VIII,IX,X,XII} (10), *Crotophaga major* ^X (2), Falconiformes: Falconidae: *Falco sparverius* ^X (1), Galbuliformes: Galbulidae: *Galbula tombacea* ^{VIII} (3), Bucconidae: *Bucco macrorhynchus* ^X (1), *Bucco tamatia* ^X (1), Galliformes: Odontophoridae: *Colinus cristatus* ^{VIII,X} (4), Phasianidae: *Coturnix coturnix* ^{VI} (2), *Gallus gallus* ^{IV,V,VI,VIII,X} (13), Gruiformes: Rallidae: *Fulica ardesiaca* ^{IV} (3), *Gallinula galeata* ^{XII} (2), Pelecaniformes:

Ardeidae: *Bubulcus ibis*^{XII} (4), *Egretta rufescens*^{II} (2), *Egretta tricolor*^{I,III} (3), *Egretta thula*^{I,XII} (4), *Nyctanassa violacea*^{II} (1), *Nycticorax nycticorax*^I (7), Threskiornithidae: *Platalea ajaja*^I (5), *Phimosus infuscatus*^{XII} (6), *Plegadis falcinellus*^{XII} (1), Piciformes: Capitonidae: *Capito aurovirens*^{XI} (4), Picidae: *Colaptes punctigula*^{VI,VIII} (2), *Colaptes rivolii*^{IV} (7), *Melanerpes cruentatus*^{VIII} (1), *Melanerpes rubricapillus*^X (2), *Picumnus olivaceus*^V (1), *Picumnus squamulatus*^X (2), Ramphastidae: *Andigena nigrirostris*^{IV} (1), *Andigena hypoglaucha*^{IV} (4), *Aulacorhynchus albivitta*^{VII} (2), *Aulacorhynchus haematopygus*^V (1), *Aulacorhynchus prasinus*^V (1), *Pteroglossus pluricinctus*^{IX,XI} (2), *Pteroglossus castanotis*^{VI} (1), Podicipediformes: Podicipedidae: *Podilymbus podiceps*^{XII} (2), Psittaciformes: Psittacidae: *Amazona amazónica*⁻ (4), *Amazona ochrocephala*⁻ (4), *Ara macao*⁻ (1), *Brotogeris versicolurus*⁻ (2), *Eupsittula pertinax*^X (1), *Psittacidae*^{VIII} (1), *Pyrhura calliptera*^{IV} (2), *Touit huetii*^{IX} (4), Strigiformes: Strigidae: *Athene cunicularia*^{IX,X} (3), *Megascops* sp.⁻ (1), Suliformes: Phalacrocoracidae: *Phalacrocorax brasilianus*^{XII} (1), Sulidae: *Sula nebouxii*^{XII} (2), Tinamiformes: Tinamidae: *Nothocercus julius*^{IV} (1), Trogoniformes: Trogonidae: *Trogon collaris*^V (1).

Regarding the avian orders, the largest number of available samples of non-passerine birds in the GERPH biological collection corresponded to the Apodiformes and the Columbiformes, while less than five individuals were from Falconiformes, Tinamiformes, Podicipediformes, and Suliformes (Tables 1, S1 and S2).

Thirteen haemosporidian morphological species (Figures 3 and 4) were identified in the positive specimens (n = 108 specimens). In addition, four specimens should be studied in more detail for taxonomic confirmation, since they do not fit into previously described morphospecies (Table 1). To date, twenty-two parasite *cytb* gene lineages and eight partial mtDNA genomes (including the five new mtDNA genomes reported in this research) were obtained from non-Passeriformes blood samples deposited in the biological collection GERPH. Such sequences were associated with ten of the thirteen morphospecies identified in this study. We also report for the first time two new lineages of *Plasmodium* and three of *Haemoproteus* in Strigiformes, Pelecaniformes, and Caprimulgiformes (Table 1, Figures 5–8). Detailed information on the infections detected in each order explored is presented below.

3.1. Accipitriformes

Six individuals from three species were analyzed (Table 1), and only two infections caused by *Haemoproteus* were found. One of them was in a Roadside Hawk (*Rupornis magnirostris*, n = 1) from the eastern plains, and the other in a specimen of a Broad-winged Hawk (*Buteo platypterus*, n = 1) from URRAS (Table 1). The infection registered in the *Rupornis magnirostris* was caused by an *Haemoproteus buteonis*-like parasite (Figure 3A,B) that needs to be further studied in more detail (when more positive samples are available) in order to determine if it corresponds to a new species, since this parasite differed from *H. buteonis* in one of its main diagnostic characters. Young parasites adhere to the host cell nucleus, growing longitudinally along it. Outlines are usually even; then, they may form angular terminals at the tips of the gametocytes (Figure 3A). Also, wavy or ameboid margins were observed (Figure 3A). Host-cell nucleus displacement was observed in nearly mature or mature gametocytes. Although mature gametocytes are closely appressed to the envelope and host cell nucleus, they do not encircle it completely.

Buteo platypterus showed infection by *Haemoproteus nisi* [40]. The parasite's cytoplasm was granular; outlines were ameboid developing small projections (Figure 3C). Growing gametocytes do not or only slightly touch the erythrocyte membranes (Figure 3D), and parasites are closely appressed to both envelope and nucleus, displacing it (Figure 3E). Fully grown gametocytes encircling the host cell nucleus were not observed. Unfortunately, we could not obtain the *cytb* lineages from these *Haemoproteus* species infecting these raptors (n = 2).

