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Population Structure of Avian Malaria Parasites

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INTRODUCTION

The ubiquity of avian malaria in the global avifauna makes it an accessible pathogen to investigate how the geographic distribution of parasite genetic diversity relates to patterns of virulence, the evolution of host-resistance, and the population structure and demography of hosts. Although more than 40 morphospecies of avian Plasmodium have been described (Valkiūnas, 2004) and hundreds of genetic lineages have been identified utilizing variation at the partial cytochrome b gene (Bensch, Hellgren, & Pérez-Tris, 2009), little is known of the genetic diversity within avian malaria parasite populations, or even concerning the boundaries of these populations. Whether individuals of a particular parasite lineage vary genetically relative to their hosts or to geographic location is poorly understood, but assessments of lineage distributions through time reveal dynamic patterns that differ among both location and host taxa (Fallon, Bermingham, & Ricklefs, 2003; Fallon, Ricklefs, Swanson, & Bermingham, 2003). Previous work in the West Indies has revealed disjunctions in the geographic distributions of parasite lineages (Ricklefs, Soares, Ellis, & Latta, 2016) and dynamic parasite
community assembly processes, including differentiation of geographically separated parasite assemblages in as little as 2,500 years (Soares, Latta, & Ricklefs, 2017). However, information about the genetic diversity and structure of parasite populations, including effective population sizes and intra-lineage variation related to host species or geographic location, has not been reported. This lack of information reflects, in part, the difficulty of obtaining suitable genetic markers.

Genetic variation and population structures of two *Plasmodium* parasites infecting humans, *P. vivax* (Pv) and *P. falciparum* (Pf), have been characterized in detail, particularly at immunogenic loci. Among these loci is the apical membrane antigen 1 (AMA1), which plays a critical role in forming the junction between *Plasmodium* merozoites and host red blood cells (reviewed in Bai et al., 2005). AMA1 is also thought to interact directly with host immune systems; analyses of PfAMA1 have located one T-cell epitope and a second B/T-cell epitope in Domain I (Escalante et al., 2001), as well as several erythrocyte binding sites (Zakeri, Sadeghi, Abouie Mehrizi, & Dinparast Djadid, 2013). Analyses of variation in the three-dimensional structure of the protein suggest that the acquisition of several highly variable loops in Domain I is related to evasion of the host immune response (Collins, Withers-Martinez, Hackett, & Blackman, 2009). These direct interactions support Domain I of AMA1 as a marker related to host-specific immune pressures. Accordingly, the rapid accumulation of mutations as a result of diversifying selection provides information about fine-scale population structure. Assessments of PvAMA1 and PfAMA1 reveal distinct demographic patterns: PvAMA1 typically exhibits a differentiated structure consistent with an endemic pathogen (Neafsey et al., 2012; Taylor et al., 2013), while PfAMA1 most often exhibits an epidemic population structure with reduced diversity and little geographic differentiation (Arnott et al., 2014; Mueller, Kaiok, Reeder, & Cortés, 2002; Ord, Tami, & Sutherland, 2008), typical of frequent clonal outbreaks. *Plasmodium vivax* and *P. falciparum* exhibit these demographic differences despite infecting the same hosts in many of the same locations and being transmitted by the same vectors. Therefore, populations of avian malaria parasites might be expected to exhibit substantial variation in the degree of population structure within lineages as a result of the more complex relationships among hosts, vectors, and locations.

To develop a better understanding of the influence of host species and geographic location on parasite genetic diversity, we assess the distribution of genetic diversity in the partial Domain 1 of AMA1 of three mitochondrial lineages of avian *Plasmodium* commonly infecting birds in the West Indies and eastern North America. We assess genetic diversity and phylogenetic relationships within mitochondrial lineages and test whether parasite populations exhibit genetic structure related to hosts, location, or geographic distance.

