



FREQUENCY OF AVIAN HAEMOSPORIDIAN PARASITES IN BIRDS FROM MARGARITA AND COCHE ISLANDS, VENEZUELA

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Abstract · By amplifying and sequencing a mitochondrial DNA barcode (cytb), we screened the frequency of haemosporidian parasites (*Haemoproteus*, *Plasmodium*, *Leucocytozoon*) in 366 birds (23 species) from Margarita and Coche islands, Venezuela. In Coche (N = 24), none of the birds were infected, while in Margarita (N = 342) 13 individuals were (3.8%). The frequency of these parasites in endemic bird subspecies was more than double than in non-endemic forms (endemics = 7.3% vs non-endemic = 3.1%, N = 342), but it was not statistically significant. We found eight parasite lineages, four of which are novel (COLPAS09, COLSQU03, COLSQU04, HYPRUF01). COLSQU03, recorded in a dove (*Columbina squammata*), falls within the *Haemoproteus* (*Parahaemoproteus*) subgenus. This was unexpected because a strong signal of co-speciation has been found between Columbidae and the *Haemoproteus* (*Haemoproteus*) subgenus, suggesting a host-switching event.

Resumen · Frecuencia de parásitos haemosporideos en aves de las islas Margarita y Coche, Venezuela

Mediante la amplificación y secuenciación de un código de barras mitocondrial (cytb), se evaluó la frecuencia de parásitos haemosporideos aviarios (*Haemoproteus*, *Plasmodium*, *Leucocytozoon*) en 366 aves (23 especies) en las islas de Margarita y Coche, Venezuela. En Coche (N = 24), ninguna de las aves estuvo infectada, mientras que en Margarita (N = 342) 13 individuos (3,8%) lo estuvieron. La frecuencia de estos parásitos en subespecies de aves endémicas fue más del doble que en las no endémicas (endémicas = 7,3% vs no endémicas = 3,1%, N = 342), pero no fue significativamente distinta. Encontramos ocho linajes, de los cuales cuatro fueron nuevos (COLPAS09, COLSQU03, COLSQU04, HYPRUF01). COLSQU03, encontrado en una paloma (*Columbina squammata*), se agrupó con el subgénero *Haemoproteus* (*Parahaemoproteus*). Este hallazgo fue inusual, ya que existe señal de coespeciación entre Columbidae y el subgénero *Haemoproteus* (*Haemoproteus*), lo que sugiere un evento de cambio de hospedador.

Key words: Caribbean · *Haemoproteus* · Host-switching · *Leucocytozoon* · Malaria · Neotropics · *Plasmodium*

INTRODUCTION

Avian haemosporidian parasites (genera *Haemoproteus*, *Plasmodium* and *Leucocytozoon*; phylum Apicomplexa, order Haemosporida) comprise diverse groups of vector-transmitted parasites that infect mainly red blood cells, as well as mononuclear leukocytes and other internal organs of vertebrates, which causes malaria disease (Valkiūnas 2005). Their prevalence in the Caribbean islands ranged from 0% to 100% among 53 avian species from the Lesser Antilles (Fallon et al. 2005) and the same range was reported for 50 avian species on Hispaniola Island (Latta & Ricklefs 2010). These studies and others (Martínez-Gómez & Matías-Ferrer 2020) show that the prevalence of haemosporidian parasites varies widely amongst host species; thus, avian species inhabiting islands are not equally susceptible to infection. The introduction of haemosporidian parasites to islands can cause negative impacts in native and endemic avian species (Van Riper et al. 1986, Van Riper et al. 2002, Parker 2018); therefore, monitoring this and other diseases is essential for their conservation (Padilla et al. 2004, Carlson et al. 2013, Paxton et al. 2018).

