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Multiple Lineages of Avian Malaria Parasites (*Plasmodium*) in the Galapagos Islands and Evidence for Arrival via Migratory Birds

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Abstract: *Haemosporidian parasites in the genus Plasmodium were recently detected through molecular screening in the Galapagos Penguin (Spheniscus mendiculus). We summarized results of an archipelago-wide screen of 3726 endemic birds representing 22 species for Plasmodium spp. through a combination of molecular and microscopy techniques. Three additional Plasmodium lineages were present in Galapagos. Lineage A–infected penguins, Yellow Warblers (Setophaga petechia aureola), and one Medium Ground Finch (Geospiza fortis) was detected at multiple sites in multiple years. The other 3 lineages were each detected at one site and at one time; apparently, they were transient infections of parasites not established on the archipelago. No gametocytes were found in blood smears of infected individuals; thus, endemic Galapagos birds may be dead-end hosts for these Plasmodium lineages. Determining when and how parasites and pathogens arrive in Galapagos is key to developing conservation strategies to prevent and mitigate the effects of introduced diseases. To assess the potential for Plasmodium parasites to arrive via migratory birds, we analyzed blood samples from 438 North American breeding Bobolinks (Dolichonyx oryzivorus), the only songbird that regularly migrates through Galapagos. Two of the ephemeral Plasmodium lineages (B and C) found in Galapagos birds matched parasite sequences from Bobolinks. Although this is not confirmation that Bobolinks are responsible for introducing these lineages, evidence points to higher potential arrival rates of avian pathogens than previously thought.*

Keywords: Bobolink, Galapagos, Haemosporida, migratory birds, *Plasmodium*

Linajes Múltiples de Parásitos de Malaria Aviar (*Plasmodium*) en las Islas Galápagos y Evidencia de su Arribo por Medio de Aves Migratorias

Resumen: *Parásitos de la familia Haemosporidia, del género Plasmodium recientemente fueron detectados por medio de análisis moleculares en el pingüino de las Galápagos (Spheniscus mendiculus). Resumimos los resultados de un análisis, extendido a lo largo del archipiélago, de 3726 aves endémicas representando a 22 especies para Plasmodium spp. mediante la combinación de técnicas moleculares y de microscopía.*

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Tres linajes adicionales de Plasmodium estuvieron presentes en las Galápagos. El linaje A infectó pingüinos, individuos Setophaga petechia aureola y a un individuo de Geospiza fortis en sitios múltiples y en varios años. Los otros 3 linajes fueron detectados cada uno en un sitio y en un tiempo específico; aparentemente, fueron infecciones transitorias de parásitos no establecidos en el archipiélago. No se encontraron gametocitos en los frotis de sangre de individuos infectados; por esto, las aves endémicas de las Galápagos pueden ser hospederos finales de estos linajes de Plasmodium. Determinar cuándo y cómo llegaron los parasitos y los patógenos a las Galápagos es clave para desarrollar estrategias de conservación para prevenir y mitigar los efectos de las enfermedades introducidas. Para evaluar el potencial de llegada de Plasmodium en aves migratorias, analizamos muestras de sangre de 438 Dolichonyx oryzivorus en época reproductiva. Esta ave es la única canora que migra regularmente a través de las Galápagos. Dos de los linajes efímeros de Plasmodium (B y C) que se hallaron en las aves de las Galápagos coincidieron con secuencias de parásitos del ave canora. Aunque esto no confirma que Dolichonyx oryzivorus sea responsable de introducer estos linajes, la evidencia apunta a un potencial más alto de tasas de llegada de patógenos aviares de lo que se pensaba previamente.

Palabras Clave: aves migratorias, *Dolichonyx oryzivorus*, Galápagos, Hameosporida, *Plasmodium*

Introduction

Species on remote island archipelagos are thought to be isolated from parasites and pathogens compared with their mainland congeners. Consequently, they are potentially more vulnerable to introduced diseases (van Riper et al. 1986; Dobson & Foufopoulos 2001). The Galapagos Islands support large numbers of sea and land birds and have had no known extinctions of endemic bird species (Parker et al. 2006). Despite the geographic isolation of the islands, pathogens such as avian pox (*Avipoxvirus* sp.), have arrived and established in Galapagos in the last century (Parker et al. 2011). Several haemosporidian parasites have been detected in Galapagos birds (Valkiunas et al. 2010; Levin et al. 2011; Levin et al. 2012), including a parasite in the genus *Plasmodium* that infects the endangered Galapagos Penguin (*Spheniscus mendiculus*) (Levin et al. 2009). *Plasmodium* was first detected in Galapagos in this species. The discovery raised questions about the parasite's mode and time of arrival, pathogenicity, possible vectors, and transmission dynamics within the archipelago.