## 2 MATERIALS AND METHODS

### 2.1 DNA extraction and sequencing

Samples for this study were obtained over several years from diverse localities in the Americas (see Figure 1). Birds were captured with mist-nets and ca. 10 μl of blood was collected by sub-brachial venipuncture (field techniques described in Latta & Ricklefs, 2010). DNA was extracted from blood using the isopropanol precipitation technique described in Svensson and Ricklefs (2009), and all samples were screened for avian malaria using primers 343F and 496R (Fallon et al., 2003, 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, Bensch, Hasselquist, and Östman (2004). For samples infected by *Plasmodium* lineages OZ01 (equivalent to PAĐOM11 in the MalAvi database; Bensch et al., 2009), OZ04 (MalAvi ICTCAY01), and OZ14 (MalAvi CARCAR11), approximately 400 bp (length varies by lineage) of the partial Domain 1 of AMA1 were amplified using nested primers Pg_AMA1F1/Pg_AMA1R1 and Pg_AMA1F2/Pg_AMA1R2 and PCR protocols described in Lauron et al. (2014). Negative controls...
were included in each PCR reaction, and products were verified by visualization on 1% TBE 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 Mega6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013), and chromatograms were checked by eye to confirm polymorphisms. Heterozygous positions were denoted with IUPAC ambiguous base codes, and haplotypes were reconstructing using PHASE in DNAsp v. 5 (Librado & Rozas, 2009).

2.2 | Statistical analyses of variation

Summary statistics describing genetic variation within parasite populations, based either on geography or host species, were calculated using DNAsp v.5 (Librado & Rozas, 2009). These statistics include number of haplotypes (h), haplotype diversity (Hd), and nucleotide diversity (π). Hd is the probability that two haplotypes chosen at random differ (Nei, 1987), while π is the average number of nucleotide differences per site for two sequences chosen at random (Nei & Li, 1979). We calculated π for the entire sequence, and to examine patterns of nucleotide substitution within the AMA1 gene, we additionally calculated π using a sliding window of 30 bp and step size of 9 bp (Lauron et al., 2014). Synonymous and nonsynonymous substitutions, and minimum number of recombination events (Rm, using the four-gamete test after Hudson & Kaplan, 1985), were also calculated in DNAsp v.5. Pairwise FST values were calculated in Arlequin v. 3.5 (Excoffier & Lischer, 2010) for a reduced dataset containing only sequences from locations or hosts with 10 or more samples.

2.3 | Phylogeographic analysis

A median-joining haplotype network was generated using Popart (Leigh & Bryant, 2015) to compare and visualize relationships among haplotypes at each sample location and within each host species or family. Pairwise FST values were calculated with confidence intervals based on 1000 permutations (Arlequin v.3.5, Excoffier & Lischer, 2010) for all locations with more than 10 sequences and for the two clusters identified in the haplotype networks of lineages OZ01 and OZ14. A Mantel test comparing pairwise FST and geographic distance was employed to determine whether the population structure detected among some locations in lineage OZ01 is consistent with isolation-by-distance. The Mantel test was implemented in the R package “ade4” version 1.7-11 with 9,999 permutations.

TABLE 1 AMA1 sample information and summary statistics for lineages OZ01, OZ04, and OZ14. Major OZ04 includes only the major cluster, and Minor OZ04 includes only the apparently introgressed samples

| Lineage   | Number of sequences | Number of haplotypes | Haplotype diversity | Number of host species | Number of locations |
|-----------|---------------------|----------------------|--------------------|------------------------|--------------------|
| OZ01      | 232                 | 18                   | 0.607              | 31                     | 18                 |
| OZ04      | 170                 | 22                   | 0.717              | 16                     | 11                 |
| Major OZ04| 108                 | 10                   | 0.362              | 12                     | 9                  |
| Minor OZ04| 62                  | 12                   | 0.804              | 12                     | 8                  |
| OZ14      | 134                 | 23                   | 0.430              | 23                     | 12                 |

3 | RESULTS

3.1 | Statistical analyses of variation

We sequenced 389–407 base pairs (depending on lineage, Table 1) of the partial Domain 1 of AMA1 corresponding to amino acid positions 169–295/300 of Plasmodium falciparum (3D7 isolate, Genbank accession U33274.1). A maximum likelihood phylogeny of AMA1 amino acid sequences (Figure 2), including representatives of four avian and three mammalian Plasmodium species in addition to the three lineages assessed here, is concordant with the mitochondrial CYTB gene tree reported in Ricklefs et al. (2014, figure S2), recovering OZ04 and OZ14 as being closely related to each other relative to OZ01.