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In the present study, we searched for the three most common avian haemosporidian genera (*Haemoproteus*, *Plasmodium*, and *Leucocytozoon*) at community level (22 bird species) in continental Margarita (952 km²) and Coche (55 km²) islands, Venezuela. Particularly, Margarita Island comprises an interesting study location and is of conservation relevance because: i) several avian species are locally at risk of extinction, or their populations are declining due to anthropogenic changes that have occurred during the last 45 years (Sanz et al. 2011); ii) as a result of insularity and habitat loss, bird populations might be more vulnerable to negative impacts by pathogens; iii) the island is close enough to the continent (about 23 km) for the mainland to be a source of host and parasite populations, yet isolated enough for the evolution of endemic forms (there are 13 endemic avian subspecies on the island; Sanz et al. 2010); and iv) in recent years, non-native bird species (mainly psittacids and aquatic birds, but also passerines) have been increasingly recorded on the island, and they could be involved in parasite introductions (Sanz et al. 2010, Sanz et al. 2016).

This study aimed to explore avian haemosporidian parasite frequency on the islands by amplifying and sequencing a mitochondrial DNA barcode (cytb). BLAST, as implemented in MalAvi, was used to identify parasite genera and subgenera. Then, phylogenetic analyses were used to identify the observed parasite species or their sister taxa. The avian haemosporidian parasite frequency in endemic vs non-endemic avian host was also compared.

MATERIALS AND METHODS

Study area. The samples were collected between July 2009 and October 2011 in six localities on Margarita Island, Nueva Esparta state (Venezuela): i) Chacaracual, CH (10°57'16"N - 64°17' 20"W); ii) Murrión, MU (11°00'52"N - 64°11'52"W); iii) Orinoco, OR (10°54'29"N - 64°01'41"W); iv) Copey, CP (11° 01'46"N - 63°53'03"W); v) Matasieta, MA (11°02'04"N - 63° 50'51"W); and vi) Tragaplata, TR (11°07' 08"N - 63°53'36"W). Vegetation in CH, MU and OR is open xerophytic thorn scrub with cacti, while CP, MA, and TG comprise a more humid woodland. In October 2010, we held an additional sampling in Coche Island, CO (55 km², 10°46'45" - 10°45'30"N, and 63° 55'03" - 63°59'38"W), located 7 km from Margarita. The vegetation there is also xerophytic scrub, but sparser than in Margarita Island.

Bird blood samples. Blood samples were taken in different months, covering both dry (April, May, October) and wet (July and February) seasons. A total of 342 blood samples were obtained in Margarita Island from 19 species, and 24 samples from 8 species in Coche Island. Birds were captured using mist nets (36 mm mesh size) and blood samples were taken only from adult birds by puncturing the ulnar vein (wing) with sterilized needles. Every bird was banded with a unique numbered aluminum ring. About 30 µL of blood were placed on filter paper (3MM® Whatman, Brentford, UK) for subsequent DNA extraction.

Parasite screening. Total genomic DNA was extracted from blood samples using the protocol described by Berezky et al. (2005). PCR and sequencing of 479 bp of the cytochrome b region were carried out using the protocol described by

Mijares et al. (2012). Samples that were not amplified by the first primer set were subjected to amplification with another primer set using a nested PCR design, according to Perkins & Schall (2002). We followed the steps suggested by Outlaw & Ricklefs (2014), applying BLAST in the MalAvi database for identifying parasite lineages. All sequences were compared with a local BLAST against the Malavi database to identify parasite genera and determine whether novel lineages (i.e., unique DNA sequence) were found. Lineages were named after the host of origin following a standard protocol (Bensch et al. 2009) and DNA sequences were deposited within the GenBank database (MW505913- MW505920).

Since our work is at community level, we estimated the frequency of birds infected with haemosporidian parasites, instead of prevalence, applying Sterne's method with a 95% confidence interval. The frequency of infected birds depends on the sample size per host population (Jovani & Tella 2006). Since several species were sampled in low numbers, we calculated the overall frequency of birds positive for infection considering all species sampled, and only using the species with seven or more individuals. The frequencies of birds infected with haemosporidian parasites of endemic and non-endemic forms were compared through Fisher's exact test. Statistical analyses were done with the software QPweb (Reiczigel et al. 2019).

