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Alpha-globin variation does not predict avian malaria infection in the West Indian Bananaquit (*Coereba flaveola*)

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Cover Page: Bananaquit (*Coereba flaveola*) on 12 January 2008 in Campinas, Sao Paulo, Brazil. Photograph by Carlos Henrique L.N. Almeida.

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Abstract

Infection by lineages of avian malaria parasites (Apicomplexa: Haemosporida) varies geographically, and some lineages exhibit disjunct distributions. These patterns might be related to differential resistance among host populations reflecting intrinsic characteristics of both the host and the pathogen. In hematophagous parasites in particular, the structure of host hemoglobin can influence parasite development and reproduction. Though variation in avian hemoglobin has been documented across altitudinal gradients, little is known about hemoglobin variation as it relates to infection by the parasites causing avian malaria. We sequenced the α^A-globin subunit of the Bananaquit (*Coereba flaveola*), and related sequence variation to avian malaria infection frequency and parasite lineage identity. We found no association between α^A-globin haplotype and infection by particular parasite lineages among all locations, nor any protective association between globin haplotype frequency and the proportion of individuals infected within populations. Phylogeographic structure and genetic variation at the α^A-globin locus, including a highly variable intron, is largely concordant with the mitochondrial cytochrome *b* locus for these same populations, supporting this marker as an independent and variable target with potential application in biogeographic analyses.

Keywords

alpha-globin, avian malaria, Bananaquit, *Coereba flaveola*, West Indies

Resumen

La variación de la globina alfa no predice la infección por malaria aviar en *Coereba flaveola* • La infección por linajes de parásitos de la malaria aviar (Apicomplexa: Haemosporida) varía geográficamente y algunos linajes exhiben distribuciones discontinuas. Estos patrones podrían estar relacionados con la resistencia diferencial entre las poblaciones de huéspedes, y reflejan las características intrínsecas tanto del huésped como del patógeno. En los parásitos hematófagos, en particular, la estructura de la hemoglobina del huésped puede influir en el desarrollo y la reproducción del parásito. Aunque se ha documentado la variación de la hemoglobina aviar a través de gradientes altitudinales, se sabe poco sobre su variación en relación con la infección por los parásitos que causan la malaria aviar. Secuenciamos la subunidad de globina α^A de *Coereba flaveola* y relacionamos la variación de la secuencia con la frecuencia de infección por malaria aviar y la identidad del linaje del parásito. No encontramos ninguna asociación entre el haplotipo de globina α^A y la infección por determinados linajes de parásitos entre localidades; ni ninguna asociación protectora entre la frecuencia del haplotipo de globina y la proporción de individuos infectados dentro de las poblaciones. La estructura filogeográfica y la variación genética en el locus de la globina α^A, que incluye un intrón muy variable, concuerdan en gran medida con el locus del citocromo *b* mitocondrial para estas mismas poblaciones; lo que respalda a este marcador como un objetivo independiente y variable con aplicación potencial en análisis biogeográficos.

Palabras clave

Caribe Insular, *Coereba flaveola*, globina alfa, malaria aviar

Résumé

La variation de l'alpha-globine ne permet pas de prédire l'infection par le paludisme aviaire chez le Sucrier à ventre jaune (*Coereba flaveola*) • L'infection

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par des lignées de parasites responsables du paludisme aviaire (Apicomplexa: Haemosporida) varie géographiquement, et certaines lignées présentent des répartitions disjointes. Ces schémas pourraient être liés à une résistance différentielle entre les populations d'hôtes reflétant des caractéristiques intrinsèques à la fois de l'hôte et du pathogène. Dans le cas particulier des parasites hématophages, la structure de l'hémoglobine de l'hôte peut influer sur le développement et la reproduction du parasite. Bien que la variation de l'hémoglobine aviaire ait été documentée en fonction des gradients altitudinaux, peu de choses sont connues sur la variation de l'hémoglobine en relation avec l'infection par les parasites responsables du paludisme aviaire. Nous avons séquencé la sous-unité α^A-globine du Sucrier à ventre jaune (*Coereba flaveola*), et nous avons mis en relation la variation de la séquence avec la fréquence d'infection du paludisme aviaire et l'identité de la lignée parasitaire. Nous n'avons trouvé aucune association entre l'haplotype α^A-globine et l'infection par des lignées parasitaires particulières en fonction des sites, ni aucune association protectrice entre la fréquence de l'haplotype globine et la proportion d'individus infectés au sein des populations. La structure phylogéographique et la variation génétique au locus de l'α^A-globine, y compris un intron hautement variable, est largement concordante avec le locus du cytochrome *b* mitochondrial pour ces mêmes populations, indiquant ce marqueur comme une cible indépendante et variable pouvant avoir une application potentielle dans les analyses biogéographiques.

Mots clés
alpha-globine, Antilles, *Coereba flaveola*, paludisme aviaire, Sucrier à ventre jaune

Avian malaria is a ubiquitous, globally distributed pathogen (except Antarctica) known to infect a wide variety of avian host orders, but it is primarily detected among passerines (Videvall 2019). The impacts of malaria infection on host fitness and population dynamics are poorly understood, but previous research indicates that the impacts are more pronounced in cases of naïve hosts that lack an evolutionary history with the parasite lineage in question (LaPointe *et al.* 2012). Effects of infection are known to range from sublethal impacts on individual host fitness to population decline and extinction, as was observed in the case of Hawaiian forest birds following the introduction of *Plasmodium relictum* and its vector (LaPointe *et al.* 2012). Additionally, host switching appears to be a common occurrence among avian malaria parasites and leads to the evolution of