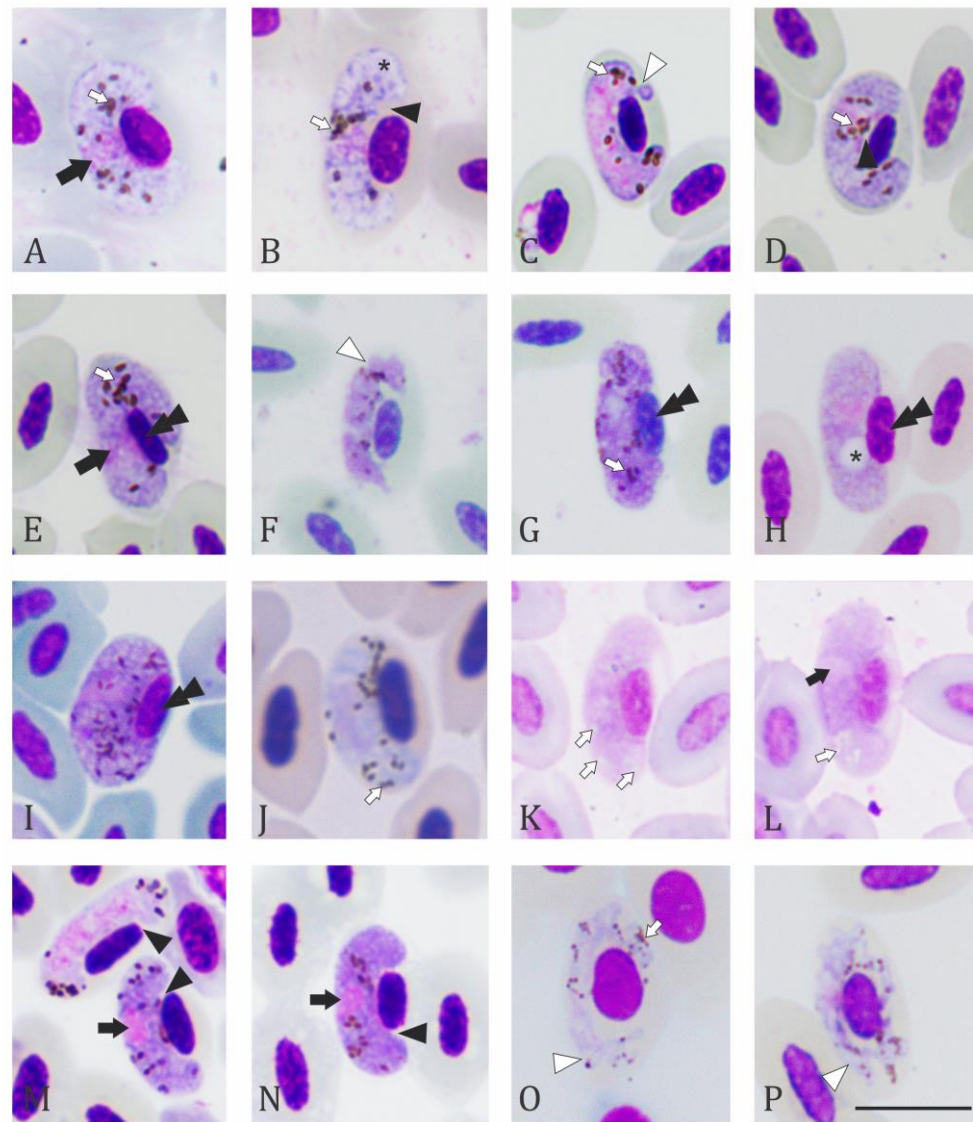


Figure 3. Haemoproteidae of non-passerine birds of Colombia: (A) macrogametocyte; (B) microgametocyte of *Haemoproteus* (*H.*) *buteonis*-like in *Rupornis magnirostris*; (C,D) immature microgametocytes; (E) nearly mature macrogametocyte of *H. nisi* of *Buteo platypterus*; (F) young gametocyte and (G) mature gametocyte of *H. gabaldoni* in *Cairina moschata*; (H) macrogametocyte of *H. macrovacuolatus* in *Dendrocygna autumnalis*; (I) fully grown gametocyte of *H. nettionis* in *Anas discors*; (J) microgametocyte of *H. witti* in *E. vestita*; (K) micro and (L) macrogametocyte of *H. caprimulgi* from *Lyncornis macrootis* in the specimen IRCAH G403126_9396; (M) nearly mature macro and microgametocytes and (N) mature macrogametocyte of *H. caprimulgi* from *Nyctidromus albicollis*; and (O,P) gametocytes of *H. contortus* in *Numenius phaeopus*. Black arrowheads: cleft between parasite and nucleus membranes; black arrows: parasite nucleus; white arrow: hemozoin granules; white arrowhead: parasite membrane; asterisk: vacuole; black double arrowheads: host cell nucleus. Giemsa-stained blood films. Scale bar = 10 μ m.

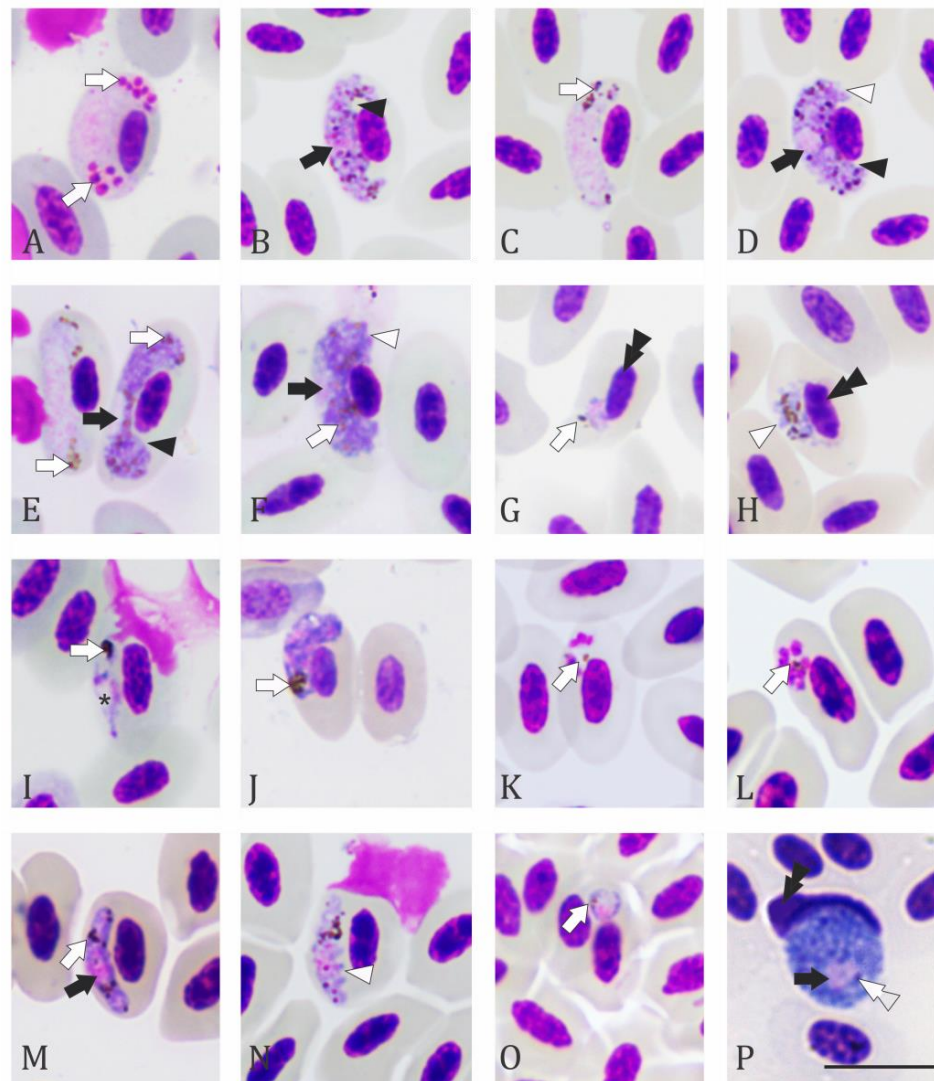


Figure 4. Haemosporidia of non-passerine birds of Colombia: (A) mature microgametocyte of *Haemoproteus* (*H.*) *columbae* in *Columba livia*; (B,C) young macro and microgametocytes; (D) nearly mature macrogametocyte of *H. paramultipigmentatus* from *Columbina talpacoti*; (E,F) young gametocytes of *H. syrni*-like in *Asio clamator*; (G) Erythrocytic meront; and (H) gametocyte of *Plasmodium* (*P.*) *nucleophilum* from *Glaucidis hirsutus*; (I) young gametocyte of *Plasmodium* sp. from *Nyctidromus albicollis*; (J) erythrocytic meront of *P. tejerai* in *Galbula ruficauda*; (K) fan-shaped and (L) roundish erythrocytic meronts; (M,N) gametocytes of *P. elongatum* in *Porphyrio martinica*; (O) parasite stage of *Plasmodium* sp. in *Butorides striata*; (P) macrogametocyte of *Leucocytozoon* (*L.*) *quynzae* from *Heliangelus exortis*. Black arrowheads: cleft between parasite and nucleus membranes; black arrows: parasite nucleus; white arrow: hemozoin granules; white arrowhead: parasite membrane; asterisk: vacuole; black double arrowheads: host cell nucleus; white double arrowheads: volutin granules. Giemsa-stained blood films. Scale bar = 10 μ m.

3.2. Anseriformes

Currently, there are 82 individuals of nine species of Anatidae deposited in the GERPH biological collection (Tables 1 and S2). According to González et al. [41], two individuals are infected with *Haemoproteus gabaldoni* (Ref. [19]—Figure 3F,G), fourteen with *Haemoproteus macrovacuolatus* (Ref. [20]—Figure 3H), and two with *Haemoproteus nettionis* (Ref. [42]—Figure 3I). Unfortunately, for the specimen infected with *H. nettionis*, there was no blood available for molecular diagnosis. *Haemoproteus gabaldoni* (lineage OL456193-CAIMOS01) and *H. macrovacuolatus* (KJ592828-DENAUT01) were placed in a well-supported clade

Regarding the mtDNA genome analysis, three partial parasite mtDNA genomes were obtained for *H. macrovacuolatus* (n = 1, KJ592828-DENAUT01) and *H. gabaldoni* (n = 2, OP701681 and OP701682-CAIMOS01). Both parasites appeared as a sister taxon and shared a common ancestor with a *Haemoproteus* sp. (Figure 8) detected in *Colinus virginianus ridgwayi* from Arizona (KY653798-COLVIR01; Ref. [43]). The genetic distance between *H. macrovacuolatus* and *H. gabaldoni* was 0.008 (Table S5). Although both partial *cytb* sequences (480 bp) for *H. gabaldoni* were identical and identified as the CAIMOS01 lineage, the mtDNA genome genetic distance between both was 0.002, indicating some population differences.