Analysis of lineage OZ01 included 232 nucleotide sequences containing 18 unique haplotypes from 31 host species and 18 locations distributed in the eastern United States and the West Indies (Table 1, Figure 3). Analysis of the geographically more restricted lineage OZ04 included 170 nucleotide sequences containing 22 unique haplotypes from 16 host species and 11 locations distributed in the Lesser Antilles and in the Ozarks region of Missouri (Table 1, Figure 3). Lineage OZ14 comprised 134 nucleotide sequences representing 10 unique haplotypes from 23 host species and 12 locations distributed in the eastern United States and the West Indies (Table 1, Figure 3). Estimates of overall nucleotide diversity (π) were 0.004, 0.173, and 0.007 for OZ01, OZ04, and OZ14, respectively, and estimates of Hd were 0.607, 0.717, and 0.430 for the same sequences (Table 2). Lineage OZ01 exhibited the highest heterozygosity with 13 individuals possessing at least one heterozygous position; lineages OZ04 and OZ14 contained 3 and 7 such individuals, respectively (Table 3).

Sliding window analysis of π revealed congruence in the distribution of diversity among lineages, particularly within the range of nucleotides from positions 1-130 (Figure 4). We detected the highest polymorphism at nucleotide positions (nt) 94-130. This region aligns with PfAMA1 amino acid residues 259–271 (Escalante et al., 2001), which constitute a hypervariable region encompassing a T-cell epitope. Lineage OZ14 exhibits three additional peaks in π; one is shared with OZ04 (nt 204–249 in both lineages) and corresponds to a B/T-cell epitope in PfAMA1 at amino acid residues 279–288, a third is at OZ14AMA1 nt 150–170, and the final peak is at OZ14AMA1 nt 276–303. The latter two are not known to have immunogenic functions. These lineages share an amino acid insertion detected in other avian malaria parasites at PfAMA1 amino acid residue 188 (Lauron et al., 2014), and lineage OZ01 and a subset of OZ04 exhibit a second
amino acid insertion at the same position. Most of the nucleotides that encode a hydrophobic trough hypothesized to contain a critical structural component of the molecule are conserved; amino acid residues at PfAMA1 tyrosine Y251, valine V169, and leucine L357 (Bai et al., 2005) are conserved in all samples, and PfAMA1 phenylalanine F183 is conserved in all samples of lineages OZ01 and OZ04, although we detected three haplotypes with a serine at this position in lineage OZ14.

3.2 | Phylogeographic analysis

Haplotype networks (Figure 5) reveal that lineages OZ04 and OZ14 each contain a lineage-specific common and widespread AMA1 haplotype, and that OZ01 contains two common haplotypes. However, in OZ01 these haplotypes are shared with individuals belonging to the mitochondrial OZ04 lineage (visualized in Figure 5A). One of the OZ01 haplotypes corresponds to a group of parasites infecting many hosts in many locations, and a second corresponds to a group infecting predominantly individuals of the avian genus Passerina (buntings): 42 of 68 infections in this group were detected in P. cyanea, and 6 of 68 were detected in P. ciris, representing 70.5% of the infections in this group and 87.5% of all Passerina OZ01 infections.

Much of the diversity of OZ04 can be attributed to the presence of a single deep division within the lineage (visualized in Figure 5B). One subdivision of OZ04 consists primarily of a single common, widespread AMA1 haplotype; the other subdivision includes haplotypes identical or nearly identical to several haplotypes detected in lineage OZ01 (Figure 6). Inspection of 21 (of 31 total) of the CYTB sequences of discordant samples showed no nucleotide variation from the reference sequence for lineage OZ04, indicating that the samples were correctly assigned to the lineage and supporting the absence of this divergence in the mitochondrial genome, though SNP variation for the remaining 10 samples was unavailable.
The *Passerina*-dominant group within lineage OZ01 is significantly differentiated from the other group \( F_{ST} = 0.74, p < 0.001 \), but pairwise \( F_{ST} \) values among the parasites of other well-sampled hosts were not significant in any of the lineages assessed. The smaller group of *AMA1* haplotypes within OZ14 (visualized in Figure 5C) is significantly differentiated from the larger group in this lineage \( F_{ST} = 0.88, p < 0.001 \) and does contain a preponderance of hosts in the family Icteridae (30% of this group, comprising 57% of the icterid infections sampled) but is broadly geographically distributed and exhibits no statistically significant differentiation in association with any one host species.