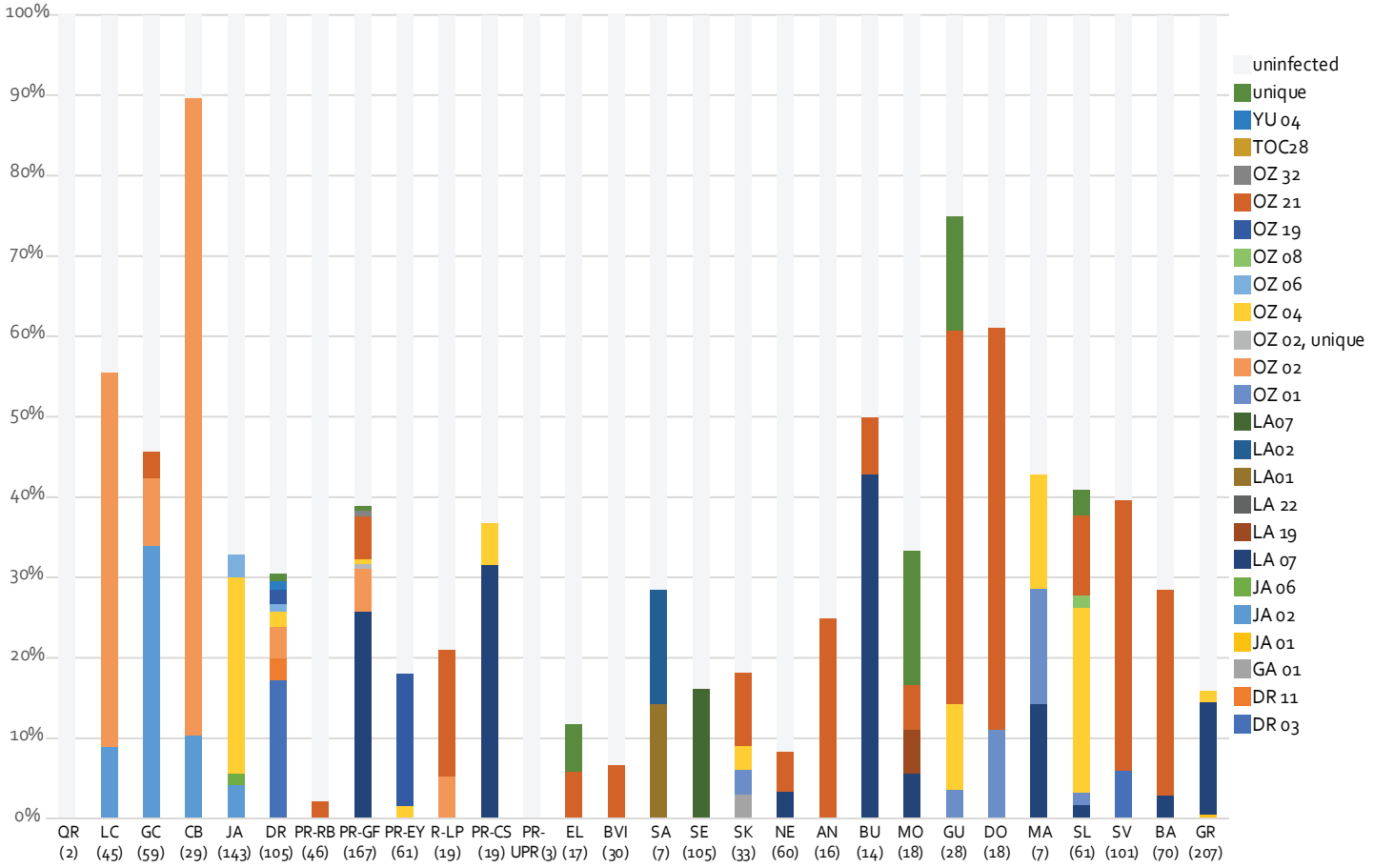

Fig. 1. Bananaquit malaria infection by location as a percent of total captures (these analyses include more individuals than those in the *HBA/CYTB* analyses). Colored portions represent infection by a particular lineage, and sample sizes are in parenthesis on the x-axis. Location abbreviations available in Appendix 1.

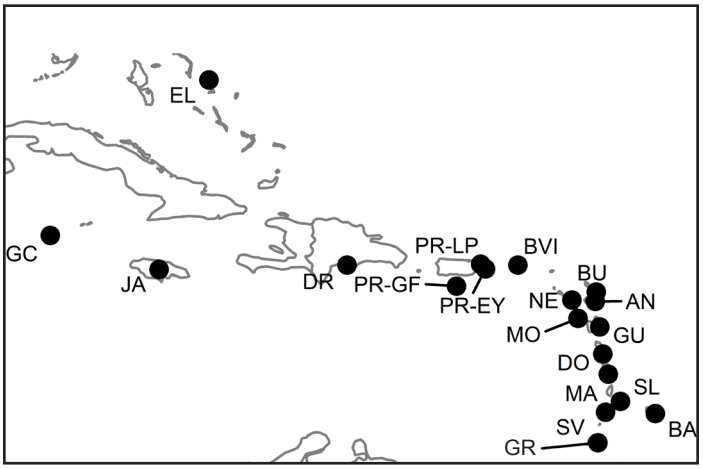

Fig. 2. Sampling localities in the West Indies. Location abbreviations available in Appendix 1.

new parasite lineages, which may then have differential impacts on hosts (Ricklefs *et al.* 2014). This general propensity for host switching may also pose a threat to humans and livestock, as the majority of emerging human diseases are zoonotic in origin (Slingenbergh *et al.* 2004).

The frequency of infection by a particular lineage of avian malaria parasite (Haemosporida: *Plasmodium* spp. and *Haemoproteus* spp.) in a single host varies geographically, and some lineages exhibit disjunct distributions (Fallon *et al.* 2005, Ricklefs *et al.* 2016). The host species *Coereba flaveola* (Thraupidae), familiarly known as the Bananaquit, is widely distributed throughout the Caribbean Basin and continental tropical America (Bellemain *et al.* 2008). Despite its ubiquity, nearly three-quarters of described parasite lineages infecting this species each occur on only one or two islands (16 of 22 lineages detected more than once). Moreover, most local populations of Bananaquits sustain three or fewer lineages of parasite (Fig. 1). These patterns might be related to variation in resistance among host populations to particular parasite lineages. In humans, 10–15% of the risk of malaria infection and severity is determined by genetic factors that cause variations in either the immune response or in red blood cell (RBC) structure or function (Mackinnon *et al.* 2000). The latter has been widely investigated in humans and is considered to have substantial influence on susceptibility to malarial infection (de Mendonça *et al.* 2012).

Hemoglobin (Hb) variation is one type of RBC variation that is especially important in interactions with hematophagous parasites (van Dooren *et al.* 2012). Hemoglobin is partly composed of heme, which is an iron-rich compound required by hematophagous parasites for growth, development, and reproduction (van Dooren *et al.* 2012). Heme is required by most life on earth, including parasites, for a variety of cellular processes including antioxidant defense, electron transfer in mitochondrial cellular respiration, and the detection and transfer of gases, including oxygen (van Dooren *et al.* 2012). Because heme is highly reactive and can be toxic, intracellular levels must be carefully regulated. Hematophagous parasites process large amounts of this compound in the course of metabolizing hemoglobin, and excess heme molecules are polymerized and sequestered as hemozoin (Toh *et al.* 2010, de Mendonça *et al.* 2012, van Dooren *et al.* 2012). Consequently, variation in the structure of host hemoglobin molecules that affect these processes can influence parasite fitness and disease progression (van Dooren *et al.* 2012).

The heme structure within the hemoglobin molecule is surrounded by globin protein subunits. Each hemoglobin molecule incorporates two α-type and two β-type globins (Hardison 2012). Most bird species have retained an identical complement of three tandemly linked alpha-type globin genes (α^{E}-, α^{D}-, and α^{A}-globin) and four tandemly linked β-type globin genes (β^{H}-, β^{A}-, ρ-, and ε-globin), although some of these genes, especially ε-globin, are truncated or exist as pseudogenes in some species (Opazo *et al.* 2015). The α-type globin genes are expressed differentially during the course of development: α^{A}- and α^{D}-globins are typically unevenly co-expressed postnatally, with α^{A}-globin comprising the majority of the expressed protein. Of the β-type globins, only β^{A}-globin is expressed at appreciable levels postnatally (Opazo *et al.* 2015).

Functionally consequential variation in hemoglobin can result from as little as a single amino acid change in one of the globin genes, while other variants result from deletions or premature stop codons (Weatherall 2008, de Mendonça *et al.* 2012). In humans, a single amino acid substitution causes the structural change known as sickle cell (HbS), and additional human thalassemia variants exhibit either a deletion (HbE) or a point mutation (HbC) causing a premature end to transcription (de Mendonça *et al.* 2012). Hemoglobin variants have also been documented in orangutans (Steiper *et al.* 2006), macaques, and baboons (Barnicot *et al.* 1966). Human thalassemia and sickle cell genes in heterozygotes have been shown to confer protection against severe malaria progression, or resistance to *Plasmodium falciparum*, though the homozygous genotype is deleterious (Weatherall 2008, de Mendonça *et al.* 2012). Hemoglobin variation is similarly thought to confer resistance to malaria in orangutans (Steiper *et al.* 2006), but no adaptive benefit has been identified in other non-human primates.

Table 1. Sample information for *HBA* sequence data.

	Bananaquits (*n*)
Individuals	382
Infections	77
Locations	19
Haplotypes (CDS)	152 (22)
Parasite lineages	11

Previous intraspecific surveys of avian globin molecular diversity have revealed adaptive modification of oxygen-binding affinity related to altitude (Galen *et al.* 2015, Natarajan *et al.* 2015). If hemoglobin variation additionally influences the susceptibility of birds to avian malaria parasites, or the severity of infections, then contemporary globin variation among avian populations might explain variation in the prevalence or intensity of infection. In the present study, we assessed genetic variation of the α^{A}-globin subunit (*HBA*) in Bananaquit populations throughout