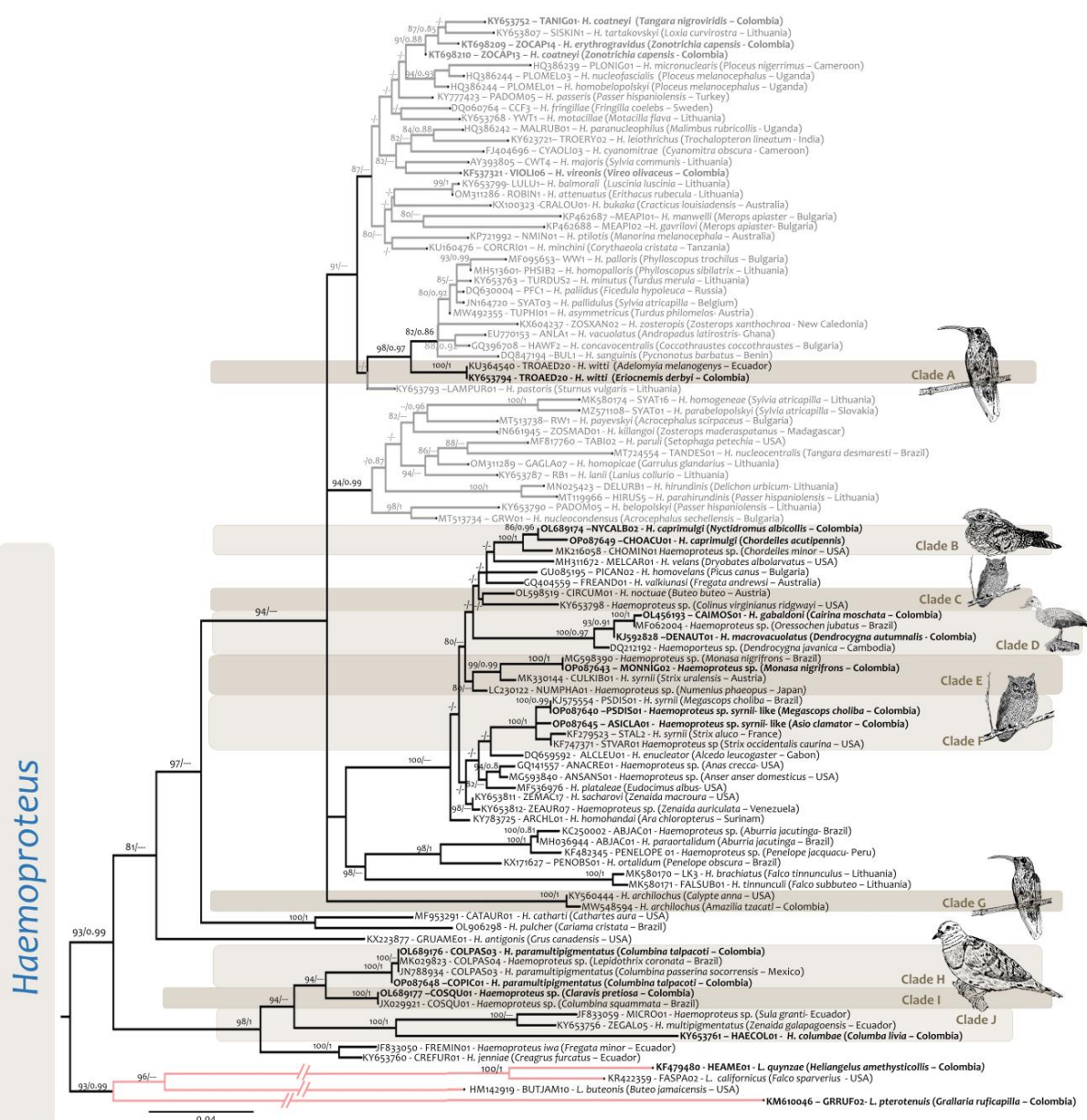


Figure 5. Phylogenetic reconstructions of *Haemoproteus* sp. obtained with the *Cytochrome b* (*cytb*) barcode fragments of 479 bp. Bold tip labels correspond to sequences obtained from specimens of GERPH biological collection. Parasites isolated from passerines are indicated by grey tip labels, while lineages for non-passerine birds are in black. The nodal support values are indicated above the branches as BI/ML. Nodal supports below 0.8/80 not shown.

3.3. Apodiformes

There are 546 samples from 59 Trochilidae species deposited in the biological collection GERPH, and 14 of them are infected with Haemosporidians. Previously, a specimen of the White-vented Plumeleteer (*Chalybura buffonii*) was reported infected with *Haemoproteus* sp. [44]. Unfortunately, for this sample there was no blood for molecular diagnosis (Tables 1 and S2). *Haemoproteus witti* [23] morphology was characterized in the Black-thighed Puffleg (*Eriocnemis derbyi*) that was associated with the TROAED02 lineage in González et al. [45]. In the same report, this species was found infecting two more species of the same genus—the Coppery-bellied Puffleg (*E. cupreiventris*) and the Glowing Puffleg (*E. vestita*) (Table S2).

In this study, three more species were positive for *H. witti* (Figure 3J): the Buff-tailed Coronet (*Boissonneaua flavescens*), the Sword-billed Hummingbird (*Ensifera ensifera*), and the Mountain Velvetbreast (*Lafresnaya lafresnayi*) (Tables 1 and S2). Furthermore, a *Haemoproteus* that could not be identified by morphology or molecular methods because of the low parasitemia, and an absence of mature parasites was reported for Rufous-breasted Hermit (*Glaucis hirsutus*) (Tables 1 and S2).

Haemoproteus witti (KY653794-TROAED02) has been identified in other hummingbirds as well as in passerine birds [13,46] (Table S6). In the phylogenetic reconstructions, this species (Clade A, Figures 3 and 8) is located basal to other lineages from parasites isolated from passerine species.

Currently, three valid species of haemoproteids infect hummingbirds: *H. witti*, *H. archilochus* and *H. trochili* [23]. Recent records of *H. archilochus* (Clade G, Figure 5) are for Colombia and the United States [47,48] associated with the lineages KY560444, and MW548594-SETGRA01 (Table S2). *Cytb* genetic distances between *H. witti* and *H. archilochus* were estimated as 0.073 (Table S4).

In addition, the Rufous-breasted Hermit was infected with *P. nucleophilum* (Ref. [49]—Figure 4G,H). Although scarce, trophozoites were seen in a subpolar position. Such structures had tiny pigment granules and two small vacuoles with even contours. Further stages were observed to adhere to the host cell nucleus growing longitudinally from subpolar positions. Pigment granules increased to four, and outlines became wavy. Scanty nearly mature meronts with up to eight merozoites and pigment granules were clumped in a big spot at the periphery of the meront. The outline was ameboid (Figure 4G). Gametocytes were slender and macrogametocytes with irregular cytoplasm were located lateral to the parasite nucleus to which they discontinuously adhere, as well as to the host cell membrane, possibly due to the scalloped edges of the parasite. The pigment granules were rounded to ovoid and numbered about 10. They were grouped towards the center of the gametocyte (Figure 4H). In contrast, in microgametocytes the cytoplasm seemed more homogeneous, with wavy contours and pigment granules clumped in two or three spots of different sizes. All the development was observed in mature erythrocytes. The lineage obtained from this hummingbird (OP087644-DENPET03) was identical to that reported by Chagas [50] in the Egyptian Goose (*Alopochen aegyptiaca*—JX467689-DENPET03) and was placed in clade B (Figure 6) along with the *Plasmodium* parasites MK695452-AMABRA01 from Anseriformes (Genetic distance 0.006) and MN458617-COLTAL05 from Columbiformes (Genetic distance 0.01) (Table S4).

Leucocytozoon quynzae (Ref. [21], Figure 5P) was the only leucocytozoid detected before in the non-passerine birds deposited in the GERPH biological collection. However, in this study, in addition to the host species reported by Matta et al. [21], two more species were found infected: the Speckled Hummingbird (*Adelomyia melanogenys*) and the Tourmaline Sunangel (*Heliangelus exortis*). All infections were detected in localities that exceeded 2100 m in altitude (Tables 1 and S2).