Transmission of a haemosporidian parasite depends on the presence of a vector (mosquito in the case of avian *Plasmodium*) capable of supporting parasite development and transmitting infective sporozoites and susceptible vertebrate hosts in which the parasite completes its life cycle and forms gametocytes. A comprehensive understanding of the identities of the *Plasmodium* species, mosquito vector(s), and avian host(s) is lacking for *Plasmodium* spp. in Galapagos but under investigation. In Levin et al. (2009), *Plasmodium* parasite DNA from Galapagos Penguin blood samples was amplified by polymerase chain reaction (PCR), but microscopic evaluation of blood smears of PCR-positive individuals did not show evidence of infection; no gametocytes (the stage infective to an arthropod vector) or other blood stages were found (Travis et al. 2006; this study). Therefore, although Galapagos penguins are infected (we detected the parasite across multiple years in multiple colonies on 3 of 4 islands where penguins breed [Levin et al. 2009]), they may be dead-end hosts for the parasite and other bird species act as reservoirs for this *Plasmodium* lineage. To understand the scope and transmission of Galapagos *Plasmodium* spp. better, we screened the most common Galapagos passerine birds, additional Galapagos Penguins, and Flightless Cormorants (*Phalacrocorax harrisi*) for *Plasmodium* parasites.

Determining when and how parasites and pathogens arrive in Galapagos is key to developing conservation strategies to prevent and mitigate the effects of introduced diseases. Some parasites arrived with their hosts when they colonized the archipelago (lice [Whiteman et al. 2009] and mites [Sari et al. 2013]), whereas others apparently jumped from one host to another after arrival (Microfilariae [Merkel et al. 2007] and Haemosporida and lice [Sari et al. 2013]). For some parasites there is evidence of more recent introduction (avian pox [Parker et al. 2011] and *Philornis downsi* [Fessl & Tebbich 2002]). Migratory birds often harbor relatively high haemosporidian diversity compared with nonmigratory birds (Figuerola & Green, 2000) and may contribute to the movement of parasites among breeding, overwintering, and migratory stop-over sites (Waldenström et al. 2002; Jenkins et al. 2012). If cross-species transmission occurs at overwintering sites or along migratory routes, there is potential for geographic expansion by haemosporidian parasites. The only passerine bird regularly reported to stop in Galapagos during migration is the Bobolink (*Dolichonyx oryzivorus*) (Kramer 1965; Pettingill 1983). Bobolinks breed across northern North America and migrate to central South America. We included Bobolink samples from 3 breeding populations in North America to determine whether parasites from Bobolinks match lineages recovered from Galapagos birds.

Conservation actions, such as targeted elimination of introduced vertebrate hosts or vectors, require testing for additional *Plasmodium* lineages that may be present in Galapagos and full understanding of birds and mosquitoes

involved in their transmission. We sought to assess the extent to which *Plasmodium* parasites are being transmitted among endemic birds and determine potential reservoir hosts through the detection of gametocytes in blood smears; determine whether additional *Plasmodium* lineages occur in Galapagos birds; use spatial and temporal infection data to identify potential transmission zones; estimate genetic differences between Galapagos *Plasmodium* lineages and previously described morphospecies; and compare *Plasmodium* lineages recovered from North American breeding Bobolinks to lineages found in Galapagos to assess the potential for arrival parasites via migratory birds.

Methods

Sampling

We sampled 2923 individuals from 20 passerine bird species between June 2003 and December 2009 on multiple islands within the Galapagos archipelago (Table 1). Our survey included all 4 mockingbird species (*Mimus* spp.), 13 finch species (*Camarhynchus* spp., *Certhidea* spp., *Geospiza* spp., *Platyspiza*), 2 flycatcher species (*Myiarchus magnirostris*, *Pyrocephalus rubinus*), and the endemic subspecies of Yellow Warbler (*Setophaga petechia aureola*). Together the sample represented 87% of the passerine species commonly found in Galapagos (Jiménez-Uzcátegui et al. 2012). We captured individual passerines in mist nets and recorded standard morphometric measurements. We took blood samples from brachial or jugular veins of captured passerines. We collected no more than 1% of the bird's weight in blood volume ($\leq$0.1 mL blood/10 g). We made blood smears immediately after sampling and fixed the smears in the field with methanol. We stained them with either a modified Wright-Giemsa stain (JorVet Dip-Quick, Jorgensen Laboratories, Loveland, Colorado) or Giemsa stain following Valkiunas (2005). Remaining blood was stored in Longmire's lysis buffer for DNA analysis.

We tested blood samples from 209 Galapagos Penguins sampled in 2008 and 2009 and 594 Flightless Cormorants sampled between 2003 and 2005. The cormorant breeding colonies were very near or overlapped those of Galapagos Penguins on the islands of Isabela and Fernandina. Penguin and cormorant sampling details are in Duffie et al. (2009) and Nims et al. (2008).

We captured Bobolinks in northwestern Vermont (N 44° 23′, W 73° 16′) in 2002–2011, along the Platte River in southeastern Nebraska (N40° 48′, W 98° 26′) in 2002–2011, and at Malheur National Wildlife Refuge in Princeton, Oregon (N 43° 16, W 118°, 50′; 2011) in mist nets in areas of relatively high Bobolink density, in territories, and near known nests. We used the same blood-sampling protocol as described previously. We screened 438 Bobolinks for haemosporidian parasites (Table 2).