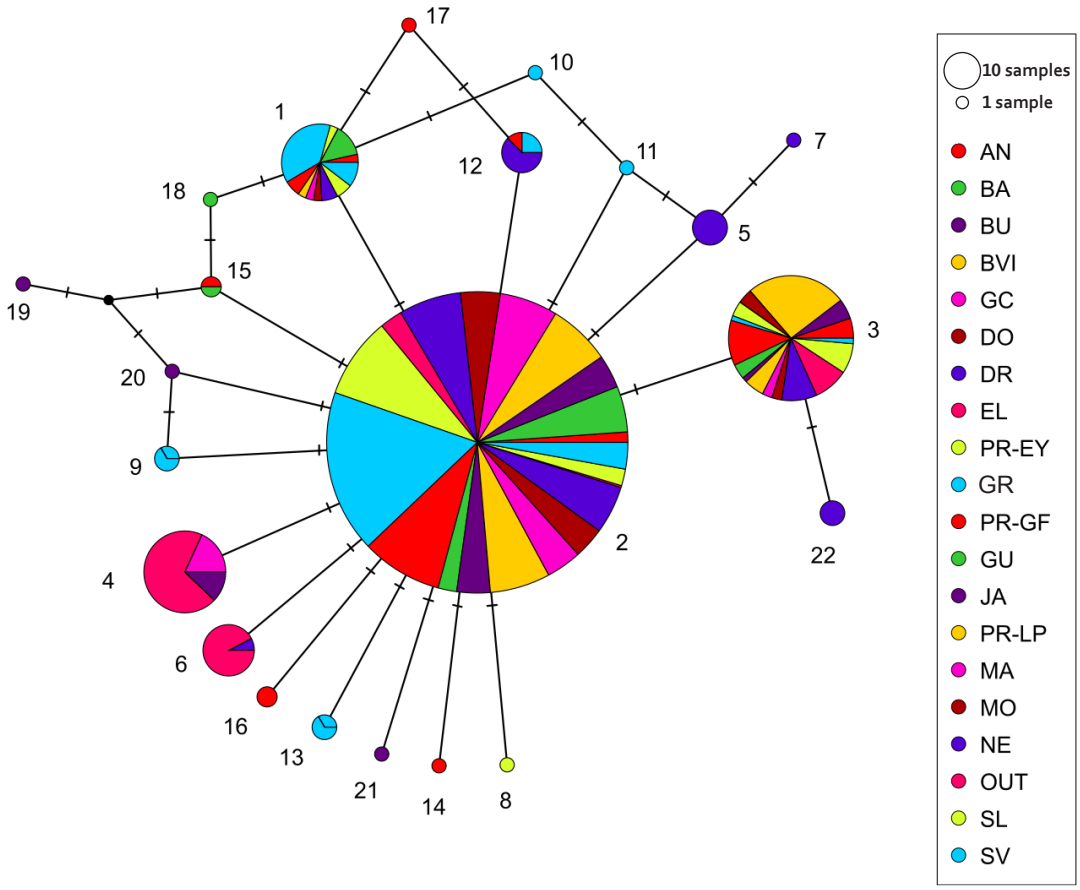

Fig. 3. Median joining haplotype network for the *HBA* coding sequence. Circle size indicates the number of individuals with a given haplotype, color indicates collection location, and black hash marks indicate mutations. Six Barbados Bullfinches (*Loxigilla barbadensis*) and four Black-faced Grassquits (*Tiaris bicolor*) from Barbados were included for outgroup comparisons, though all outgroup samples possessed haplotypes also recovered in Bananaquits. Location abbreviations available in Appendix 1. OUT = outgroup.

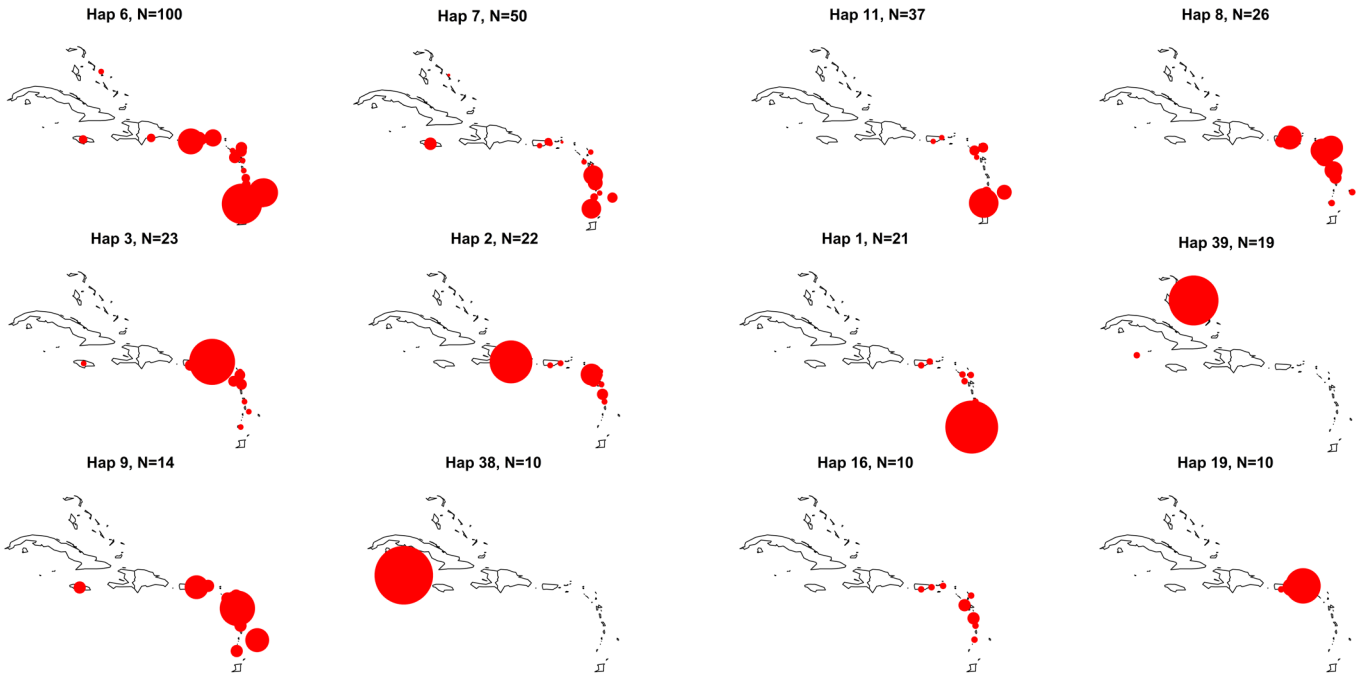

Fig. 4. Relative abundance among locations for each haplotype recovered 10 or more times. Abundances are scaled relative to the total number of detections of a particular haplotype and an upper limit to circle size was applied in some cases for clarity.

much of the species' range in the West Indies to test whether associations exist between globin variants and parasitism by avian malaria parasites. If an association exists, we would expect parasite prevalence to be inversely related to the frequency of an advantageous *HBA* haplotype.

In addition to potentially being associated with disjunctions and turnover in avian malaria infections among island populations, *HBA* provides an independent nuclear genetic locus that can inform demographic patterns inferred from mitochondrial variation. Accordingly, we compare patterns of genetic diversity and population structure inferred from *HBA* variation to patterns based on an independent mitochondrial locus, cytochrome *b* (*CYTB*) (de Oliveira Pil 2015).

Methods

We sampled Bananaquit populations throughout the West Indies (Table 1, Fig. 2) on all islands of the West Indies (except Cuba) and coastal areas of North, Central, and South America over the course of several years (2004–2017). To do this, we used mist-nets to capture birds and collected c. 10 µL of blood via sub-brachial venipuncture using field techniques described by Latta and Ricklefs (2010). In total, we recorded and sampled 456 infections from 1,527 Bananaquits. Six Barbados Bullfinches (*Loxigilla barbadensis*) and four Black-faced Grassquits (*Tiaris bicolor*; both Thraupidae) from Barbados were included for outgroup comparisons. We extracted DNA from the blood using the isopropanol precipitation technique, described in Svensson and Ricklefs (2009), and screened all samples for avian malaria using primers 343F and 496R (Fallon *et al.* 2003). Samples that screened positive were genotyped to identify the cytochrome *b* lineage of the infection (Bensch *et al.* 2009) using a variety of primers and PCR conditions described in Perkins and Schall (2002), Ricklefs *et al.* (2005), and Waldenström *et al.* (2004) (Appendix 7).