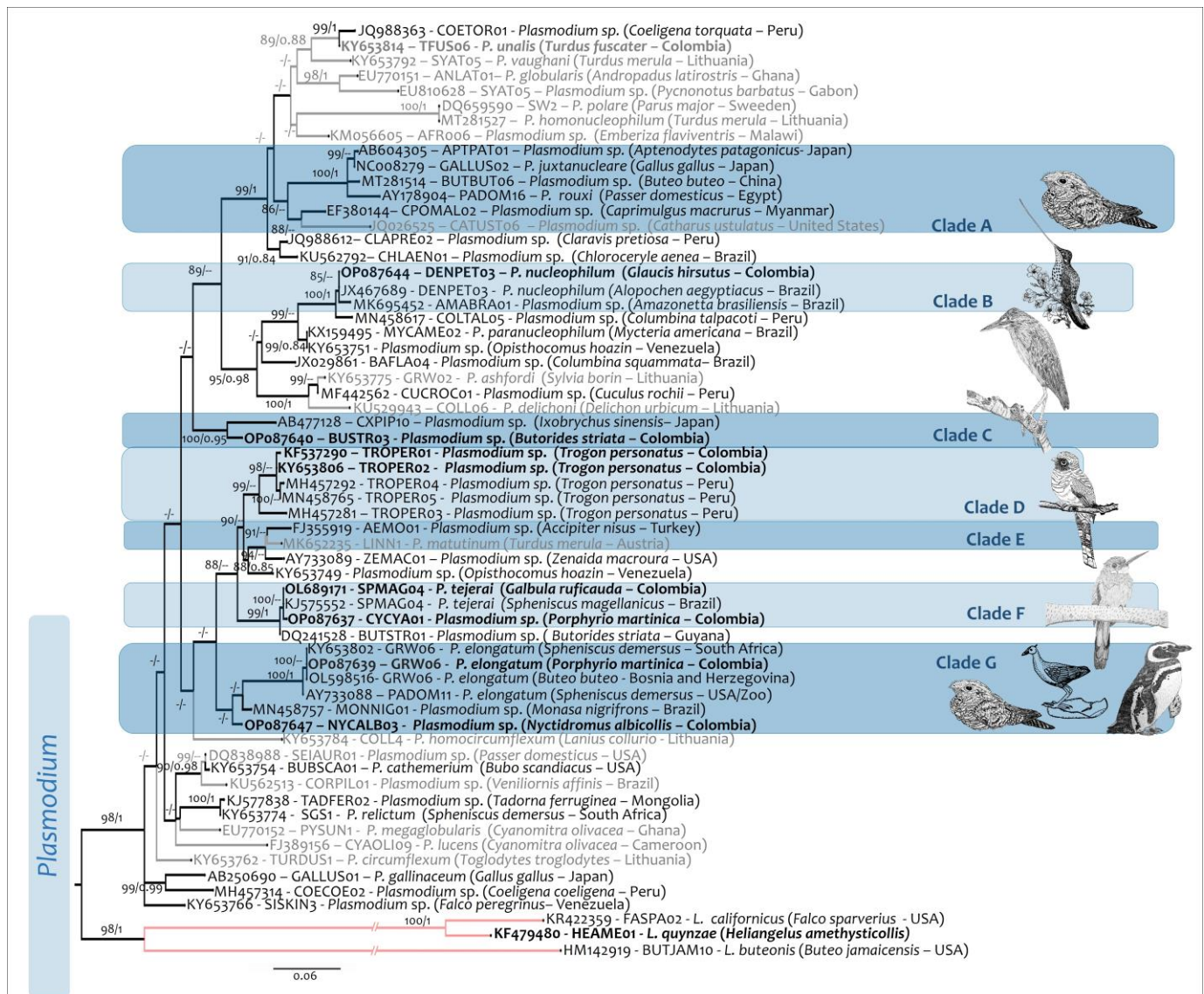


Figure 6. Phylogenetic reconstructions of *Plasmodium* sp. obtained with the Cytochrome b (*cytb*) barcode fragments of 479 bp. Bold tip labels correspond to sequences obtained from specimens from the GERPH biological collection. Parasites isolated from passerines are indicated by grey tip labels, while lineages from non-passerine birds are in black. The nodal support values are indicated above the branches as BI/ML. Nodal supports below 0.8/80 not shown.

The new infections reported here were identical to the lineage KF479480-HEAME01 isolated from the type specimen of the species Amethyst-throated Sunangel (*Heliangelus amethysticollis*) [21]. This lineage was also reported in a White-tufted Sunbeam (*Aglaeactis castelnaudii*) from Peru, while the lineage KF309189-COHEL01 from *L. quynzae* was found in the White-bearded Hermit (*Phaethornis hispidus*) from Peru [13] (Clade C, Figure 8).

Leucocytozoon quynzae was placed basal to the parasites of non-passerine birds such as ducks, raptors, toucans, and woodpeckers in clades A and B (Figure 7). The median genetic distances between those clades were 0.061 (A vs. C) and 0.062 (B vs. C). Although *Leucocytozoon polynuclearis* was placed in clade A (Gen dist KF471028-HEAME01 vs. MW626894-COLAUR01: 0.06, Table S4), the closest species to *L. quynzae* was *L. schoutedeni* in clade D (gen dist 0.05) (Table S4). In the mtDNA phylogenetic tree (Figure 8), *L. quynzae* (from *Heliangelus amethysticollis*, KF479480-HEAME01) appeared as the sister taxon of *Leucocytozoon* sp. (from *Turdus fuscater*, KT162002-TFUS14); both shared a common ancestor

with *L. majoris* (from *Zonotrichia leucophrys oriantha*, FJ168563-ZOLEU02). Genetic distances between these species are shown in Table S5.