Molecular Screening for *Plasmodium* Parasites

We extracted DNA from blood samples following a phenol-chloroform extraction protocol described in Sambrook et al. (1989). We used a PCR-based molecular screen to test for the presence of parasite DNA in the blood samples. All samples except those of Flightless Cormorants were tested by amplifying a portion of the parasite cytochrome *b* (cyt *b*) gene with primers from Waldenström et al. (2004). We followed 1 of 2 protocols: Levin et al. (2011) or Sari et al. (2013). Following Levin et al. (2009), we used cyt *b* primers (Perkins & Schall 2002) to test Cormorants. We used a consistently amplifying Galapagos Penguin infected with *Plasmodium* spp. as a positive control in each reaction. Negative controls consisted of PCR reagents and no DNA. We retested positive individuals to confirm infection and re-ran all samples if the positive control did not amplify. We followed Levin et al. (2011) for PCR reaction conditions, amplicon purification, and sequencing of 490 base pairs of parasite Cyt *b*. Rescreened individuals were also resequenced to verify *Plasmodium* parasite sequence. We deposited sequences in GenBank (accession numbers: KC867648-KC867680). We performed Pearson's chi-square tests in R (version 2.15.2) (R Foundation for Statistical Computing, Vienna) to test for temporal, spatial, and host species patterns in *Plasmodium* infection.

Phylogenetic Analyses

We assembled parasite cyt *b* sequences in Seqman 4.0 and added them to a data set containing overlapping cyt *b* data for described morphospecies obtained from GenBank. Any contig containing double peaks that indicated multiple infection was removed from the analyses. We used ClustalW (Larkin et al. 2007) implemented in BioEdit to align sequences (version 7.1.7) (Hall 1999). The best-fit model of DNA evolution was determined with jModelTest2 (Guindon & Gascuel 2003; Darriba et al. 2012). Using a GTR + I + Γ model of nucleotide substitution, we reconstructed a maximum likelihood phylogeny and bootstrap analysis (1000 pseudoreplicates) in MEGA 5 (Tamura et al. 2011). We obtained Bayesian posterior probabilities from 10 million trees with BEAST 1.7 (first 100,000 steps as burnin) (Drummond et al. 2012). Parameters included a relaxed clock: uncorrelated lognormal and lineage birth was modeled with a Yule prior. We determined the likelihood stationarity of sampled trees graphically with Tracer (Drummond et al. 2012). We calculated sequence divergence between lineages in MEGA 5 with a Jukes-Cantor substitution model. A sequence divergence of more than 2 base pairs was used to define lineages.

Table 1. Number of individual birds tested by PCR for *Plasmodium* parasites by species and island.[a]

Species	*Common Name*	*Bartoleme*	*Champion*	*Espanola*	*Fernandina*	*Floreana*	*Gardner*	*Genovesa*	*Isabela*	*Marchena*	*Pinta*	*Pinzon*	*San Cristobal*	*Santa Cruz*	*Santa Fe*	*Santiago*	*Total*
Camarhynchus pallidus	Woodpecker Finch	–	–	–	–	–	–	–	0	5	–	–	3	6	–	–	14
Camarhynchus parvulus	Small Tree Finch	–	–	–	0	9	–	–	17	-	0	0	14	81	0	0	121
Camarhynchus pauper	Medium Tree Finch	–	–	–	–	5	–	–	–	–	–	–	–	–	–	–	5
Camarhynchus psittacula	Large Tree Finch	–	–	–	–	0	–	–	0	5	0	0	–	14	0	0	19
Certhidea olivacea	Green Warbler Finch	–	–	–	–	–	–	–	–	–	–	–	0	4	–	4	8
Certhidea fusca	Grey Warbler Finch	–	–	0	–	0	–	5	–	6	0	–	1	–	0	–	12
Geospiza conirostris	Large Cactus Finch	–	–	0	–	–	–	8	–	–	–	–	–	–	–	–	8
Geospiza difficilis	Sharp-Beaked Ground Finch	–	–	–	–	–	–	7	–	–	0	–	–	0	–	–	7
Geospiza fortis	Medium Ground Finch	–	1	0	6	57	–	–	60	8	0	0	3	371 (1)	0	5	511 (1)
Geospiza fuliginosa	Small Ground Finch	–	2	–	23	103 (3)	–	–	80	25 (1)	–	–	16	389	23	29	690 (4)
Geospiza magnirostris	Large Ground Finch	–	–	–	0	0	–	1	0	8	0	–	8	50	0	16	83
Geospiza scandens	Cactus Finch	–	–	–	–	2	–	–	17	2	–	–	–	68	0	3	92
Mimus macdonaldi	Espanola Mockingbird	–	–	94	–	–	–	–	–	–	–	–	–	–	–	–	94
Mimus melanotis	San Cristobal Mockingbird	–	–	–	–	–	–	–	–	–	–	–	2	–	–	–	2
Mimus parvulus	Galapagos Mockingbird	–	1	–	35	–	–	30	72	10	27	–	–	82	0	9	266
Mimus trifasciatus	Floreana Mockingbird	–	0	–	–	–	47	–	–	–	–	–	–	–	–	–	47
Myiarchus magnirostris	Galapagos Flycatcher	–	–	26	0	35	–	0	60	3	0	0	59	106	11	22	322
Phalacrocorax harrisi	Flightless Cormorant	–	–	–	270	–	–	–	324	–	–	–	–	–	–	–	594
Platyspiza crassirostris	Vegetarian Finch	–	–	–	0	0	–	–	2	13	0	0	7	95	–	0	117
Pyrocephalus rubinus	Vermilion Flycatcher	–	–	–	0	0	–	–	1	1	0	0	–	0	–	0	2
Setophaga petechia	Yellow Warbler		1	2	104 (1)	32		23	100 (8)	2	35	9	5	184 (1)		6	503 (10)
Spheniscus mendiculus	Galapagos Penguin	5	–	–	71 (5)	2	–	–	131 (8)	–	–	–	–	–	–	0	209 (13)
		5	5	122	509 (6)	245 (3)	47	74	864 (16)	88 (1)	62	9	118	1450 (2)	34	94	3726 (28)