The gene encoding the hemoglobin alpha A subunit (*HBA*) was amplified from nucleotide positions 96–581 (which encompasses exon 2 and part of exon 3 of the coding sequence [Cheviron *et al.* 2014]), using primers HBAF (5'TTC GGC AAA ATY GGC GGC CAK GCC GA3') and HBAR (5'CCA CGG CAC ACA SGA ACT TGT CCA GG3') developed for our study using HYDEN software (available atacgt.cs.tau.ac.il/hyden/) with reference sequences from Opazo *et al.* (2015). Polymerase Chain Reaction (PCR) conditions and thermocycling protocols for *HBA* were as follows: we performed all PCRs in 25 µL volumes using Immomix Red PCR premix (Bioline product BIO-25022). Reaction volumes were composed of 12.5 µL of Immomix, 10 µL of ddH_2O, 0.5 µL of 50 mM $MgCl_2$ solution, 0.5 µL of each primer (10 mM), and 1 µL of genomic DNA (concentrations varied between 30–70 ng/ µL). Thermocycling conditions began with a hot-start at 95˚C for 10 mins, followed by 30 cycles of a 30-s denaturing step at 95˚C, a 45-s annealing step at 62˚C, and 30-s extension step at 72˚C. A final extension step was carried out at 72˚C for 5 min. Positive and negative controls were included in each PCR reaction, and products were verified by visualization on 1% TAE agarose gels with ethidium bromide. PCR product was cleaned using the ExoSAP-IT protocol (Bell 2008) and sequenced by Eurofins Genomics (Louisville, KY). Contigs were aligned and edited in Mega7 (Tamura *et al.* 2013) and chromatograms were checked by eye to confirm polymorphisms. HBA sequences were coded for ambiguous bases in Mega7 and phased for heterozygosity in DNAsp v.5 (Librado and Rozas 2009). All full-length sequences were deposited in Genbank (Accession numbers ON161139–ON161754).

To determine whether *HBA* genotypes are differentiated among sampling locations, we calculated fixation indices (pairwise F_{ST} values; Excoffier and Lischer 2010) with confidence intervals based on 1,000 permutations (Arlequin v.3.5; Excoffier *et al.* 2005) for the entire gene sequence and for the sequence with the introns removed (identified in Cheviron *et al.* [2014], query nucleotide positions 1–135 and 339–439; subject positions 96–230 and 436–536). A median-joining haplotype network for the coding sequence was generated using PopArt (available atpopart.otago.ac.nz) to compare and visualize relationships between haplotypes at each sample location. Nucleotide diversity (π) and haplotype diversity (*Hd*) for each sampling locality were calculated using DNAsp v.5 (Librado and Rozas 2009).

To determine whether particular globin genotypes are associated with higher or lower rates of parasitism by particular lineages of parasite, we performed a chi-squared test on the numbers of infected individuals that carried or did not carry the allele for all full-length haplotypes detected 10 or more times (hereafter common haplotypes) and for the four most common coding sequence haplotypes. We regressed the infection prevalence of common globin haplotypes where they are most abundant against all other haplotypes in the same locality to determine whether infection rates on common haplotypes varied from the overall rate of a given locality. Finally, to determine the degree of concordance among genetic distances observed at *HBA* and *CYTB*, we performed a Mantel test (999 permutations) and Mantel correlogram of the pairwise F_{ST} values for each marker using the R package *vegan* (Oksanen *et al.* 2020). All statistical analyses were conducted using R (R Core Team 2019).

Results

Our samples included *HBA* sequences from 382 Bananaquit individuals at 19 localities across the species range in the West Indies (Table 1). Of these individuals, 77 (20%) exhibited haemosporidian infections representing at least 11 parasite lineages. Sample sizes per locality ranged from 5 to 48 individuals. The full-length *HBA* sequence (494 base pairs) was highly variable (152 unique haplotypes), in contrast to the highly conserved coding sequence. We recovered only 22 unique coding sequence haplotypes (i.e., excluding nucleotide positions 1–135, 339–439). One *HBA* coding sequence haplotype, which comprised 75% of all sequences, was distributed in all localities sampled, and was shared with one Barbados Bullfinch sample (Fig. 3). Four additional haplotypes were somewhat common (13–77 detections), and the remaining 17 haplotypes were detected rarely. 20 of the 22 coding sequence haplotypes differed by a single mutational step from the most common haplotype, and all outgroup samples possessed haplotypes also recovered in Bananaquits. Eighteen of the 22 coding sequence haplotypes exhibited exclusively synonymous nucleotide variation and only two nonsynonymous substitutions were detected in the remaining three haplotypes: 86Valine>Alanine (shared by haplotypes 12 and 17) and 69Alanine>Threonine (haplotype 8).

Table 2. Sampling and diversity statistics for the *HBA* coding sequence. *n*, number of sequences; *H*, number of haplotypes; π, nucleotide diversity; *Hd,* haplotype diversity.

Locality	*n*	*H*	π	*Hd*
Antigua (AN)	10	3	0.049	0.644
Barbados (BA)	26	2	0.018	0.271
Barbuda (BU)	20	2	0.022	0.337
British Virgin Islands (BVI)	50	2	0.033	0.490
Grand Cayman, Cayman Islands (GC)	34	2	0.020	0.300
Dominica (DO)	22	2	0.016	0.247
Dominican Republic (DR)	38	4	0.028	0.360
Eleuthera, Bahamas (EL)	34	2	0.030	0.451
El Yunque, Puerto Rico (PR-EY)	44	4	0.015	0.214
Grenada (GR)	96	8	0.024	0.330
Guánica Forest, Puerto Rico (PR-GF)	56	8	0.039	0.494
Guadeloupe (GU)	14	4	0.051	0.571
Jamaica (JA)	24	6	0.052	0.544
Carite Forest, Puerto Rico (PR-CS)	34	3	0.018	0.266
Martinique (MA)	20	3	0.019	0.279
Montserrat (MO)	19	3	0.021	0.307
Nevis (NE)	40	5	0.057	0.631
Saint Lucia (SL)	16	3	0.049	0.633
Saint Vincent (SV)	20	5	0.050	0.626

Table 3. Chi-square and *p*-values for tests assessing infected vs. not infected for each haplotype against all other haplotypes. Tests include the four most common coding sequence haplotypes (CDS) and all full-length haplotypes (FL) detected 10 or more times. All tests have df = 1 and results with $p < 0.05$ are bolded. In all significant tests, individuals with that haplotype exhibited higher than average infection prevalence.

Haplotypes	Chi-square	*p*
CDS Hap 1	0.4236	0.5152
CDS Hap 2	23.7220	**< 0.0001**
CDS Hap 3	0.0008	0.9770
CDS Hap 4	0.5369	0.4637
FL Hap 1	0.2993	0.5843
FL Hap 2	4.3173	**0.0377**
FL Hap 3	0.1127	0.7371
FL Hap 6	2.6345	0.1046
FL Hap 7	10.2405	**0.0014**
FL Hap 8	0.2372	0.6263
FL Hap 9	0.9254	0.3361
FL Hap 11	0.3752	0.5402
FL Hap 16	0.6495	0.4203
FL Hap 19	0.0002	0.9901
FL Hap 38	5.6071	**0.0179**
FL Hap 39	1.1229	0.2893