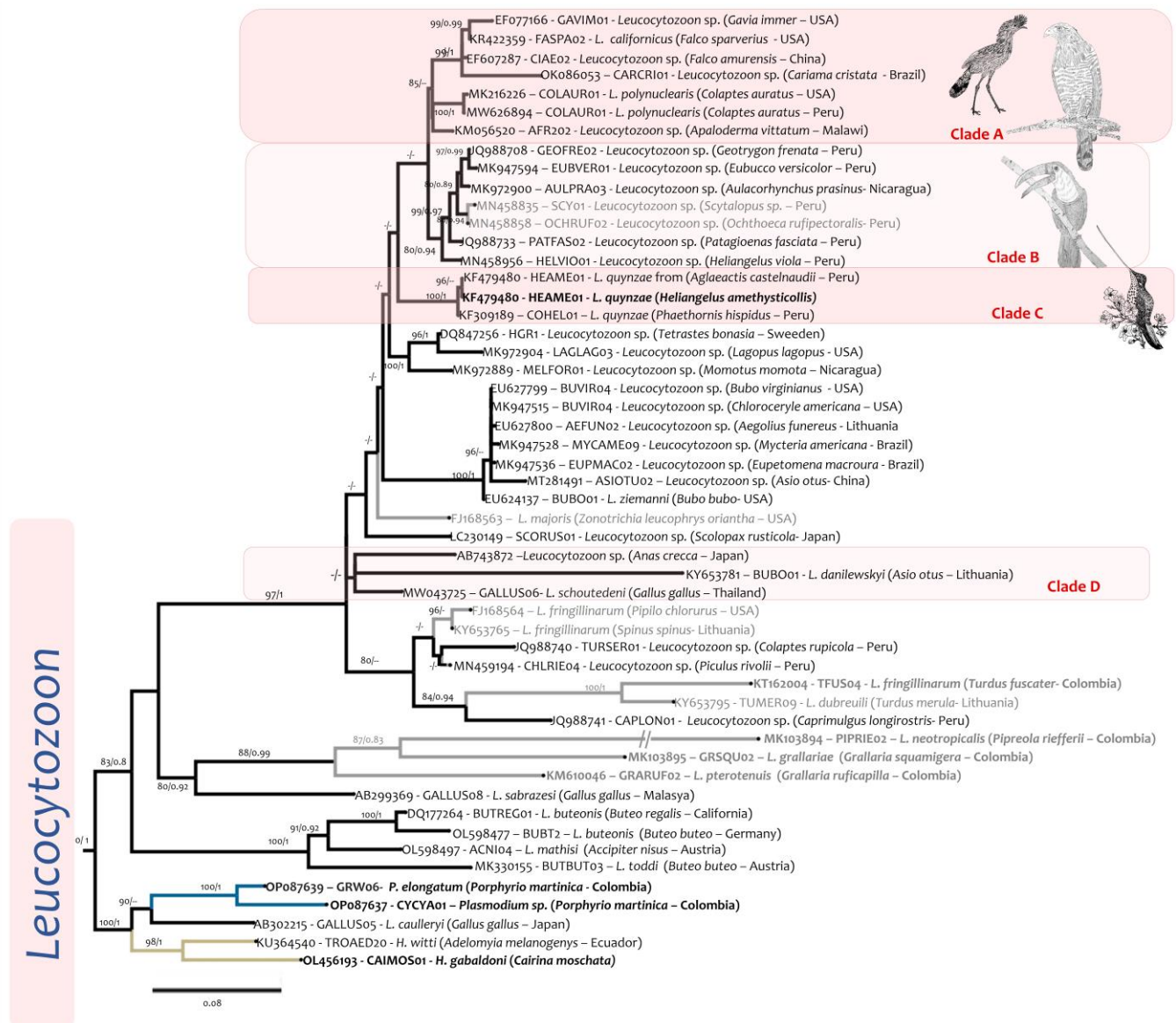


Figure 7. Phylogenetic reconstructions of *Leucocytozoon* sp. obtained with the *Cytochrome b* (cytb) barcode fragments of 479 bp. Bold tip labels correspond to sequences obtained from specimens of the GERPH biological collection. Parasites isolated from passerines are indicated by grey tip labels, while lineages from non-passerine birds are in black. The nodal support values are indicated above the branches as BI/ML. Nodal supports below 0.8/80 are not shown.

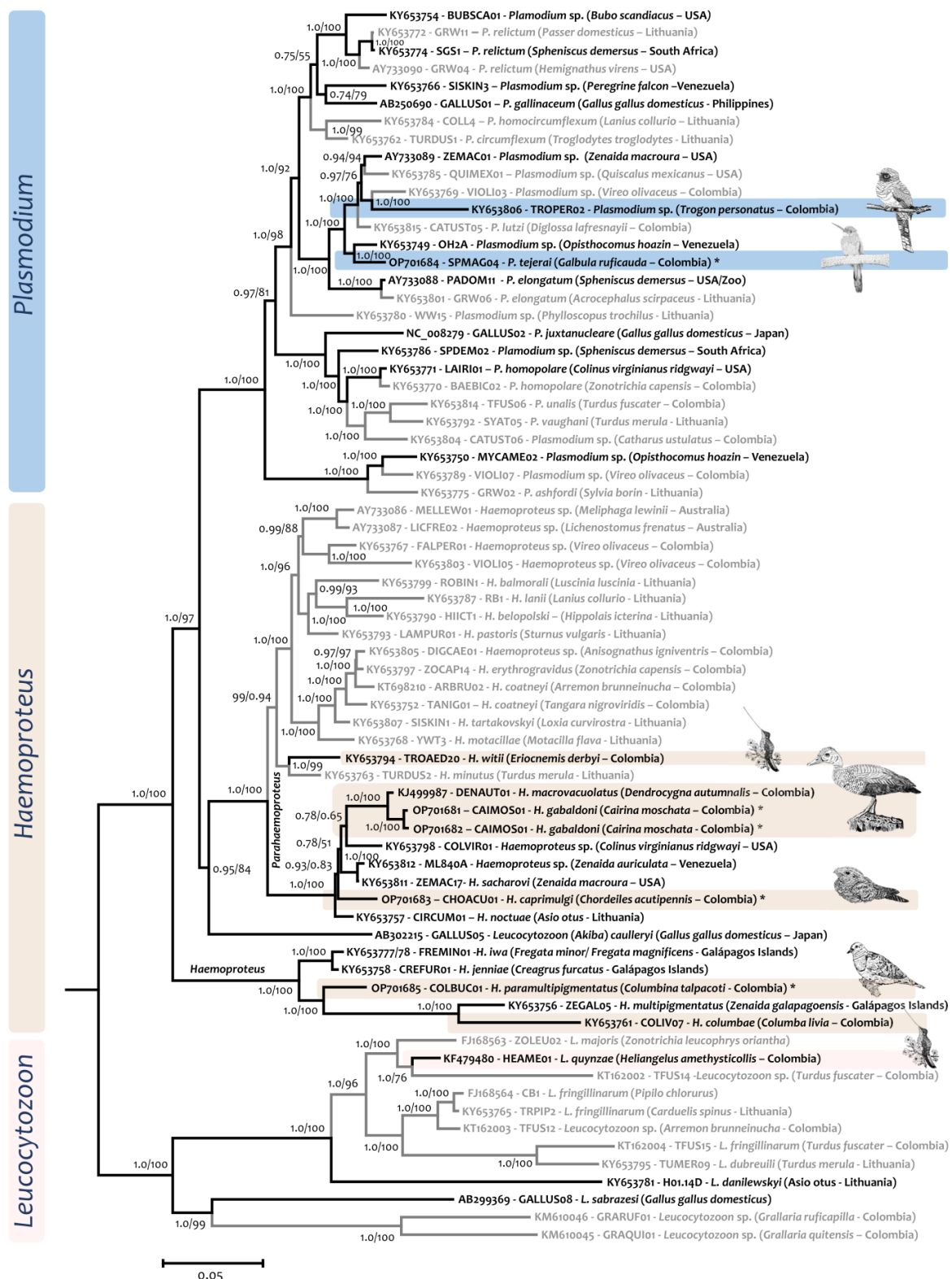


Figure 8. Phylogenetic reconstructions of Haemosporida using mtDNA genomes. The Bayesian and maximum likelihood phylogenetic analyses were performed using 70 partial avian parasite mtDNA genomes (5234 bp excluding gaps). The values above branches are posterior probabilities/bootstraps. Parasites reported in non-passerines are indicated in black and bold, and parasites in passerines are indicated in gray. The new mtDNA genomes (N = 5) reported in this study are marked with an asterisk. Sequences obtained from samples deposited in the biological collection named “Grupo de estudio relación parásito hospedero” (GERPH) are indicated by colored rectangles.

3.4. Caprimulgiformes

Fourteen individuals of six species were evaluated for haemosporidian infections. The presence of the *Haemoproteus* sp. and *Plasmodium* sp.—registered here in the blood of the Lesser Nighthawk (*Chordeiles acutipennis*), the Pauraque (*Nyctidromus albicollis*), and the Blackish or Roraiman Nightjar (*Nyctipolus nigrescens*)—constituted the first report of infection caused by haemosporidians for each species. The *Haemoproteus* observed in these three species (Figure 3M,N) fit the description of *Haemoproteus caprimulgi* [51]. Indeed, the comparison of these specimens with the paratype of *H. caprimulgi* (IRCAH specimen G403126_9396, Figure 3K,L) from the Great-Eared Nightjar (*Lyncornis macrotis*) indicated that the macrogametocytes were slightly longer in the IRCAH specimen than in the specimens in the Colombian samples (media specimen IRCAH: 16.4 ± 2.01 , media specimen *N. nigrescens*: 15.17 ± 1.42). For the reviewed specimens from the wild (Figure 3M,N), growing parasites often partially touch the host cell nucleus, but some spaces are left between those two membranes (Figure 3M macrogametocyte), particularly in gametocytes with slightly ameboid contours. Parasites in the IRCAH reference material (Figure 3K,L) showed even outlines in the different stages of the development of the parasite, while the specimens in *C. acutipennis* and *N. albicollis* sometimes showed ameboid outlines. Fully grown gametocytes were found in mature erythrocytes. Pigment granules are either scattered (see the macrogametocyte in Figure 3M) in the cytoplasm or grouped at the tips (Figure 3M microgametocytes), or different places of the parasite (Figure 3N). For the IRCAH material, small roundish white spots grouped at the tips of the parasites (Figure 3L) could correspond to what once were pigment granules.