[a]*Numbers in parentheses indicate number of individuals infected with Plasmodium; 0 denotes no samples collected; and – denotes species not known to exist in that location.*

Table 2. ***Plasmodium*** **parasite infections of bobolinks *(Dolichonyx oryzivorus)* in 3 U.S. states.**

Site in United States	*Infected*	*Sampled*	*No. of plasmodium lineages*
Nebraska[a]	56 (25.7%)	218	10
Oregon	4 (15.4%)	26	3
Vermont[a]	18 (9.3%)	194	7
Total	78 (17.8%)	438	12

[a] *A 100% sequence match found between Bobolink Plasmodium and a Galapagos Plasmodium lineage.*

Microscopy

Because PCR results cannot distinguish between sexual (infective to vectors) and asexual (not infective to vectors) parasite stages, we inspected blood smears of all birds that amplified *Plasmodium* spp. DNA by PCR and >900 of PCR-negative samples for the presence of gametocytes. At the WildCare Institute at the Saint Louis Zoo, slides were scanned at low magnification (×200) for 5 min after which 200 fields were studied at high magnification (×1000) under oil immersion with a Nikon Labophot light microscope (Nikon, Tokyo, Japan). This procedure was performed twice on each PCR-positive bird. Slides from PCR-positive birds and from 390 samples from areas where we had previously detected lineage A in penguins, were sent to the P. B. Šivickis Laboratory of Parasitology at the Nature Research Centre, Vilnius, Lithuania for additional screening. We used an Olympus BX61 light microscope (Olympus, Tokyo, Japan) to examine slides and prepare illustrations. Blood films were examined for 10–15 min at low magnification (×400), and then at least 100 fields were studied at high magnification (×1000).

Results

Galapagos *Plasmodium* Infection

Our molecular screen identified 15 (0.51%) individual passerines that amplified *Plasmodium* spp. DNA out of 2923 individuals of 20 species that were sampled in Galapagos. Of the 209 penguins we tested, 13 (6.22%) were positive and no infections were detected in 594 cormorants. Sampling location, number of infected individuals, and host species for all screened samples are in Table 1. Verification rates of PCR-positive passerines were low; 6 of 15 passerines tested positive at least once more. We tested the 9 passerines that were not positive more than once 10–33 additional times. No Galapagos birds we sampled had evidence of multiple infections. We did not find gametocytes in blood smears of any PCR-positive individuals, including the penguins. We found one developing binuclear erythrocytic meront with visible pigment in a Cactus Finch (*Geospiza scandens*)

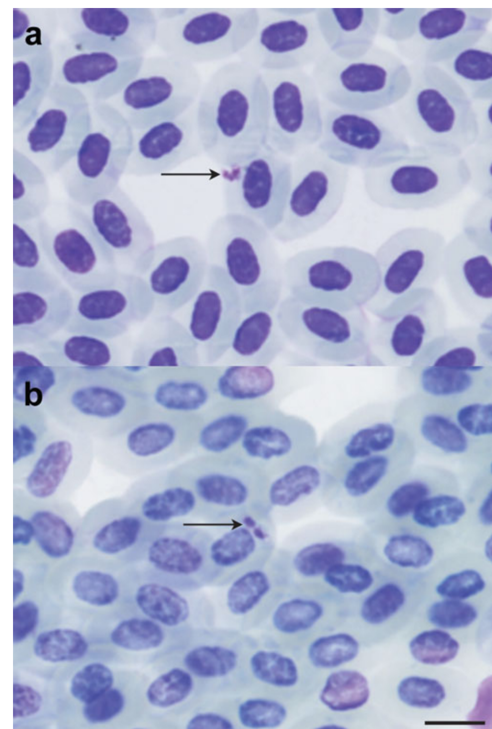

Figure 1. Parasite blood stages from 2 individual Galapagos passerines: (a) a developing binuclear erythrocytic meront of Plasmodium sp. from a Cactus Finch (Geospiza scandens) and (b) intra-erythrocytic haemosporidian trophozoites from a Vegetarian Finch (Platyspiza crassirostris) (arrows, parasites, bar = 10 μm).