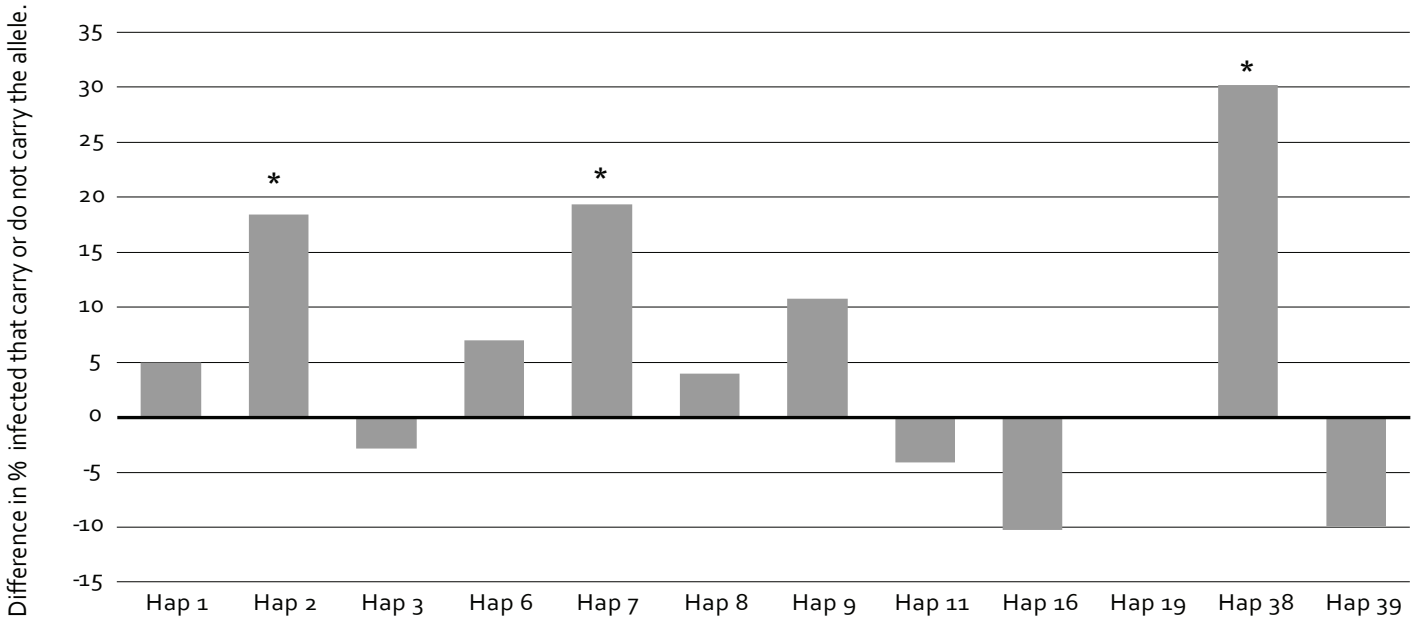

Fig. 5. For full-length sequences detected 10 or more times, the difference in percent infected that carry (+) versus do not carry (-) a given allele. * indicates Chi-square tests with $p < 0.05$. Chi-square values reported in Table 3.

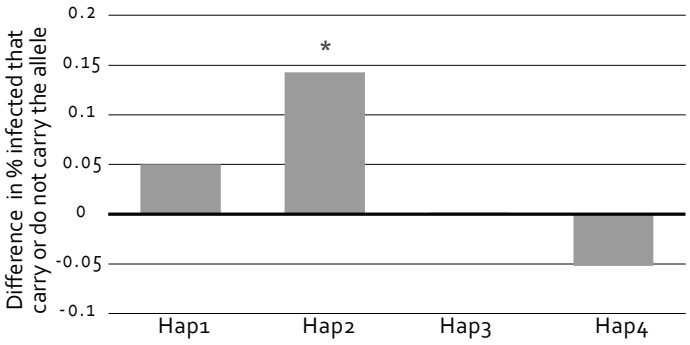

Fig. 6. For the most common *HBA* coding sequences, the difference in percent infected that carry or do not carry a given allele. * indicates Chi-square tests with $p < 0.05$. Chi-square values reported in Table 3.

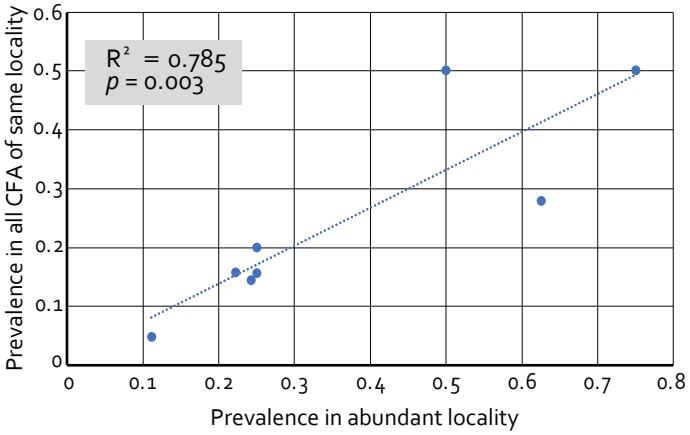

Fig. 7. Linear regression of infection prevalence of a haplotype where the haplotype is common against all other haplotypes in the same locality (includes *HBA* full-length sequence haplotypes detected at least 14 times total and 8 or more times in the 'abundant' locality). Infection rates on the abundant *HBA* haplotype are consistent with infection rates on other haplotypes in the same locality.

Comparisons of the relative abundances of the full-length haplotypes among locations revealed that 8 of the 12 haplotypes detected 10 or more times were abundant on one or two islands and were comparatively rare elsewhere (Fig. 4).

Population nucleotide diversity (π) and haplotype diversity (*Hd*) for the *HBA* coding sequence ranged from 0.015 to 0.066 and 0.214 to 0.644, respectively (Table 2). The majority of populations (12/20) contained either two or three HBA haplotypes, though as many as eight haplotypes were recovered from some localities, i.e., Guánica Forest (Puerto Rico) and Grenada. Barbados Bullfinch and Black-faced Grassquit samples revealed three coding sequence haplotypes that were shared with the Bananaquit samples. The intron sequence exhibits substantial variation among individuals, including indels that varied in size among species.

We found no difference in the probability of malaria infection for 9 of the 12 common *HBA* haplotypes when compared to the prevalence of all other haplotypes. The other three full-length haplotypes exhibited significantly higher than average rates of infection (Table 3, Fig. 5). Similarly, three of the four most common coding-sequence (CDS) haplotypes exhibited no difference in the probability of malaria infection compared to the prevalence of all other haplotypes, and the sole statistically significantly different comparison was one in which the rate of parasitism is higher than in all other haplotypes (Fig. 6). Additionally, malaria prevalence was not significantly lower on a particular haplotype where it was most common compared to all other haplotypes in the same location (Fig. 7). Finally, higher genetic diversity at *HBA* was not associated with lower rates of parasitism among locations; the overall prevalence was 29.7% while prevalence in the four locations with the highest *HBA* diversity (Guadeloupe, Jamaica, Nevis, and Saint Vincent) ranged from 8.3% to 75.0%.

Population structure inferred by F_{ST} was largely concordant between *HBA* (full-length sequence: Appendix 3; coding sequence: Appendix 4) and *CYTB* (Appendix 5), with *HBA* exhibit-

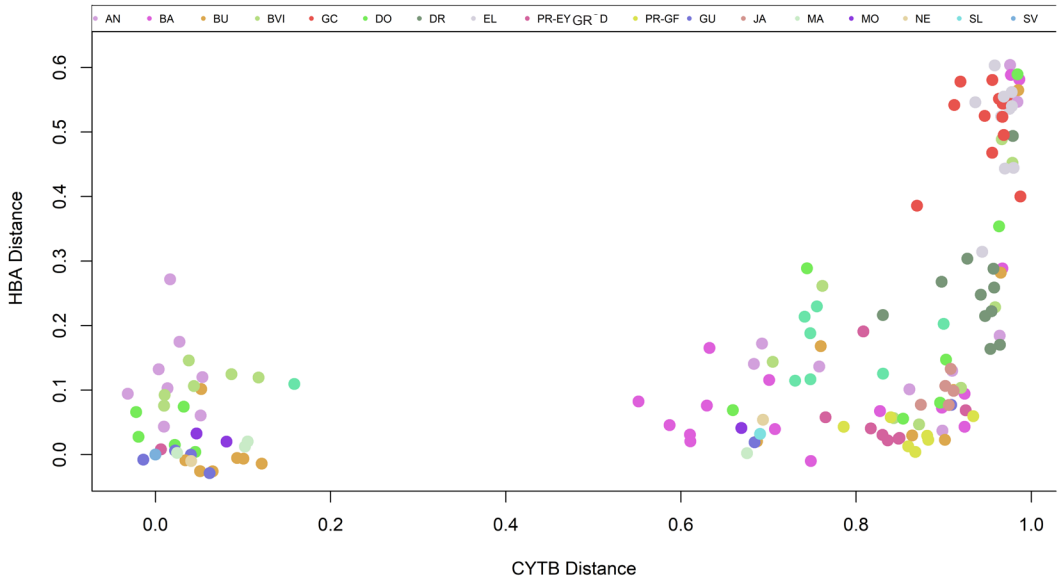

Fig. 8. Mantel Test of *CYTB* and *HBA* pairwise F_{ST} values. Mantel $r = 0.52701$; $p = 0.002$. Location abbreviations available in Appendix 1.

ing generally lower values, as expected in comparisons of nuclear and mitochondrial markers. A Mantel test of the F_{ST} matrices for *HBA* and *CYTB* revealed that the two are significantly correlated (Fig. 8; Mantel *r* statistic = 0.527, *p* = 0.002), but that the correlation breaks down at higher distance classes. This breakdown is attributed to the lower genetic distances at *HBA* for Bananaquits from Jamaica, Puerto Rico, Barbados, and Saint Vincent, relative to *CYTB* distances for the same populations (Fig. 8; Appendix 6).