Here, we reported for the first time the *cytb* and mtDNA genome sequences associated with *H. caprimulgi*. The *cytb* lineages obtained in this study for this morphospecies were placed in a well-supported clade along with the sequence CHOMIN01-MK216058 obtained from the Common or Antillean Nighthawk (*Chordeiles minor*) from the USA (clade B, Figure 5). Genetic distances between the last-mentioned sequence and the sequences obtained in this study ranged from 0.013 to 0.02 (Table S4). Interestingly, in the mtDNA genomic analysis (Figure 8), *H. caprimulgi* appeared to share a common ancestor with parasites found in other non-passerine birds (Anseriformes, Galliformes, Columbiformes, and Strigiformes). Indeed, all *Haemoproteus* (except *H. witti*) mtDNA genomes available from species found in non-Passeriformes are part of a monophyletic group with high posterior probability and bootstrap at the node (1.0/100). The genetic distance between these parasites is shown in Table S5.

Nyctidromus albicollis, additionally, was found infected with *Plasmodium* sp. Although the morphospecies of this infection could not be identified because of the low number of parasite stages in the peripheral blood (Figure 4I). Molecular tests indicated that the lineage OP087647-NYCTALB03 shared a common ancestor with the parasites of the clade G (Figure 6) that included the lineage OL598516 of *P. elongatum*, which differed by 0.037, and a lineage of *Plasmodium* sp. (AY733088) isolated from an African Penguin (*Spheniscus demersus*) separated by a genetic distance of 0.034. There is only one additional record of a Plasmodiidae infecting a Caprimulgiform species in Myanmar, the lineage COPMAL02 (EF380144) isolated from *Caprimulgus macrurus* (clade A, Figure 6) that differed from OP087647-NYCTALB03 by 0.067 (Table S4).

3.5. Charadriiformes

As for Charadriiformes, 228 individuals from six families and twenty-four species were screened for haemosporidian parasites. Only one was found infected. *Haemoproteus contortus* [52] was the only parasite detected in a Whimbrel (*Numenius phaeopus*), the same host species in which this parasite was described [52]. The cytoplasm was homogeneous and faintly stained, so the macrogametocytes were recognized by the more compact nucleus and a slightly more bluish tone of cytoplasm (Figure 3P). Growing and fully mature gametocytes showed highly ameboid outlines (Figure 3O,P) that makes the parasite touch the host cell nucleus at some points but leave the characteristic cleft between them (Figure 3P). We

did not find ring-like, fully closed parasites as published in the original description, but all mature forms did completely encircle the host cell nucleus (Figure 3O). Unfortunately for this specimen (infected Whimbrel), there was no material available for molecular analysis.

Currently, there is only one lineage from a haemoproteid species isolated from *N. phaeopus* in Japan (NUMPHA01 GenBank Acc. num LC230122) that lies basal in clade E (Figure 5).

3.6. Columbiformes

One hundred and thirty Columbiformes representing twelve species were analyzed (Table 1), from which thirty-nine individuals from eleven species were infected with *H. columbae* [53], *H. paramultipigmentatus* [54] or *Haemoproteus* sp.

The Rock Pigeon (*Columba livia*), an introduced species in Colombia, showed a prevalence of 41% (16/39, Table 1) of *H. columbae*. Most of the specimens infected with *H. columbae* (Ref. [53] Figure 4A) had high parasitemia, with 8% as the maximum parasitemia recorded for a host in our biological collection [55]. All the sampled specimens had the lineage HAECOL01 (KY653761) that share a common ancestor with *H. multipigmentatus* [54] (clade J, Figure 5), differing with a genetic distance of 0.098 (Table S4).

Haemoproteus paramultipigmentatus (Ref. [54] Figure 4B–D), was detected in the Ruddy Ground Dove (*Columbina talpacoti*). Young or nearly mature stages predominate (Figure 4B) in the individuals infected with this species of parasite. Very early stages were seen in sub-polar positions. The young parasites were located lateral to the host cell nucleus touching it in some portions (Figure 4B). Outlines were predominantly ameboid, and the cytoplasm was irregular with some small vacuoles visible. In nearly mature gametocytes, outlines were wavy. Few pigment granules were observed, particularly for microgametocytes that seem to aggregate in clumps throughout the cytoplasm or at the poles of the parasites (Figure 4C). The more mature parasites observed fill the erythrocyte to the poles, displacing the host cell nucleus considerably. However, gametocytes still showed clefts between the membranes of gametocytes and the host cell nucleus (Figure 4D), and the contours were still slightly wavy. However, from the early stages, macrogametocytes may show multiple pigment granules scattered throughout the cytoplasm that was a distinctive character of the species [54].

Four sequences were obtained from the Ruddy Ground Dove specimens sampled in Colombia (n = 13). Two of them were identical to the lineage obtained from the type specimen of *H. paramultipigmentatus* (JN788934-COLPAS03) in Mexico, while the other (OP087648-COPIC01) differed by a genetic distance of 0.002 (clade H, Figure 5; Table S4).

In addition, a *Haemoproteus* sp. was detected in a *Claravis pretiosa* (Blue Ground Dove) with low parasitemia. This lineage (OL689177-COSQU01) lay basal to *H. paramultipigmentatus*, along with JX029921-COSQU01, a *Haemoproteus* parasite that was found in the Scaled Dove (*Columbina squamata*) in Brazil, which was identical (clade I, Figure 5; Table S4). In addition, the mtDNA lineage obtained in this study (OP701685) was identified as COLBUC01 and appeared as a sister taxon of *H. columbae* from *Columba livia* (KY653761-COLIV07, Colombia). The genetic distance for all the subgenus *Haemoproteus* species are shown in Table S5.

A White-throated Quail-Dove (*Zentrygon frenata*) was found infected with *Haemoproteus* sp.; however, given the few stages observed and the small additional sample available, molecular analysis and further determination were not possible.

3.7. Galbuliformes

Only one specimen of the individuals of Galbulidae had a Haemosporidian infection (Table 1). A specimen of Rufous-tailed Jacamar (*Galbula ruficauda*) had an infection caused by *Plasmodium tejerai* [56]. Few morphological stages were found in the smear; however, the erythrocytic meront observed (Figure 4J) in a polar position of the erythrocyte was elongate with even outlines. Although appressed with the host nucleus and outer membrane, no displacement of it or perceptible deformation was evident. The large vacuole was not

evident in this species, however. Such structures fade as meronts mature. In addition, pigment granules were clumped, as in *P. tejerai*, once the large vacuoles disappeared [2]. In addition, 10 merozoites were observed, fitting the description of *P. tejerai*, since this species of parasite usually showed 10 to 15 nuclei. Yet, the parasite cytoplasm was not basophilic as has been reported for the species. In any case, the lineage obtained (OL689171-SPMAG04) was identical to KJ575552 and MF953290 (SPMAG04) (clade E, Figure 6), and both were related to *P. tejerai*, detected in *Spheniscus magellanicus* [57,58] and *Galbula ruficauda* [59] from Brazil (Table S2). These lineages were placed in a well-supported clade (clade F, Figure 6) along with *Plasmodium* species found in Gruiformes in Colombia and Pelecaniformes in Guyana. Genetic distances between these taxa were 0.0021 in both cases (Table S4). In the mtDNA phylogenetic analysis, *P. tejerai* appeared as a sister taxon of *Plasmodium* sp. found in *Opisthocomus hoazin* from Venezuela (KY653749-OH2A; Ref. [60]); it was part of the monophyletic group that contains *P. lutzi*, *P. elongatum*, and *Plasmodium* sp. of a *Trogon personatus* from Colombia (KY653806-TROPER02). The genetic distances among these parasites can be found in Table S5.

In addition to the three species of Bucconidae deposited in the GERPH biological collection (Table 1), only one infection caused by a *Haemoproteus* parasite was found in a Black-fronted Nunbird (*Monasa nigrifrons*). The morphology of this parasite must be studied in more detail since it does not fit completely with the previously described species (*Haemoproteus buconis*) for this family [2]. Yet, the lineage OP087643 was identical to the sequence MONNIG02-MG598390 found in Brazil in the same host species. Such lineages were placed as the sister group of *H. syrnnii* (clade E, Figure 5). The genetic distance between the lineage CULKIB01 of *H. syrnnii* and the lineage found in *M. nigrifrons* was 0.038 (Table S4).