Support for differentiation among at least some locations comes from pairwise comparisons in each lineage. Lineage OZ01 exhibits a complex pattern of differentiation and gene flow (Table 4) while both OZ04 and OZ14 are differentiated with respect to only a single location: OZ04 samples from Saint Lucia are differentiated from those from Dominica, Guadeloupe, and Jamaica, but no other pairwise comparisons among these locations are significant; OZ14 samples from Chicago are differentiated with respect to samples from locations in Saint Louis, the Missouri Ozarks, Michigan, and Pennsylvania, but exhibit no other significant pairwise differences among these locations. Further investigation into the complex pattern of OZ01 revealed no shared geographic boundary among pairwise haplotype comparisons and no signal of isolation-by-distance (Mantel statistic \( r: -0.188, \text{significance} \ 0.69 \)).

### Table 2: Nucleotide diversity (\( \pi \)), percentage of synonymous variation (\( S \)), and recombination estimates (\( Rm \)) for avian and human *Plasmodium* lineages

| Lineage          | \( \pi \)       | \( S \% \) | \( Rm \) |
|------------------|-----------------|---------|--------|
| OZ01             | 0.004           | 25      | 3      |
| OZ04             | 0.173           | 12.7    | 9      |
| Major OZ04       | 0.006           | 21.6    | 1      |
| Minor OZ04       | 0.004           | 21.5    | 1      |
| OZ14             | 0.007           | 20.7    | 1      |
| *P. lucens*      | 0.043/0.003\(^b\) | 22\(^a\) | 0      |
| *P. vivax*       | -0.016\(^b\)    | 24\(^d\) | 0      |
| *P. falciparum*  | 0.01\(^e\)      | 0.0098\(^f\) | 0      |

Note: Lineage OZ04 estimates of \( \pi \) are for all sequences, including suspected cases of introgression, major OZ04 includes only the major cluster, and minor OZ04 includes only the apparently introgressed samples. Locations of samples for congeneric comparisons:

\(^a\)Africa (for all sequences above, for a reduced dataset excluding seven sequences of uncertain taxonomic identity, from Lauron et al., 2014),
\(^b\)Venezuela (Grynberg et al., 2008),
\(^c\)Brazil (Figtree et al., 2000),
\(^d\)Iran (Abouie Mehrizi, Sepehri, Karimi, Djadid, & Zakeri, 2013),
\(^e\)10 locations distributed globally (Escalante et al., 2001),
\(^f\)Venezuela (Ord et al., 2008).

4 | DISCUSSION

The avian malaria parasite lineages assessed here have been defined based on their similarity at the partial cytochrome *b* gene, but whether they represent good phylogenetic species has not been determined. Broad host ranges, propensity for host switching, and difficulty in linking morphological species and genetic lineages have all contributed to the problem of delimiting species in this group (Galén, Nunes, Sweet, & Perkins, 2018; Martinsen, Perkins, & Schall, 2008; Outlaw & Ricklefs, 2014). Moreover, a cut-off based on percentage of divergence is not a useful way to define these species because it has been demonstrated through combined geographic range, host range, and corroboration with multiple loci that good phylogenetic species can exhibit low divergence at CYTB (Nilsson et al., 2016; Outlaw & Ricklefs, 2014). In the absence of morphological data, species delimitation is thought to be best addressed by comparing multiple gene trees (Bensch, Pérez-Tris, Waldenström, & Hellgren, 2004; Galen et al., 2018; Outlaw & Ricklefs, 2014).

The *AMA1* gene tree produced here contradicts the mitochondrial lineage assignment in some cases. Specifically, all parasites from mitochondrial lineage OZ01 possess *AMA1* alleles similar to one another, but 31 parasites from mitochondrial lineage OZ04 also possess *AMA1* alleles identical or very similar to OZ01 alleles; the remaining parasites of lineage OZ04 possess *AMA1* alleles that are similar to one another and more than 60 mutations divergent from the OZ01 cluster (Figure 6). We interpret these relationships as supporting