Discussion

The findings reported here suggest that while *HBA* coding sequence does vary among populations of Bananaquits, variation is consistent with findings reported here and by de Oliveira Pil (2015) for neutral mitochondrial variation and these patterns are likely not related to interactions with avian malaria parasites. We find no support for particular *HBA* variants conferring resistance to infection by particular lineages of malaria parasites, either in terms of overall rates of infection or in relation to variation in infection rates among localities. We suspected that parasite prevalence might be inversely related to the frequency of an advantageous *HBA* haplotype but found that the haplotypes with highest frequencies had average or above-average parasite prevalence. Nonetheless, the distribution of genetic variation at *HBA* among locations, particularly in the intronic regions, does support this marker as a promising independent locus for phylogeographic and population studies focused on fine-scale demographic processes. We recovered substantial individual variation and, in interspecific comparisons, we found indels of varying length, which will provide useful contemporary genetic variation with which to identify population structuring and phylogeographic relationships among populations.

We were unable to design suitable primers for the α subunit of β-globin (*HBB*) in Bananaquits with currently available data, though this marker may warrant further investigation. β-globin makes up half the tetrad of the hemoglobin molecule and therefore we would expect it to be as consequential as α-globin to hematophagous parasite fitness. As previously described, among the four tandemly linked β-globin genes in most avian species, only *HBB* is expressed at appreciable levels postnatally. Support for the response of this gene to natural selection was reported by Galen *et al.* (2015), who found functionally consequential variation in the oxygen-binding affinity of *HBB* along an altitudinal gradient.

Parasite population disjunctions and lineage turnover observed in island populations of Bananaquits appear independent of globin variation and might be better explained by variation at immune markers such as the major histocompatibility complex (*MHC*) and toll-like receptors (*TLR*). Loiseau *et al.* (2011) found evidence of local adaptation of House Sparrow (*Passer domesticus*) *MHC* diversity in relation to *Plasmodium relictum* infection and inverse associations of some alleles with parasite infection across locations. *TLR* variation is reported to impact clinical outcomes of malaria in humans (Leoratti *et al.* 2008) and these genes, along with *MHC*, have recently been annotated in Bananaquits (Antonides *et al.* 2017), making this avenue suitable for further investigation.

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Appendix 1. Location abbreviations

LC	Little Cayman
GC	Grand Cayman
CB	Cayman Brac
JA	Jamaica
DR	Dominican Republic
PR-RB	Refugio Boquerón, Puerto Rico
PR-GF	Guánica Forest, Puerto Rico
PR-EY	El Yunque, Puerto Rico
PR-LP	Las Playitas, Puerto Rico
PR-CS	Carite Forest, Puerto Rico
PR-UPR	University of Puerto Rico, Puerto Rico
EL	Eleuthera, Bahamas
BVI	British Virgin Islands
SA	Saba, Lesser Antilles
SE	Saint Eustatius, Lesser Antilles
SK	Saint Kitts
NE	Nevis
AN	Antigua
BU	Barbuda
MO	Montserrat
GU	Guadeloupe
DO	Dominica
MA	Martinique
SL	Saint Lucia
SV	Saint Vincent
BA	Barbados
GR	Grenada

Appendix 2. Samples sizes for Bananaquit infection by location represented in Fig. 1. Location abbreviations in Appendix 1.

	DR 03	DR 11	GA 01	JA 01	JA 02	JA 06	LA 07	LA 19	LA 22	LA 01	LA 02	LA 07	OZ 01	OZ 02	OZ 02, unique	OZ 04	OZ 06	OZ 08	OZ 19	OZ 21	OZ 32	TOC 28	YU 04	unique	uninfected	Grand Total
LC					4									21											20	45
GC					20									5						2					32	59
CB					3									23											3	29
JA					6	2										35	4								96	143
DR	18	3												4		2	1		2				1	1	73	105
PR-RB																				1					45	46
PR-GF							43							9	1	1				9	1			1	102	167
PR-EY																1			10						50	61
PR-LP														1						3					15	19
PR-CS							6									1									12	19
PR-UPR																									3	3
EL																				1				1	15	17
BVI																				2					28	30
SA										1	1														5	7
SE												17													88	105
SK			1										1			1				3					27	33
NE							2													3					55	60
AN																				4					12	16
BU							6													1					7	14
MO							1	1												1				3	12	18
GU													1			3				13				4	7	28
DO													2							9					7	18
MA							1						1			1									4	7
SL							1						1			14		1		6				2	36	61
SV	6																			34					61	101
BA							2													18					50	70
GR				1			29									3									174	207
Grand Total	24	3	1	1	33	2	91	1	0	1	1	17	6	63	1	62	5	1	12	110	1	0	1	12	1039	1488

Appendix 3. Pairwise F_{ST} for the full-length *HBA* sequence. F_{ST} values are below the diagonal and significance is above (+ indicates $p < 0.05$).

	AN	BA	BU	BVI	GC	DO	DR	EL	PR-EY	GR	PR-GF	GU	JA	PR-LP	MA	MO	NE	SL	SV
AN		+	+	+	+	+	+	+	+	-	-	+	+	+	-	+	-	+	+
BA	0.172		+	+	+	+	+	+	+	+	-	+	+	-	-	+	-	-	-
BU	0.132	0.040		+	+	-	+	+	+	-	-	+	-	-	-	-	-	-	-
BVI	0.103	0.165	0.102		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
GC	0.604	0.589	0.558	0.489		+	+	+	+	+	+	+	+	+	+	+	+	+	+
DO	0.272	0.116	0.000	0.146	0.546		+	+	+	+	+	+	-	-	-	-	-	+	+
DR	0.184	0.289	0.282	0.228	0.578	0.354		+	+	+	+	+	+	+	+	+	+	+	+
EL	0.547	0.582	0.565	0.452	0.400	0.589	0.494		+	+	+	+	+	+	+	+	+	+	+
PR-EY	0.137	0.067	0.168	0.261	0.669	0.289	0.304	0.603		+	+	+	+	+	+	+	+	+	+
GR	0.037	0.043	0.023	0.047	0.495	0.080	0.170	0.445	0.126		-	+	-	-	-	+	-	+	+
PR-GF	0.043	0.031	0.000	0.092	0.551	0.066	0.222	0.536	0.117	0.004		+	+	-	-	-	-	-	+
GU	0.130	0.094	0.100	0.103	0.386	0.147	0.216	0.314	0.203	0.060	0.077		+	+	+	+	+	+	-
JA	0.165	0.072	0.025	0.051	0.479	0.038	0.269	0.489	0.224	0.021	0.042	0.067		-	+	+	-	+	+
PR-LP	0.175	0.020	0.000	0.106	0.544	0.004	0.288	0.562	0.188	0.023	0.000	0.077	0.006		-	-	-	-	+
MA	0.061	0.046	0.000	0.119	0.581	0.074	0.215	0.555	0.115	0.013	0.000	0.107	0.073	0.020		-	-	-	-
MO	0.120	0.082	0.000	0.125	0.525	0.015	0.248	0.524	0.214	0.058	0.006	0.133	0.066	0.012	0.020		-	+	-
NE	0.094	0.076	0.000	0.076	0.523	0.028	0.259	0.540	0.230	0.029	0.000	0.099	0.040	0.003	0.033	0.000		-	+
SL	0.141	-0.010	0.021	0.144	0.542	0.069	0.268	0.546	0.109	0.043	0.019	0.077	0.052	0.002	0.041	0.054	0.032		-
SV	0.141	-0.010	0.021	0.144	0.542	0.069	0.268	0.546	0.057	0.109	0.043	0.019	0.077	0.052	0.002	0.041	0.054	0.032	