3.8. Gruiformes

Thirty-six individuals from three families of Gruiformes were screened for haemsporidian parasites (Table 1). Two *Plasmodium* species were detected in the American Purple Gallinule (*Porphyrio martinica*). The first of the *Plasmodium* species was compatible with *P. elongatum* (Ref. [61] Figure 4K–N). Erythrocytic meronts were found in only one specimen and developed predominantly in mature erythrocytes containing six or nine merozoites arranged in a fan (Figure 4K) or a circumference (Figure 4L) with no displacement of the host cell nucleus. Gametocytes located in a lateral position had even (Figure 4M) or highly amoeboid (Figure 4N) outlines; in contrast to those reported by [62], they were slightly separated from the envelope of the erythrocyte (Figure 4M). None or only a few medium-sized pigment granules were observed clumped in the parasite cytoplasm (Figure 4N). The amplified lineage OP087639-GRW06 was identical to OL598516, identified as *P. elongatum* and reported by Harl et al. [63]. It was closely related to the *Plasmodium* sp. lineage AY733088 from Sphenisciformes, while differed from lineages of Passeriformes and Caprimulgiformes (clade G, Figure 6) by a genetic distance ranging between 0.0021 and 0.036 (Table S4).

The second species of *Plasmodium* sp. needs to be studied in more detail, since few diagnostic characters could be seen due to the low parasitaemia. The lineage (CYACYA02-OP087637) was placed in clade F (Figure 6), closely related to the lineage SPMAG04 (OP087638) *P. tejerai* found in Galbuliformes (genetic distance of 0.0021—Table S4).

3.9. Pelecaniformes

From the ten sampled species of Pelecaniformes, only the Striated Heron (*Butorides striata*) showed infection by a *Plasmodium* sp. species. However, it could not be identified due the low parasitemia (Figure 4O), where only a few immature parasite stages were found. Yet, the lineage OP087640-BUTSTR03 was placed in a well-supported clade, along with AB477128-CXPIP10 that was isolated from a Yellow Bittern (*Ixobrychus sinensis*)—a Pelecaniform species in Japan (clade C, Figure 6). Both lineages showed a genetic distance of 0.03 (Table S4). Another, the lineage of *Plasmodium* was isolated from *B. striata* in Guyana

(DQ241528-BUTSTR01, clade F, Figure 6); however, it is distantly related to BUTSTR (OP087640), differing from it with a genetic distance of 0.052 (Table S4).

3.10. Strigiformes

Two of the three species of Strigiformes sampled were positive for haemosporidians (Table 1). A species closely similar to *Haemoproteus syrnii*, henceforth *H. syrnii*-like, was detected in the Striped Owl (*Asio clamator*) and the Tropical Screech-Owl (*Megascops choliba*). Most of the parasites in *Asio clamator* (Figure 4E,F) were immature. Early stages were observed in lateral or polar position to the host cell nucleus. Young gametocytes were identified by the presence of a space between the host cell nucleus and the parasite membrane, and by the spaces at the host cell poles (Figure 4E). Even (Figure 4E) or slightly ameboid (Figure 4F) outlines were observed in equal proportions. Parasites caused a slight to moderate displacement of the host cell nucleus without encircling it completely (Figure 4F) as has been reported by [64]. The specimen in *M. choliba* was slightly different from that of *Asio clamator*, so it needs to be studied in more detail to confirm the parasite species' identity.

Two closely related *cytb* lineages were obtained from the infected owls—OP087645-ASICLA010 from *A. clamator* and OP087641-PSDIS01 from *Megascops choliba*. (genetic distance 0.0084—Table S4)—the last one identical to the lineage KJ575554 identified as *H. syrnii*, isolated from *Megascops choliba* in Brazil [58].

The lineage of *H. syrnii* was located in two distantly related clades (clade E and clade F, Figure 5). The sequences OP087645-ASICLA01 and OP087641-PSDIS01 were placed in the well-supported clade F (Figure 6) along with the lineages KF279523-STAL2 (*H. syrnii*) and KF747371-STVAR01 (*Haemoproteus* sp.). Both infections were isolated from owls from Europe and North America. The genetic distances between the lineages in the Colombian hosts and the group comprising KF279523-STAL2 and KF747371-STVAR01 were 0.0127 and 0.0084 (Table S4). Meanwhile, the genetic distance with the lineage MK330144-CULKIB01 in clade E was 0.0322.

The lineages OP087645-ASICLA01 and OP087641-PSDIS01 differed from the two other recognized species of *Haemoproteus* infecting Strigiformes, *H. noctuae* and *H. syrnii* (lineage CIRCUM01, clade C, and KF279523-STAL2, clade F; Figure 5) by 0.032 (Table S4).

3.11. Trogoniformes

Only one out of the six Trogoniformes samples analyzed was found infected. The sample showed the two *Plasmodium* species (Table 1) already reported by González et al. [45]. A blood smear of a Masked Trogon (*Trogon personatus*) showed parasite stages of *Plasmodium* (*Huffia*) sp. along with a *Plasmodium* (*Novyella*) sp. which was found in coinfection. The sequences TROPER01 (KF537290) and TROPER02 (KY653806) obtained for this specimen have no other known host. Such sequences were placed in clade D (Figure 6; see also Figure 8 for comparison), along with the other lineages isolated from Peruvian *T. personatus* (MH457281-TROPER03, MH457292-TROPER04, and MN458765-TROPER05) that differed by genetic distances ranging from 0.0021 to 0.023. The most closely related species identified in the phylogenetic tree was *P. matutinum* in clade E (MK652235-LINN1), separated by genetic distances of 0.034 and 0.036 (Table S4).

4. Discussion

At a global scale, the diversity patterns of avian haemosporidian species seem similar to those of their hosts [9,65]. Thus, the Neotropics, as the world's most diverse area for birds, is expected to harbor diverse haemosporidian parasite communities. However, at the regional level, biodiversity patterns are also driven by ecological and biogeographical contexts resulting from the local and regional evolutionary histories. Thus, as expected, the diversity of these hematozoa is unevenly distributed throughout the continent and along elevational gradients in mountainous areas [13,14,45,59]. In general, such patterns have been studied using data from passerine birds. Thus, due to the scarcity of information, estimates of diversity, distribution, and life cycles, as well as the characterization of prevalence

and their relationship with life histories, environmental conditions, and specific traits, are unknown for the vast majority of parasites found in non-passerine birds (see [66]).

There are five major results from this study. (1) It added valuable information on the hosts, morphology, barcode sequences, and phylogenetic relationships of parasites infecting non-passerine birds of 11 bird orders and 41 species, of which 34 are endemic in the Neotropical region. In addition, we reported the barcode sequence for *H. caprimulgi* while reporting the host range expansion for *P. elongatum*, and *P. nucleophilum*, reported for the first time infecting *Porphyrio martinica* (Gruiformes) and *Glaucidium hirsutum*. (2) From the 22 lineages isolated from samples deposited in the biological collection at GERPH, five are newly reported in this study. In this repository, we have identified thirteen morphological species, and four more need to be investigated in the future since they seem to constitute new species. (3) Furthermore, 14 of the 22 lineages amplified in this study were identical to 78 *cytb* sequences previously reported in GenBank (Table S6), which have been isolated from 274 host species around the globe. Due to the tremendous volume of information published today, compilations such as the one presented here greatly aid to avoid the duplication of information released in public databases. (4) Also, we are reporting for the first time five new parasite mtDNA genomes for the morphospecies *P. tejerai*, *H. gabaldoni* ($n = 2$), *H. caprimulgi*, and *H. paramultipigmentatus* and their phylogenetic relationships. (5) In this study, we present a summary of all the reports on non-passerine birds examined for haemosporidians over the last 40 years for the Neotropics (Tables S2 and S3).