Alignment Construction and Phylogenetic Analyses. All sequences were aligned using ClustalW, as implemented in MEGA 7 (Kumar et al. 2016), with manual editing. The closest cyt b available sequences of known morphospecies and sequences that showed 100% of identity (BLAST analyses) were included in the alignment. Based on the sequences' overlap, an alignment of 347 bp was selected for the phylogenetic analyses, which were inferred using the Bayesian methods implemented in MrBayes v3.2.6, with the default settings and applying the GTR+I + Γ model (Ronquist & Huelsenbeck 2003) and the Neighbor-joining method in MEGA 7.0 (Kumar et al. 2016).

RESULTS

Overall, the frequency of birds positive for haemosporidian parasites in the avian community was low (3.6%, 2.0-6.0% CI, N = 366). The frequency of birds infected was almost the same (3.7%) when considering only the species with more than seven sampled individuals (14 species, N = 326 individuals). In Coche Island (N = 24), none of the sampled individuals were infected, while in Margarita Island (N = 342) 13 individuals were (3.8%, 2.1-6.4% CI): 11 were infected with a single *Haemoproteus* lineage (3.2%, 1.7-5.7% CI), 1 with a single *Plasmodium* lineage (0.3%, 0-1.7% CI), and 1 migratory species with a single *Leucocytozoon* lineage (0.3%, 0-1.7% CI) (Table 1). The parasite frequency in endemic subspecies was more than double compared to non-endemic forms (endemics = 7.3% vs non-endemic = 3.1%, N = 342), but the difference was not statistically significant (Fisher's test, $P = 0.1952$). The pathogens were spread all over the lowlands of Margarita island. Infected birds were found at every locality sampled, except Matasieta.

We found eight different lineages, four novel, and four previously described (Table 1). The phylogenetic analysis (Figure 1) indicated that within the *Haemoproteus*