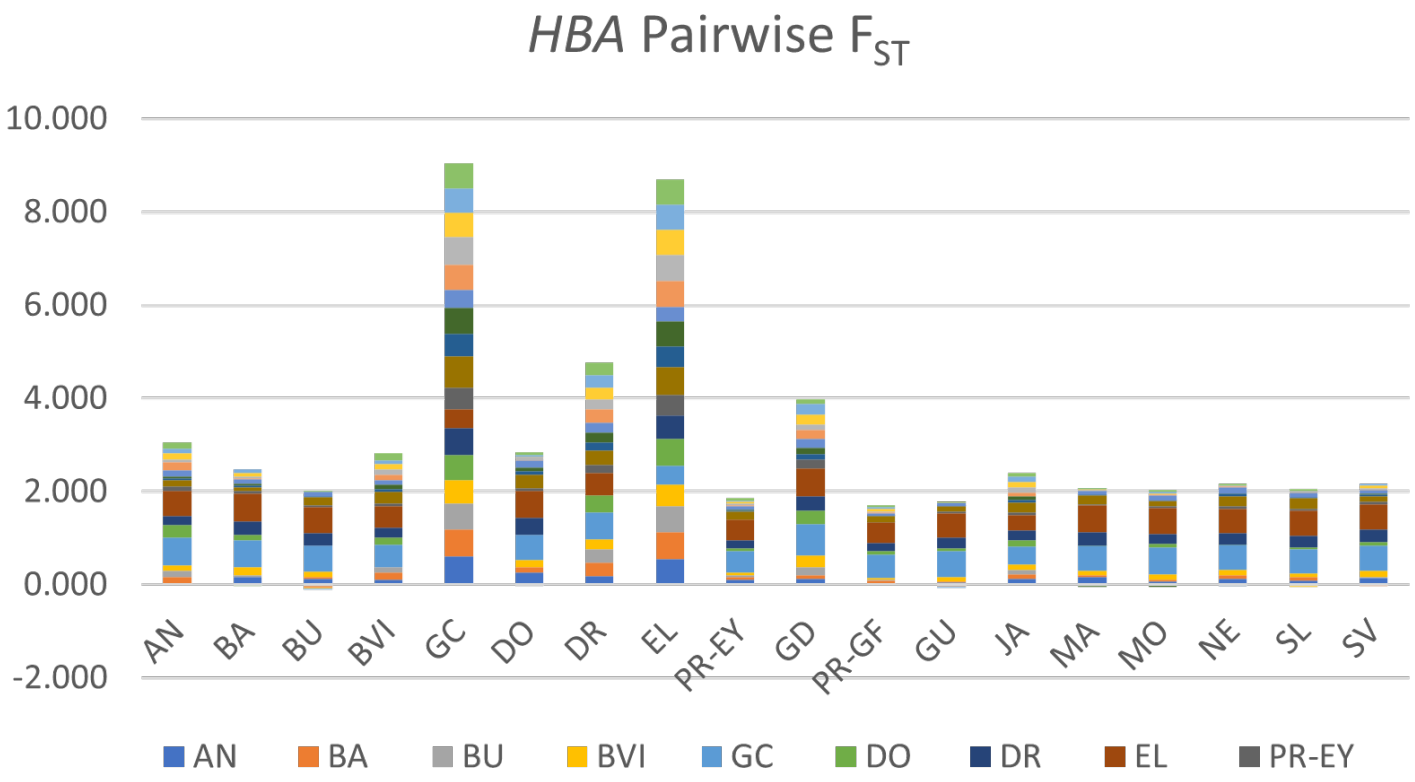
Appendix 4. Pairwise F_{ST} for the *HBA* coding sequence. F_{ST} values are below the diagonal and significance is above (+ indicates $p < 0.05$).

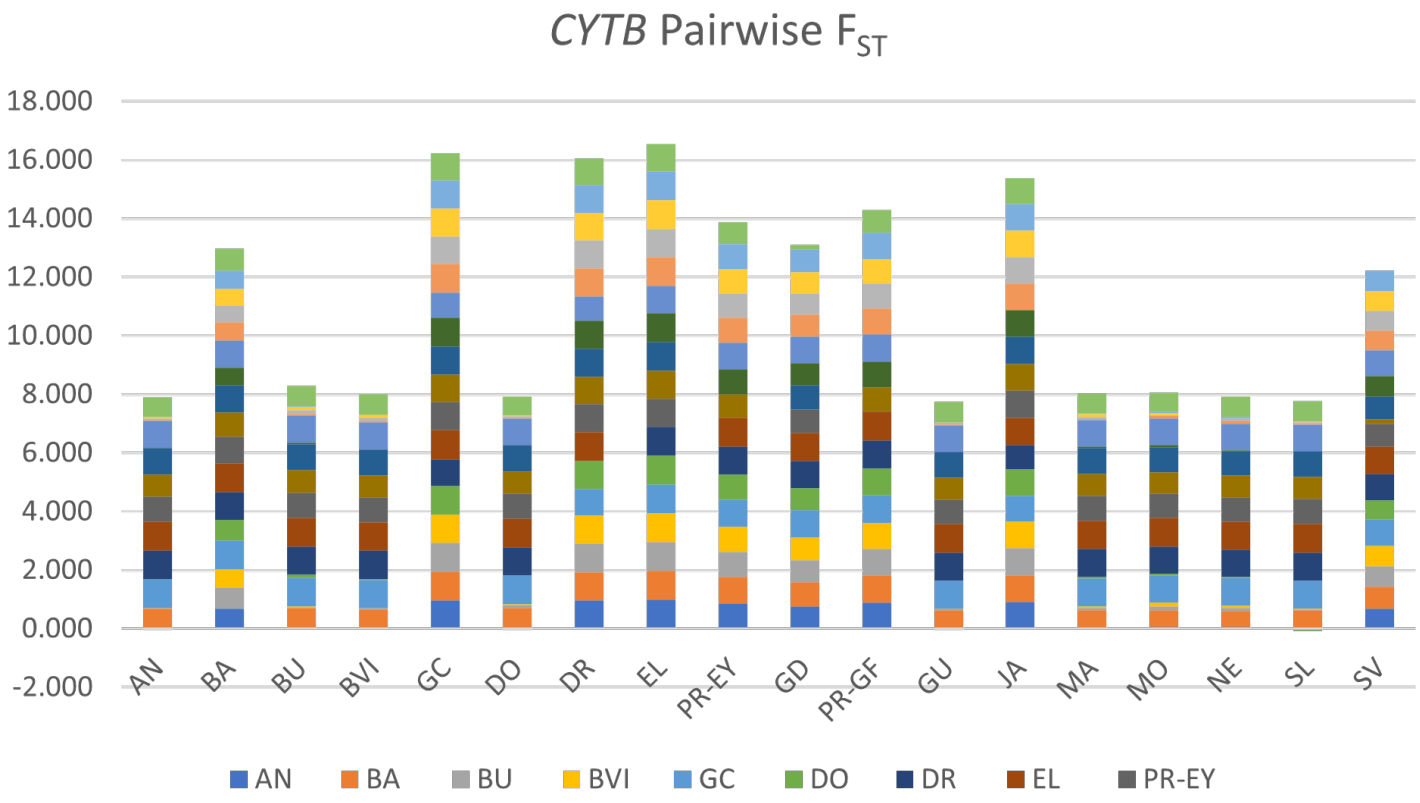
	AN	BA	BU	BVI	GC	DO	DR	EL	PR-EY	GR	PR-GF	GU	JA	PR-LP	MA	MO	NE	SL	SV
AN		+	-	-	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+
BA	0.257		+	+	+	+	+	+	+	-	+	+	+	+	-	-	+	+	-
BU	0.032	0.144		-	+	-	+	+	-	+	-	-	+	-	-	-	-	-	+
BVI	0.000	0.292	0.052		+	+	+	+	+	+	+	-	+	+	+	+	+	-	+
GC	0.309	0.136	0.158	0.305		+	+	+	+	+	+	+	-	+	+	+	+	+	+
DO	0.115	0.108	0.000	0.118	0.123		+	+	-	+	-	-	+	-	-	-	-	+	+
DR	0.269	0.118	0.135	0.288	0.136	0.104		+	+	+	+	+	+	+	+	+	+	+	+
EL	0.538	0.557	0.540	0.561	0.389	0.554	0.527		+	+	+	+	+	+	+	+	+	+	+
PR-EY	0.240	0.059	0.034	0.221	0.106	0.000	0.097	0.586		+	-	+	+	-	-	-	+	+	+
GR	0.248	0.000	0.104	0.284	0.105	0.062	0.098	0.546	0.030		+	+	+	+	-	-	+	+	-
PR-GF	0.042	0.049	0.000	0.089	0.097	0.000	0.097	0.470	0.010	0.051		-	+	-	-	-	-	-	-
GU	0.000	0.100	0.000	0.060	0.157	0.012	0.139	0.481	0.075	0.106	0.000		-	-	-	-	-	-	-
JA	0.139	0.080	0.062	0.205	0.000	0.047	0.095	0.301	0.064	0.092	0.057	0.048		+	-	-	+	+	+
PR-LP	0.140	0.069	0.000	0.149	0.116	0.000	0.104	0.561	0.000	0.042	0.000	0.025	0.058		-	-	-	+	-
MA	0.122	0.029	0.000	0.151	0.097	0.000	0.083	0.537	0.000	0.011	0.000	0.009	0.035	0.000		-	-	-	-
MO	0.096	0.028	0.000	0.135	0.099	0.000	0.083	0.529	0.000	0.012	0.000	0.000	0.032	0.000	0.000		-	-	-
NE	0.000	0.107	0.001	0.047	0.145	0.022	0.137	0.441	0.077	0.123	0.012	0.004	0.088	0.038	0.027	0.018		-	+
SL	0.000	0.211	0.033	0.000	0.278	0.097	0.250	0.522	0.203	0.222	0.045	0.000	0.143	0.119	0.100	0.079	0.008		+
SV	0.107	0.003	0.065	0.206	0.111	0.052	0.103	0.462	0.057	0.014	0.036	0.035	0.048	0.046	0.013	0.007	0.069	0.102	