(Fig. 1a) and 2 intra-erythrocytic trophozoites in a Vegetarian Finch (*Platyspiza crassirostris*) (Fig. 1b), both of which were sampled on Santa Cruz but did not test positive in our PCR screen for *Plasmodium*.

The majority of infected individuals were Yellow Warblers (10 of 503, 2%). Infected Yellow Warblers were highly localized on southern Isabela, where 8 out of 100 (8%) individuals tested positive (Fig. 2). This infection rate was significantly greater than all other species combined across all locations where infected birds were found (7/787; Yeat's corrected Pearson chi square, $\chi^2 = 22.876$, $p < 0.0001$). One infected Yellow Warbler was found on Fernandina ($n = 104$, 1%) and another on Santa Cruz ($n = 184$, 0.5%). Other infected birds included a Medium Ground Finch from Santa Cruz ($n = 371$, 0.3%) and 4 Small Ground Finches, 3 from Floreana ($n = 103$, 2.9%) and 1 from Marchena ($n = 25$, 4%) (Fig. 2). Increasing sample size helped in the detection of infections when considering either sampling site (island) or species (infections per site: $p = 0.001$; infections per species: $p = 0.008$). However, the relatively high concentration of positive Yellow Warblers on Isabela was not explained solely by increased sampling. Fewer

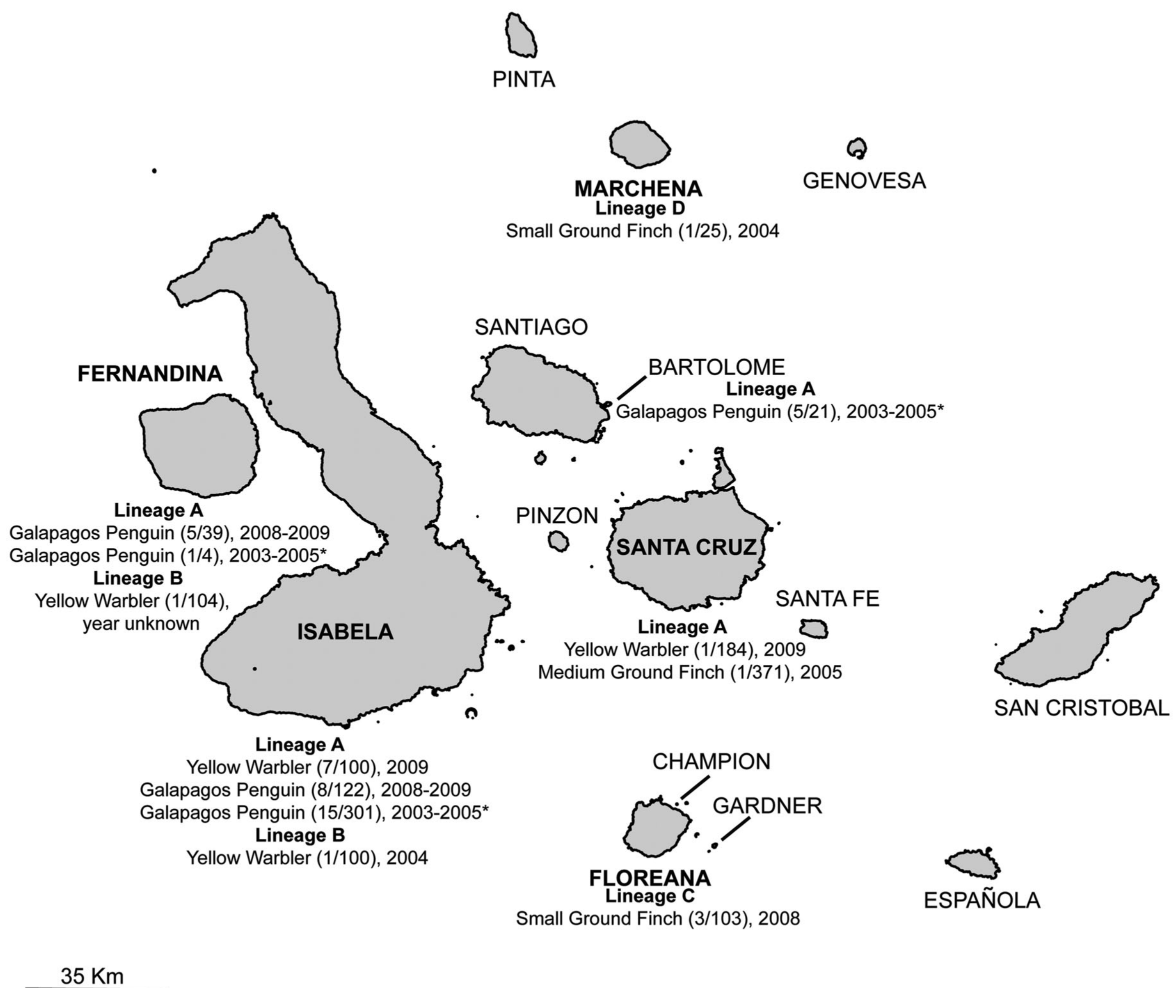

*Figure 2. Map of the Galapagos Islands indicating locations of Plasmodium parasite-positive individuals from 3726 tested birds (letters A-D, parasite lineages; birds, host species; numbers in parentheses, ratio of positive individuals to total tested within species at each location; year, year infections were detected; *, Galapagos Penguin samples tested and reported in Levin et al. [2009]). For samples sizes on other islands with no infected individuals, see Table 1.*

infections were detected in many well-sampled species (e.g., ground finches) at sites where we had over 100 individuals per site (e.g., Santa Cruz, Floreana). Of the penguin samples tested ($n = 209$), parasite DNA amplified in 8 samples from Isabela and 5 from Fernandina.