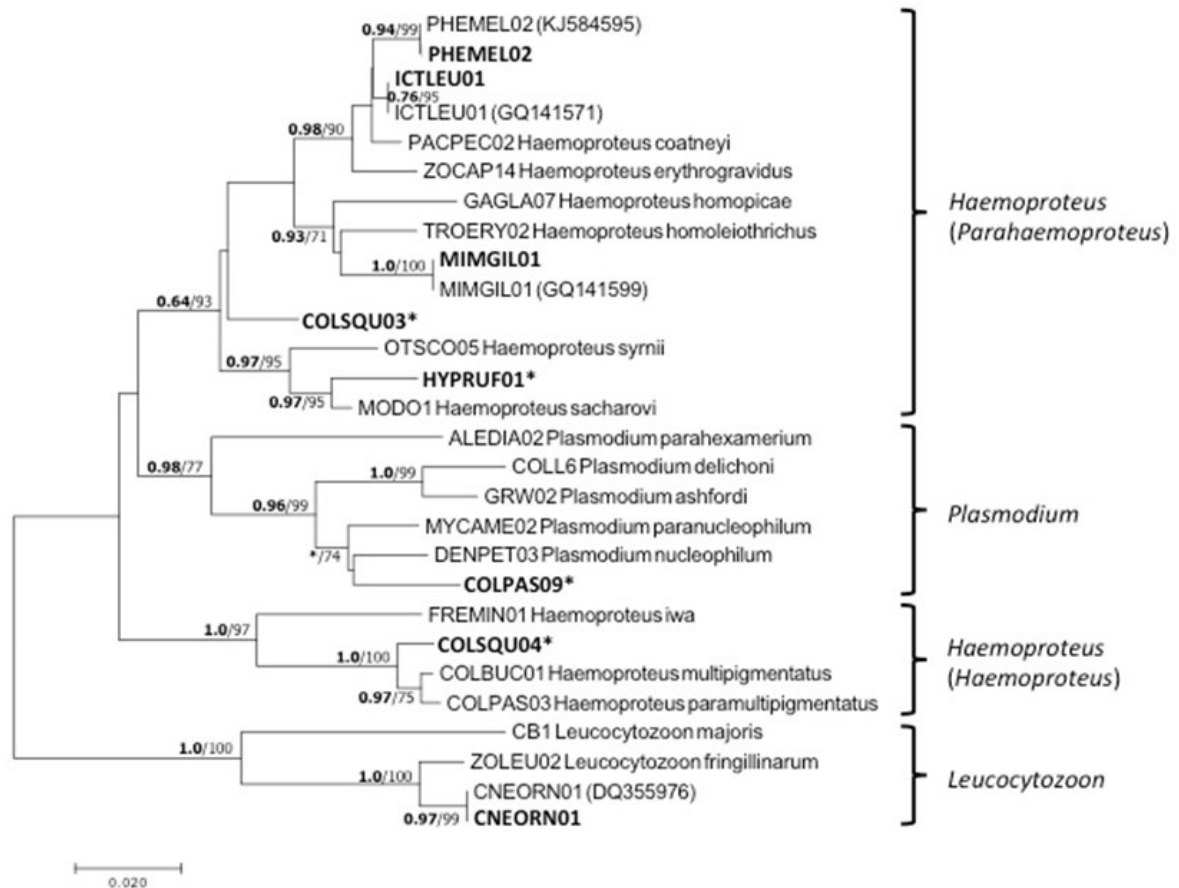


Figure 1. Neighbor-joining (NJ) tree depicting phylogenetic relationships between cytb lineages. Statistical support for Bayesian searches (posterior probability, bold) and for the NJ (1000 bootstrap) are reported on branches. Lineages from this study are indicated in bold and novel lineages in bold with an asterisk.

(*Parahaemoproteus*) subgenus clade there are five of the eight lineages identified in this study; one of them (COLSQU03, novel) was found in a *Columbina* dove, (*Columbina squammata*) while the rest were found in passerine birds. Within the *Haemoproteus (Haemoproteus)* subgenus clade there is one novel lineage (COLSQU04), also found in a *Columbina* dove (*Columbina squammata*). The *Plasmodium* lineage COLPAS09 (novel) and the *Leucocytozoon* lineage CNEORN01 were placed in their respective genera.

DISCUSSION

The frequency of birds infected with haemosporidian parasites (*Haemoproteus*, *Leucocytozoon*, and *Plasmodium*) in Margarita Island was low (3.8%). In other Caribbean islands, the average frequency of infected individuals has been as high as 41% in Hispaniola (Latta & Ricklefs 2010) and 28% in several Lesser Antilles islands (Fallon et al. 2005). However, the comparison from different studies should be made cautiously. The frequency of birds positive for infection by haemosporidian parasites shows variability depending on many factors, which include the identity and abundance of the bird species sampled, as well as their age, sex, and physiological conditions. Other factors to consider are abiotic conditions and seasonality, which affect vector abundance and activity, the particular evaluated habitats, and the sampling

effort in such habitats (Bennett et al. 1980, van Riper et al. 1986, Apanius et al. 2000, Jovani & Tella 2006, Svensson-Coelho et al. 2013, Scordato & Kardish 2014, Calero-Riestra & Garcia 2016). Even that the frequency of birds infected in endemic forms almost doubled that of the non-endemic subspecies, the difference was not statistically significant. Other Neotropical studies found that birds of endemic species were more infected than other residents; for instance, Latta & Ricklefs (2010) found on Hispaniola island that the prevalence of haemosporidian parasites differed significantly among migratory, endemic resident, and non-endemic resident species, with endemics having the highest rates of infection. González-Quevedo et al. (2016) found evidence of higher overall prevalence of haemosporidian parasites among endemic rather than nonendemic birds on an isolated coastal massif in northern Colombia. As our sample size for endemic subspecies was low ($N = 41$), it would be necessary to confirm our results with a greater sampling effort of this group, combining diagnostic methodologies such as PCR, microscopy, and serology, for a better estimation of the frequency (Valkiūnas & Atkinson 2020).