Appendix 5. Pairwise F_{ST} of *CYTB* from de Oliveira Pil (2015). F_{ST} values are below the diagonal and significance is above (+ indicates $p < 0.05$).

	AN	BA	BVI	BU	GC	DR	DO	EL	PR-EY	GR	PR-GF	GU	JA	MA	MO	NE	SL	SV
AN		+	-	-	+	+	-	+	+	+	+	-	+	-	-	-	-	+
BA	0.693		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BVI	0.014	0.633		-	+	+	-	+	+	+	+	-	+	-	+	+	-	+
BU	0.004	0.707	0.052		+	+	-	+	+	+	+	+	+	+	+	+	+	+
GC	0.976	0.977	0.966	0.977		+	+	+	+	+	+	+	+	+	+	+	+	+
DR	0.964	0.967	0.959	0.965	0.919		+	+	+	+	+	+	+	+	+	+	+	+
DO	0.017	0.701	0.038	0.093	0.977	0.963		+	+	+	+	-	+	-	-	-	-	+
EL	0.984	0.986	0.979	0.985	0.988	0.979	0.984		+	+	+	+	+	+	+	+	+	+
PR-EY	0.861	0.898	0.843	0.864	0.955	0.953	0.854	0.970		+	-	+	+	+	+	+	+	+
GR	0.758	0.827	0.761	0.760	0.940	0.927	0.744	0.958	0.808		+	+	+	+	+	+	+	+
PR-GF	0.899	0.924	0.872	0.901	0.969	0.964	0.896	0.980	0.006	0.831		+	+	+	+	+	+	+
GU	0.010	0.610	0.010	0.051	0.963	0.955	0.000	0.975	0.836	0.748	0.868		+	+	+	-	-	+
JA	0.910	0.924	0.920	0.911	0.870	0.831	0.903	0.944	0.925	0.900	0.934	0.909		+	+	+	+	+
MA	0.028	0.611	0.044	0.065	0.967	0.957	0.046	0.978	0.849	0.748	0.882	0.040	0.906		+	+	-	+
MO	0.052	0.587	0.118	0.121	0.956	0.947	0.032	0.969	0.830	0.730	0.860	0.062	0.902	0.105		+	+	+
NE	0.054	0.552	0.087	0.101	0.947	0.942	0.022	0.966	0.817	0.741	0.840	0.022	0.908	0.102	0.081		-	+
SL	0.000	0.630	0.010	0.034	0.967	0.958	0.000	0.978	0.850	0.755	0.881	0.000	0.911	0.025	0.047	0.041		+
SV	0.683	0.748	0.705	0.687	0.912	0.898	0.659	0.936	0.765	0.159	0.786	0.684	0.874	0.675	0.669	0.694	0.690	

Appendix 6. Stacked histograms of pairwise F_{ST} values for *HBA* (above) and *CYTB* (below). Values are lower than expected for *HBA* on Saint Vincent, Barbados, Dominican Republic, Jamaica, and Puerto Rico.





Appendix 7. Primers and thermocycling conditions for cytochrome *b* genotyping of avian malaria parasites.

Perkins and Schall (2002) primers and thermocycling protocols

Outer reaction primers	DW2 5′-TAA TGC CTA GAC GTA TTC CTG ATT ATC CAG-3′	DW4 5′-TGT TTG CTT GGG AGC TGT AAT CAT AAT GTG-3′
Inner reaction/ sequencing primers	DW1 5′-TCA ACA ATG ACT TTA TTT GG-3′	DW6 5′-GGG AGC TGT AAT CAT AAT GTG-3′
Alternative sequencing primers	DW3 5′-TGC TGT ATC ATA CCC TAA AG-3′	DW8 5′-GCA CAA ATC CTT TAG GGT ATG ATA C-3′

Outer Protocol: 35 cycles of 94°C for 20 s, 60°C for 20 s, and 72°C for 1.5 min.
Inner Protocol: 40 cycles of 94°C for 20 s, 50°C for 20 s, and 72°C for 1 min and then 72°C for 7 min.

Ricklefs *et al.* (2005) primers and thermocycling protocols

Primer pair 1	543F 5′-AAA AAT ACC CTT CTA TCC AAA TCT-3′	926R 5′-CAT CCA ATC CAT AAT AAA GCA T-3′
Primer pair 2	413F 5′-GTG CAA CYG TTA TTA CTA A-3′	1162R 5′-TTG TTC YGC TCA ATA CTY AGA-3′
Primer pair 3	413F 5′-GTG CAA CYG TTA TTA CTA A-3′	926R 5′-CAT CCA ATC CAT AAT AAA GCA T-3′

Thermocycling protocol: initial denaturation of 2 min at 94°C followed by 35 cycles with denaturation for 50 s at 94°C, annealing for 50 s at 48°C (543F/926R) or 46°C (413F/1162R and 413F/926R), 1 min 10 s extension at 72°C. Final extension of 3 min at 72°C.

Waldenstrom *et al.* (2004) primers and thermocycling protocols

Outer reaction primers	HAEMNF 5′-CATATATTAAGAGAAT-TATGGAG-3′	HAEMNR2 5′-AGAGGTGTAGCATATCTATCT-AC-3′
Inner reaction primers	HAEMF 5′-ATGGTGCTTTCGATATA-TGCATG-3′	HAEMR2 5′-GCATTATCTGGATGTGATAATGG-T- 3′

Outer protocol: 3 min of denaturation at 94°C, 20 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, and an elongation step at 72°C for 10 min.
Inner protocol: 3 min of denaturation at 94°C, 35 cycles at 94°C for 30 s, 50°C for 30 s, 72°C for 45 s, and an elongation step at 72°C for 10 min.