### Table 3: *AMA1* genotype heterozygosity for lineages OZ01, OZ04, and OZ14, and for the suspected introgressed group

|                    | OZ01 | OZ04 | OZ14 | Introgressed group |
|--------------------|------|------|------|--------------------|
| Heterozygous Ind.  | 13   | 3    | 7    | 13                 |
| No. of ind. With 1 | 12   | 1    | 3    | 13                 |
| Heterozygous pos.  |     |      |      |                    |
| No. of ind. With 2 | 1    | 1    | 4    | 0                  |
| Heterozygous pos.  |     |      |      |                    |
| No. of ind. With 3 | 0    | 1    | 0    | 0                  |
| Heterozygous pos.  |     |      |      |                    |
mitochondrial introgression from OZ04 to OZ01, but an alternative explanation of undetected coinfections cannot be ruled out with currently available data. Avian malaria coinfections are known to occur and are known to be underestimated by PCR as a result of preferential primer binding on variations of the template sequence (Bernotiene, Palinauskas, Iezhova, Murauskaite, & Valkiunas, 2016). Therefore, it is possible that the suspected introgressed parasites are instead OZ01/OZ04 coinfections in which our CYTB primers only amplified templates from OZ04 and our AMA1 primers only amplified templates from OZ01.

Individual CYTB SNP data were available for 21 (of 31) samples with suspected introgressed AMA1 variants and revealed no difference from the reference sequence for lineage OZ04 (Genbank accession GQ395669) confirming appropriate lineage assignment and

**FIGURE 4** Sliding window analysis of nucleotide diversity for OZ01, OZ04, OZ14, and Plasmodium falciparum from Escalante et al. (2001). Window, 30 bp; step size, 9 bp

**FIGURE 5** Median-joining haplotype networks of AMA1 variation for mitochondrial lineages OZ01 (A), OZ04 (B), and OZ14 (C). Circle size indicates the number of individuals with a given haplotype, color indicates host family/superfamily, and black hash marks indicate mutations. The gray oval in panel A encloses the group primarily infecting Passerina
FIGURE 6  Median-joining haplotype networks of AMA1 depicting combined lineages OZ01, OZ04, and OZ14. Circle size indicates the number of individuals with a given haplotype, color indicates mitochondrial lineage designation, and black hash marks indicate mutations.

TABLE 4  Pairwise $F_{ST}$ among locations for lineage OZ01 (only locations with 20 or more detections are included in this analysis). $N$ = sample, $p$-value < 0.05 indicated by + and $p$-value > 0.05 indicated by −. in upper diagonal. Note: negative $F_{ST}$ value reported as 0.
12 haplotypes) cluster together more than 60 mutational steps from the other haplotypes. The pairwise nucleotide differences among haplotypes within the major cluster vary between one and three substitutions, suggesting uncertain taxonomic identity of the minor cluster. If the divergent sequences are removed from analysis, the estimate of $\pi$ is 0.003 (reported in Table 2), consistent with findings for OZ01 and OZ14. The estimate for lineage OZ04 provided here is also exceedingly high, but expectedly so because it includes the highly divergent introgressed group. Removal of the divergent group in this case produced an estimate of $\pi = 0.006$ for lineage OZ04.

We found no support for isolation-by-distance in the widespread lineage OZ01, and lineages OZ04 and OZ14 are genetically undifferentiated with respect to host species. OZ04 does, however, exhibit overall reduced host breadth compared to OZ14, with the former infecting mainly West Indian tanagers such as bananaquits (Coereba flaveola), grassquits (Tiaris bicolor), and bullfinches (Loxigilla spp.), while the latter infects extremely diverse hosts. The lack of population differentiation among some locations in OZ01 and OZ14 is likely related to host dispersal as these lineages infect primarily migratory species. In contrast, we detected OZ04 most commonly in resident species. The presence of genetic differentiation among locations within lineages might be promoted by geographic isolation related to migratory paths of hosts or vector dispersal, but we lack information to assess these possibilities at present.
**TABLE 5** Host and geographic location of introgressed parasites