Following the general tendencies observed in the last 40 years of research from Neotropical countries, *Haemoproteus* parasites were the most prevalent in the study. Indeed, these parasites were found in most of the biomes sampled, with Columbiformes being the most infected birds, followed by the Anseriformes and the Apodiformes (Trochilidae). This data should be interpreted with precaution since the Apodiformes and Columbiformes are the non-passerine birds most sampled using mist nets.

According to our results, the Columbiformes sampled in the Amazon-Orinoco peino-biome (VIII) were in the bird order where *Haemoproteus* is the most frequent parasite genus, similar to what has been reported by [59]. In that article, the prevalence in doves was high in Amazonia and in the tropical and subtropical grassland great biome located close to the area of the Amazon-Orinoco peino-biome [5,59,67]. Most of the species of Columbiformes exhibit a gregarious behavior that may facilitate the vector to easily locate and feed on different individuals of the group [55,68].

In the case of Trochilidae, our results indicate the highest frequency of infections by *Haemoproteus* in the high altitude orobiomes of the Andes (IV). Fecchio et al. [59] reported the Peruvian Andes as one of the biomes with the highest prevalence of these parasites in hummingbirds; McNew et al. [13] reported the highest prevalence at altitudes that fit with the high and middle altitude orobiomes of the Andes (IV and V respectively).

Meanwhile, *Plasmodium* was found in biomes under 2500 m asl. In all these places, plenty of breeding places for Culicidae vectors are available, from bromeliads in the middle altitude orobiomes of the Andes to large tracts of flooded land in the helobiomes of the Orinoco and the Amazon. Temperatures in these areas exceed 12 °C, which is suitable for *Plasmodium* development [69].

As for *Leucocytozoon*, our results contrast the recent findings where this parasite has been reported to infect resident birds under 2100 m asl in Brazil [70,71]. Gametocytes from in situ captured birds have not corroborated these infections, and they may correspond to non-patent chronic infections that were probably overlooked on blood smears, or they may correspond to abortive infections. However, the fact that those reports are in territorial species suggests the presence of a competent vector that is maintaining the infection from either migratory birds or resident chronically infected hosts in such places, defying the proposed theory of the existence of an altitudinal gradient of distribution of these parasites [21,27,72].

Current research of haemosporidian diversity, particularly in non-passerine birds, faces three methodological challenges to be resolved. First, mist nets of medium-size mesh

reaching less than three meters of height above the ground (i.e., ref. [73,74]) are the usual method for host sampling [3]. Such methodology biases the capture towards small non-passerine birds such as Columbiformes and Trochilidae. There is a paucity of studies using alternative methods like bow-nets [75] rocket nets, and swim-in bait traps [76] that are used by hand at nests during the breeding season [3,77], as opposed to samples obtained from animals collected by guns [78] and collisions with different objects [79]. They may bias the information obtained towards the orders under investigation that can be collected using these methods (i.e., raptors and ducks). The joint work with animal protection entities such as rescue centers represents an alternative for obtaining samples of rare specimens that are not regularly captured in mist nets, or for which there is some restriction on sampling. Valuable information has been obtained from these specimens in different recent studies (i.e., ref. [21,61,62]). However, parasite transmission can occur at these facilities, and the captive birds can become “accidental hosts” of parasites with which they are not infected in their natural environment [80–83]. So, it is essential to settle protocols of sampling that lead us to detect the infections truly acquired in the wild.

Second, the low parasitemias common in birds with chronic infections represent a challenge for estimating the prevalence and diversity of haemosporidians in wild birds using morphology. Although the association of parasite lineages with a morphological parasite species was the main aim of this investigation, particularly for this study, such values were underestimated since the molecular determination was made only on samples whose smear was positive. As PCR methods are more sensitive than microscopy [25,84], they can detect infections that are non-patent and might be part of the transmission cycles [25]. This is common in other vertebrates, such as humans, where asymptomatic patients with submicroscopic infections can infect malaria vectors [85]. However, detection based only on PCR may compromise the reliability of the host–parasite relationships since such methodologies can detect the parasite DNA at any development stage, even if the life cycle was interrupted before the parasite reaches the gametocyte stage [86]. Despite the fact that molecular methods to detect specific Haemosporidian developmental stages such as gametocytes are more reliable than microscopy [87], those are not available in avian Haemosporida due to limited genomic information.

Third, according to recent molecular characterizations, the haemosporidian parasites infecting non-passerine birds are highly diverse [22,64,68,88,89]. The Neotropics may harbor a great diversity of haemosporidian lineages that might be overlooked due to the mispriming of the oligonucleotides routinely used for the detection of these parasites [63,90,91]. Indeed, in this study we failed to amplify the *Haemoproteus* parasites of raptors, probably due to a mismatch between the primers and the *cytb* sequence of the parasites. To overcome this issue different primer sets have been developed aiming to characterize the genetic diversity of particular areas and hosts [31,63,91,92].

Biological collections constitute large information banks on the specimens deposited in these repositories [93]. In this sense, the specimens stored in collections such as the “GERPH collection” have allowed for the obtaining of informative molecular data linked to morphospecies. Furthermore, multiple parasite mtDNA genomes from morphospecies found in non-passerine and passerine birds deposited in biological collections have yielded phylogenies with a better resolution (Figure 8). Although mtDNA phylogenetic hypothesis lacks sequences of species that are available only for *cytb* fragments, which may affect branch length in some of the taxa, the main topologies are similar to those obtained with 478 bp. lineages (for example, the clade of *Haemoproteus* (*Haemoproteus*) parasites and their position regarding the *Haemoproteus* (*Parahaemoproteus*) species or lineages included in both analyses). Thus, future directions of research with this repository will encompass the molecular screening of all samples and will increase the efforts to get more mitochondrial genomes. These perspectives will provide new insights into diversity and more robust phylogenetic hypotheses of haemosporidian parasites [33,94].

For the Neotropics, 1779 species of non-passerine birds have been reported [95]. To date, about 30% of these species have been explored for haemosporidian infections. In the

case of Colombia, 195 of a total of 878 non-passerine bird species have been tested for avian malaria and related parasites. Many host taxa have been tested with only a handful of individuals that do not represent the species' geographic range nor allow for the estimation of the prevalence of haemosporida parasites. This number of taxa is equivalent to only 22% of the biodiversity of non-passerine hosts in the country. Although significant advances have been made in the last twenty years, there are still nine bird orders under-sampled. Then, for a more precise understanding of parasite diversity and dynamics in Colombia and the Neotropics, deeper sampling of the great host biodiversity reported for this area will be needed.

We confirmed infections using morphology that were previously reported using only PCR in other neotropical countries. So, the use of collections allows the implementation of integrative approaches prevailing in modern taxonomy. Although limited in number of sampled individuals per host species, our findings exemplify the importance of the information deposited in repositories and collections as data sources to support the study of the geographic distribution of host-parasite diversity associations. Indeed, surveys directed to inform biodiversity assessments of vertebrates can better provide insights into regional processes by considering parasites and other symbionts in order to document host-symbiont species assemblages. Biological collections where specimens are curated with their associated metadata are indispensable for incorporating neglected symbiont taxa such as haemosporidian parasites into biodiversity science.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/d15010057/s1>: Table S1: Biomes and specific localities of origin of the non-passerine bird samples deposited in the GERPH biological collection included in this study. Table S2: Non-passerine species found infected with Haemosporidian parasites in the Neotropics after the comprehensive review of White et al. [17]. Table S3: Non-passerine species explored and found negative for Haemosporidian parasites in the Neotropics after the comprehensive review of White et al. (1978). Table S4: Kimura-two-parameters pairwise genetic distances between lineages in Figures 5–7. Table S5: Estimates of evolutionary divergence between haemosporidian parasites' mtDNA genome sequences (5234 bp excluding gaps). Table S6: Lineages identified in this study as identical with others previously reported. Table S7: Database of papers reviewed for data compilation of Haemosporidian parasite infections in neotropical birds after 1978. References [96–108] are cited in Supplementary Materials.

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Data Availability Statement: The sequence data produced in this study are available in the GenBank database under accession numbers OL689174, OL689176, OL689177, OP087637-OP087649, and OP701681-OP701685. Also, this lineage information was submitted to the MalAvi database.

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