Bobolink *Plasmodium* Infection

We identified 92 of 438 Bobolinks infected with haemosporidian parasites, and after discarding samples containing multiple infections and removing *Haemoproteus* lineages, we had 78 *Plasmodium* sequences (17.8% prevalence) (Table 2). Prevalence of *Plasmodium* parasites in Bobolinks ranged from 9.3 to 25.7% across the 3 sites (Table 2). Overall prevalence of *Plasmodium* spp. varied significantly by site ($\chi^2 = 13.4$, $p = 0.001$), and prevalence varied by year in Nebraska, where we had the best temporal sampling ($\chi^2 = 38.5$, $p < 0.0001$). In total, we recovered 11 *Plasmodium* lineages in Bobolinks from all sampling sites combined. There was no difference in the number of *Plasmodium* lineages in Bobolinks after correcting for sample size differences per site ($\chi^2 = 2.9$, $p = 0.24$). Similarly, there were no significant differences in the frequencies of lineages among sites ($\chi^2 = 96$, $p = 0.26$).

Phylogenetic Analyses and Lineage Description

There was little support for many of the relations within *Plasmodium* (Fig. 3). Four lineages of *Plasmodium* were found in Galapagos birds (lineages A, B, C, D, i [Fig. 3]). These 4 lineages were genetically distinct. The smallest pair-wise genetic divergence, 5.99% (27 base pairs), was between lineages A and B and the greatest, 8.74% (40 base pairs), was between lineages A and D (Table 3). In all cases, Galapagos lineages formed