|                | JAM | OZ | GU | DO | CF-PR | RB-PR | SK | SL | Sum |
|----------------|-----|----|----|----|-------|-------|----|----|-----|
| Coereba flaveola | 7   |    |    |    |       |       |    |    | 9   |
| Tiaris bicolor   | 2   | 3  | 1  | 1  | 1     | 1     |    |    | 8   |
| Euneornis campestris | 3 |     |    |    |       |       |    |    | 3   |
| Passerina cyanea | 2   |    |    |    |       |       |    |    | 2   |
| Vireo olivaceus  | 2   |    |    |    |       |       |    |    | 2   |
| Cinclocerthia ruficauda | 1 |     |    |    |       |       |    |    | 1   |
| Setophaga plumbea | 1   |    |    |    |       |       |    |    | 1   |
| Icteria virens   |     | 1  |    |    |       |       |    |    | 1   |
| Loxipasser anoxanthus | 1  |     |    |    |       |       |    |    | 1   |
| Loxigilla noctis |     | 1  |    |    |       |       |    |    | 1   |
| Mniotilta varia |     |    |    |    |       |       |    |    | 1   |
| Turdus plumbeus  |     |    |    |    |       |       |    |    | 1   |
| Sum             | 13  | 6  | 6  | 2  | 1     | 1     | 1  | 1  | 31  |

Location abbreviations are as follows: JAM, Jamaica; OZ, Ozarks region of Missouri; GU, Guadeloupe; DO, Dominica; CF-PR, Carite Forest, Puerto Rico; RB-PR, Refugio Boqueron, Puerto Rico; SK, Saint Kitts; SL, Saint Lucia.

**FIGURE 9** Median-joining haplotype networks of AMA1 for only introgressed samples. Circle size indicates the number of individuals with a given haplotype, color indicates location, and black hash marks indicate mutations.

**FIGURE 10** Median-joining haplotype network of 51 Plasmodium lucens AMA1 sequences depicting the main group (red) and the divergent group (blue). All samples were recovered from a population of olive sunbirds (Cyanomitra olivacea) in Cameroon (Lauron et al., 2014)

### 4.1 Congeneric comparisons

*Plasmodium vivax* and *P. falciparum* represent contrasting demographic patterns and impacts on hosts, and these parasites may provide an informative context in which to consider the findings presented here. In general, *P. vivax* causes less mortality and is more likely to persist at low densities and with a lower transmission rate than *P. falciparum* (Neafsey et al., 2012). *Plasmodium vivax* has more diverse populations, exhibits dormancy and relapse, and dominates the interepidemic periods when sympatric with *P. falciparum* (Arnott et al., 2014; Neafsey et al., 2012). *Plasmodium falciparum* exhibits a contrasting demographic pattern typified by dramatic clonal outbreaks (Mueller et al., 2002; Razakandrainibe et al., 2005) and more severe host illness. The avian parasites with the three AMA1 lineages described here exhibit population patterns inconsistent with either the epidemic pathogen structure of *P. falciparum* or the endemic pathogen structure of *P. vivax*. That is, our lineages present comparatively low diversity at AMA1, as in *P. falciparum*, but nonetheless exhibit local differentiation and recombination as in *P. vivax*. Little is known of other avian malaria parasite populations, but analysis of a taxonomically conservative subset of *P. lucens* AMA1 from a population of olive sunbirds (*Cyanomitra olivacea*) in Cameroon (Lauron et al., 2014) is consistent with our finding that genetic diversity in the avian parasites is lower than both *P. vivax* and *P. falciparum*.
(Figtree et al., 2000; Grynberg, Fontes, Hughes, & Braga, 2008) and *P. falciparum* (Escalante et al., 2001).

## 5 | CONCLUSIONS

Analyses at more loci and with wider taxon sampling will be necessary to uncover the complexities of these relationships, but findings here provide a glimpse into the host distribution, spatial distribution, and diversity of avian malaria AMA1 in natural host communities. The complex spatial patterns and differentiation in relation to host genus described here suggest several possible influences on population structure, including host immune pressure, host dispersal, and migratory movements, as well as vector dispersal and host feeding preferences. Moreover, the mitonuclear discordance detected here warrants further investigation to assess the role of coinfections and to determine the frequency of such introgression events and implications for defining parasite lineages based on mitochondrial genetic variation.

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## CONFLICT OF INTEREST

All authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

RER and MBH designed the research; RER collected samples; MBH and MTS performed laboratory and statistical analyses; MBH wrote the manuscript with contributions and revisions by all authors. All authors approved the final manuscript.

## DATA ACCESSIBILITY

All sequences deposited to Genbank, accession numbers MK965548-MK965653 and MK929797-MK930264.

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This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at Genbank https://www.ncbi.nlm.nih.gov/genbank/, accession numbers MK965548-MK965653 and MK929797-MK930264.

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