Four of the lineages we found have been previously reported (Table 1), exhibiting differences concerning their host's specificity. The *Leucocytozoon* lineage CNEORN01 shows low host specificity as it has been reported in several bird families (Fecchio et al. 2019). The other three previously reported lineages (MIMGIL01, PHEMEL02, ICTLEU01), be-

Table 1. Bird species, sample size and localities surveyed for haemosporidian parasites in Margarita and Coche islands, Nueva Esparta State (Venezuela). * indicates endemic sub-species from Margarita Island. Novel lineages are in bold. ^a Codes for locations: CH– Chacaracual, CO– Coche, CP– Copey, MA– Matasi-ete, MU– Murrión, OR– Orinoco, TR–Tragaplata. ^b *Haemoproteus* subgenus: *P*– *Parahaemoproteus*, *H*– *Haemoproteus*.

Family/Species	Locality ^a (sample size)	Number of infected birds (locality)	Lineage name, parasite genus (subgenus ^b)	Closest BLAST hit (identities, % match)	Other hosts reported (reference)
Columbidae					
<i>Columbina passerina</i>	CH(13), CO(3), MA(8), MU(27), OR(10)	1 (MU)	COLPAS09 , <i>Plasmodium</i>	MITOM01 (455/456, 99%)	
<i>Columbina squammata</i>	CH(17), CO(1), MU(7), OR(30), TR(1)	1 (CHA)	COLSQU03 , <i>Haemoproteus</i> (<i>P.</i>)	MAFUS02 (463/464, 99%)	
		1 (OR)	COLSQU04 , <i>Haemoproteus</i> (<i>H.</i>)	COSQU02 (478/479, 99%)	
<i>Leptotila verreauxi</i>	CH(3), CP(1), MA(2), OR(9), TR(3)	0			
Psittacidae					
<i>Eupsittula pertinax margaritensis</i> *	CH(2), MU(7)	0			
Cuculidae					
<i>Coccyzus americanus</i>	MU(2), OR(1), TR(1)	0			
<i>Crotophaga ani</i>	CO(1)	0			
Bucconidae					
<i>Hypnelus ruficollis stolicus</i> *	CH(3), CP(4), MU(1), OR(1), TR(3)	1 (CP)	HYPRUF01 , <i>Haemoproteus</i> (<i>P.</i>)	NYSMAC03 (431/437, 99%)	
Picidae					
<i>Melanerpes rubricapillus</i>	CH(1), OR(1)	0			
Furnariidae					
<i>Dendroplex picus longirostris</i> *	CH(3), CP(3), MU(4), OR(2), TR(1)	0			
Mimidae					
<i>Mimus gilvus</i>	CH(15), CO(10), CP(5), MA(7), MU(13), OR(11), TR(3)	2 (OR)	MIMGIL01, <i>Haemoproteus</i> (<i>P.</i>)	MIMGIL01 (361/361, 100%)	<i>Mimus gilvus</i> (Outlaw & Ricklefs 2009)
Tyranidae					
<i>Myiarchus tyrannulus</i>	CH(7), MU(3), OR(2), TR(1)	0			
<i>Sublegatus arenarum</i>	CO(1)	0			
Pipridae					
<i>Chiroxiphia lanceolata</i>	CP(9)	0			
Vireonidae					
<i>Cyclarhis gujanensis</i>	CP(1)	0			
Thamnophilidae					
<i>Thamnophilus doliatus</i>	CP(2), TR(5)	1 (TR)	MIMGIL01, <i>Haemoproteus</i> (<i>P.</i>)	MIMGIL01 (361/361, 100%)	<i>Mimus gilvus</i> (Outlaw & Ricklefs 2009)
Parulidae					
<i>Setophaga striata</i>	CH(2), CO(5), MU(1)	1 (CH)	CNEORN01, <i>Leucocytozoon</i>	CNEORN01 (457/457, 100%)	21 bird species from 5 families, (Fecchio et al. 2019)
Thraupidae					
<i>Thraupis glaucocolpa</i>	CP(7), MA(22), OR(3), TR(14)	0			
<i>Thraupis palmarum</i>	CP(3), MA(2), TG(1)	0			
<i>Coereba flaveola</i>	CH(1), CO(1)	0			
<i>Melospiza bicolor</i>	CO(2)	0			
Cardinalidae					
<i>Cardinalis phoeniceus</i>	CH(6), MA(3), MU(5), OR(6), TR(2)	1 (MU)	PHEMEL02, <i>Haemoproteus</i> (<i>P.</i>)	PHEMEL02 (362/362, 100%)	<i>Pheucticus melanocephalus</i> and <i>P. ludovicianus</i> , Cardinalidae family (Barrow et al. 2021 and unpublished)
		1 (CHA)	PHEMEL02, <i>Haemoproteus</i> (<i>P.</i>)	PHEMEL02 (362/362, 100%)	
Icteridae					
<i>Icterus nigrogularis helioeides</i> *	CH(2), MU(2), OR(2), TR(1)	1 (MU)	ICTLEU01, <i>Haemoproteus</i> (<i>P.</i>)	ICTLEU01 (479/479, 100%)	<i>Icterus leucopteryx</i> and <i>I. galbula</i> (Outlaw & Ricklefs 2006 and unpublished)
		1 (CHA)	ICTLEU01, <i>Haemoproteus</i> (<i>P.</i>)	ICTLEU01 (479/479, 100%)	
<i>Icterus icterus</i>	CH(1), MU(1)	1 (MU)	ICTLEU01, <i>Haemoproteus</i> (<i>P.</i>)	ICTLEU01 (479/479, 100%)	