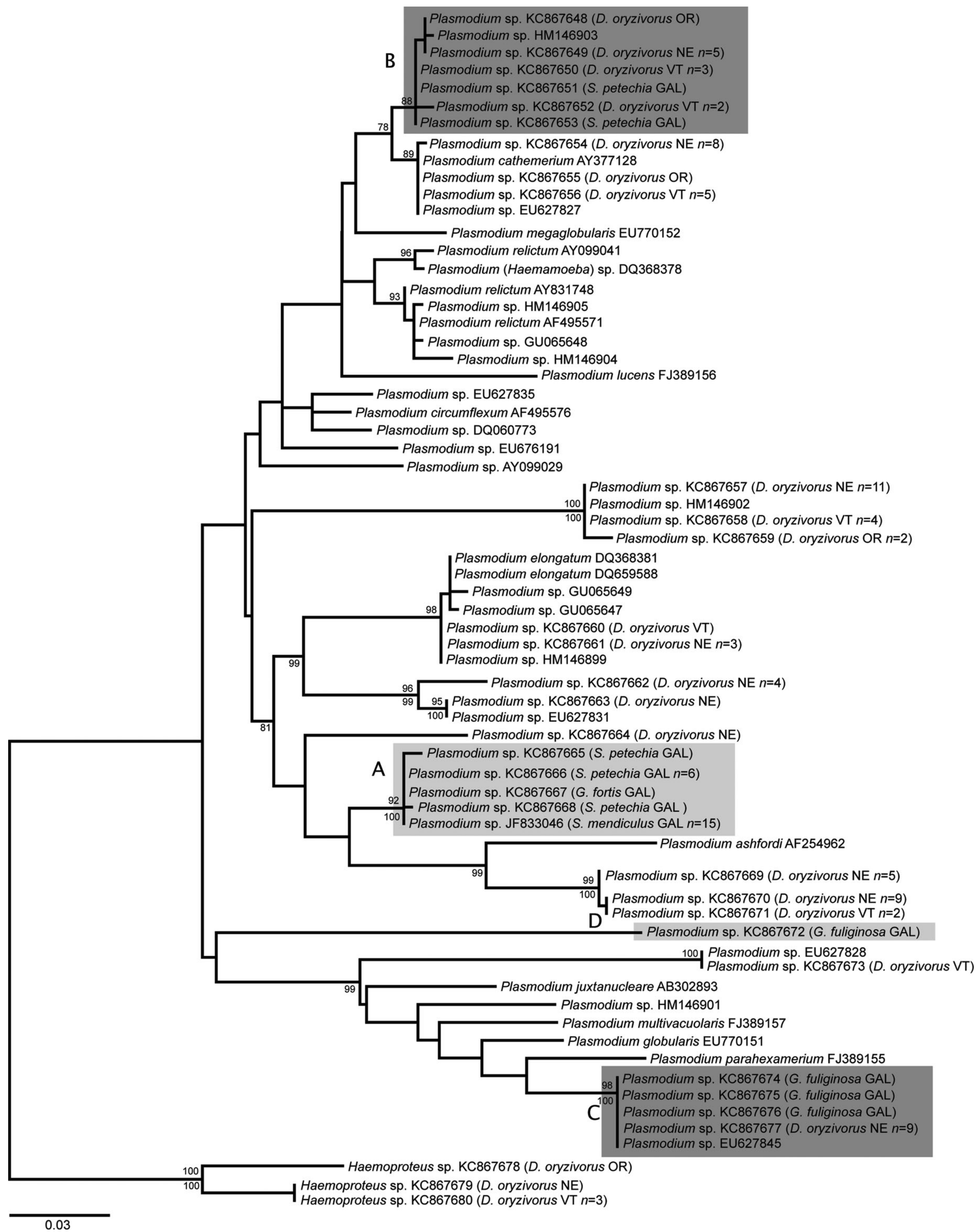

Figure 3. Maximum likelihood (ML) phylogenetic hypothesis of Plasmodium parasite sequence constructed from 490 base pairs of the mitochondrial gene, cytochrome b. The ML bootstrap values appear above the nodes where support values >75 were found. Numbers below the nodes correspond to Bayesian posterior probabilities >95. All gray shading indicates sequences found in Galapagos birds and dark gray corresponds to sequences found in Galapagos birds that also match sequences recovered from North American breeding Bobolinks (Dolichonyx oryzivorus). Galapagos lineages are labeled A–D. GenBank accession numbers are listed for all sequences, and geographic location and sample size are indicated for Galapagos and Bobolink sequences (GAL, Galapagos; VT, Vermont; NE, Nebraska; OR, Oregon).

Table 3. **Percent nucleotide difference (below diagonal; $n = 456$ base pairs after deletion of characters with missing data) and average number of nucleotide differences (above diagonal) within (†) and between (above and below the diagonal) the sequences found in the 4 Galapagos *Plasmodium* lineages, previously identified *Plasmodium* morphospecies, and 2 *Haemoproteus* lineages used as an outgroup in our analysis.**[a]

	Galapagos lineage A	*Galapagos lineage B*	*Galapagos lineage C*	*Galapagos lineage D*	*Plasmodium ashfordi*	*Plasmodium cathemerium*	*Plasmodium circumflexum*	*Plasmodium elongatum*	*Plasmodium gallinaceum*	*Plasmodium globularis*	*Plasmodium (haemamoeba)*	*Plasmodium juxtanucleare*	*Plasmodium lucens*	*Plasmodium megaglobularis*	*Plasmodium multivacuolais*	*Plasmodium parahexam-erium*	*Plasmodium relictum AY099041*	*Plasmodium relictum AY831748*	*Plasmodium relictum AF495571*	*Haemoproteus KC867678 OR*	*Haemoproteus KC867679 NE*
Galapagos lineage A	0.300†	26.3	28.6	37.6	29.2	25.1	20.6	25.2	25.5	35.1	24.3	28.4	28.5	29.6	33.1	29.2	25.4	25.4	26.5	41.3	39.1
Galapagos lineage B	5.99	0.200†	32.2	34.2	32.1	5.3	18.7	24.4	19.6	34.7	14.2	34.7	24.8	14.2	32.5	35.3	15.1	13.4	14.1	40.8	42.3
Galapagos lineage C	6.55	7.41		37.0	28.0	30.0	29.0	35.0	43.0	19.0	33.0	28.0	35.0	37.0	27.0	18.0	34.0	32.0	31.0	44.0	48.0
Galapagos lineage D	8.74	7.90	8.59		40.0	33.0	33.0	38.0	39.0	42.0	37.0	45.0	44.0	37.0	46.0	42.0	38.0	36.0	35.0	47.0	47.0
Plasmodium. Ashfordi	6.65	7.37	6.41	9.33		31.0	29.0	31.0	39.0	34.0	31.0	41.0	37.0	33.0	38.0	37.0	32.0	32.0	33.0	33.0	54.0
Plasmodium Cathemerium	5.69	1.11	6.89	7.61	7.13		15.0	26.0	21.0	32.0	14.0	32.0	24.0	15.0	33.0	35.0	15.0	13.0	14.0	41.0	43.0
Plasmodium Circumflexum	4.52	4.06	6.65	7.61	6.65	3.36		26.0	23.0	33.0	18.0	34.0	27.0	20.0	33.0	31.0	19.0	19.0	18.0	40.0	40.0
Plasmodium Elongatum	5.69	5.46	5.46	8.83	7.13	5.93	5.93		29.0	38.0	25.0	36.0	32.0	30.0	37.0	37.0	26.0	28.0	29.0	26.0	47.0
Plasmodium Gallinaceum	5.69	4.29	10.08	9.08	9.08	4.75	5.22	6.65		46.0	25.0	39.0	31.0	26.0	40.0	42.0	26.0	26.0	27.0	43.0	45.0
Plasmodium Globularis	8.10	7.85	4.29	9.83	7.85	7.37	7.61	8.83	10.83		34.0	25.0	36.0	38.0	22.0	19.0	35.0	35.0	34.0	49.0	53.0
Plasmodium (haemamoeba)	5.46	3.14	7.61	8.59	7.13	3.14	4.06	5.69	5.69	7.85		33.0	24.0	18.0	36.0	36.0	3.0	9.0	10.0	42.0	44.0
Plasmodium Juxtanucleare	6.41	7.85	6.41	10.58	9.58	7.37	7.85	8.34	9.08	5.69	7.61		34.0	37.0	27.0	27.0	34.0	34.0	35.0	50.0	55.0
Plasmodium Lucens	6.41	5.46	8.10	10.33	8.59	5.46	6.17	7.37	7.13	8.34	5.46	7.85		26.0	39.0	35.0	25.0	24.0	25.0	53.0	50.0
Plasmodium Megaglobularis	6.65	3.14	8.59	8.59	7.61	7.37	4.52	6.89	5.93	8.83	8.83	8.59	5.93		35.0	38.0	19.0	15.0	16.0	46.0	45.0
Plasmodium Multivacuolaris	7.61	7.37	6.17	10.83	8.83	7.61	7.61	8.59	9.33	4.99	8.34	6.17	9.08	8.10		24.0	37.0	35.0	36.0	46.0	48.0
Plasmodium parahexamerium	6.65	8.10	4.06	9.83	8.51	8.10	7.13	8.59	9.83	4.29	8.34	6.17	8.08	8.83	5.46		37.0	35.0	34.0	45.0	51.0
Plasmodium relictum AY099041	5.69	3.36	7.85	8.83	7.37	3.36	4.29	5.93	5.93	8.10	0.66	7.85	5.69	4.29	8.59	8.59		10.0	11.0	39.0	41.0
Plasmodium relictum AY831748	5.69	2.91	7.37	8.34	7.37	2.91	4.29	6.41	5.93	8.10	2.00	7.85	5.46	3.36	8.10	8.10	0.66		1.0	42.0	43.0
Plasmodium relictum AF495571	5.93	3.14	7.13	8.10	7.67	3.14	4.06	6.65	6.17	7.85	2.23	8.10	5.69	3.59	8.34	7.85	2.45	0.22		43.0	44.0
Haemoproteus KC867678 OR	9.58	9.33	10.33	11.09	11.6	9.58	9.33	10.58	10.08	11.60	9.83	11.85	12.63	10.83	10.83	10.58	9.08	9.83	10.08		23.0
Haemoproteus KC867679 NE	9.08	9.83	11.34	11.09	12.90	10.08	9.33	11.09	10.58	12.63	10.33	13.15	11.85	10.58	11.34	12.11	9.58	10.1	10.33	5.22	