longing to the *H.* (*Parahaemoproteus*) subgenera, have been found in birds from the same host families reported herein (MIMGIL01 in Mimidae, PHEMEL02 in Cardinalidae, ICTLEU01 in Icteridae; Table 1) (Outlaw & Ricklefs 2009, Barrow et al.

2021). These results corroborate earlier findings of *Haemoproteus* co-speciation at the host family taxonomic level (Beadell et al. 2004, Santiago-Alarcon et al. 2014), while *Leucocytozoon* shows a more generalist ability (Lotta et al.

2016). The other four lineages from this study were novel, highlighting COLSQU03, a *H. (Parahaemoproteus)* lineage found in a dove. This is the fourth report of *Parahaemoproteus* cyt b lineages infecting doves (Križanauskienė et al. 2013, Santiago-Alarcon et al. 2014, Pacheco et al. 2018), as doves and pigeons are generally parasitized by species of the *Haemoproteus* subgenus (Santiago-Alarcon et al. 2010). The evolution of avian haemosporidians is related to their Diptera vectors (Martinsen et al. 2008, Križanauskienė et al. 2013, Pacheco et al. 2018), suggesting that these few *Parahaemoproteus* lineages found in doves are likely transmitted by biting midges (Ceratopogonidae). The feeding preferences of Diptera vectors do not seem to be sufficiently specialized to isolate populations of parasites on different bird species (Ferreira et al. 2020), resulting in local host-switching events (e.g., Ferreira-Junior et al. 2018). In combination with subsequent reproductive parasite isolation, this might lead to the formation of new evolutionary lineages of parasites (Ricklefs et al. 2014, Ellis et al. 2019). Our finding of a *H. (Parahaemoproteus)* lineage in a dove indicates that the parasite found the opportunity to colonize a new host, a direct proof of a host-switching event and a normal mechanism of haemosporidian diversification (Ellis et al. 2019). Such dynamic host-parasite associations are relevant to the epidemiology of emerging infectious diseases because low parasite specificity increases the spread risk to novel hosts (Santiago-Alarcon et al. 2014).

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