[a] *See Fig. 3 for GenBank accession numbers.*

well-supported, monophyletic groups, with support for a sister relationship for lineage B with a group including *Plasmodium cathemerium* (Fig. 3). No genetic variation was found in lineage C (3 Galapagos sequences); however, both lineages A and B showed small amounts of within-lineage variation (0.3% or 1.4 base pairs, 0.2% or 0.9 base pairs average sequence divergence, respectively) (Table 3).

Lineage A, which was first described only from Galapagos Penguins (Levin et al. 2009), infected more recently sampled penguins ($n = 15$), Yellow Warblers ($n = 8$), and one Medium Ground Finch. This lineage was detected in all years for which we had samples (2003–2009), mostly on Isabela (around Puerto Villamil) and Fernandina. Lineage A was also detected in passerines in Santa Cruz in 2005 and 2009 (Fig. 2). The sister relationship of lineage A remains unresolved.

Lineage B was detected in 2 Yellow Warblers, one captured in 2004 on Isabela and the other in 2004 from Fernandina. Lineage B matched perfectly to *Plasmodium* sequences recovered from 3 Vermont Bobolinks (Fig. 3).

Lineage C occurred in 3 Small Ground Finches sampled in 2008 on Floreana and matched sequences recovered from 9 Bobolink samples from Nebraska. A sister relationship could not be determined for lineage C; however, this lineage was in a clade containing several described morphospecies: *Plasmodium juxtanucleare*, *Plasmodium multivacuolaris*, *Plasmodium globularis*, and *Plasmodium parahexamerium*.

Lineage D occurred on a long branch with unresolved placement within *Plasmodium*. This lineage occurred in 1 Small Ground Finch from Marchena sampled in 2004. Lineages B and C matched several lineages in GenBank. Lineage A matched 1 accession from *Aedes taeniorhynchus* sampled from Socorro Island, Mexico (HQ853668) (Carlson et al. 2011), and lineage D did not match any sequence in the database. Reamplification of positive samples varied greatly from an inability to reamplify to 77.8% repeatability in others. Despite the high variance in repeatability, there were no indications of contamination and Galapagos and non-Galapagos birds were never tested in the same reactions.

Discussion

We found 3 additional avian *Plasmodium* lineages infecting Galapagos birds beyond the one described in Levin et al. (2009). Infections were clustered on islands that had fresh water, which is important for the development of some mosquito vectors. The newly described lineages (B, C, and D) appeared in only 1–3 individuals at 1 or 2 sites in a single year, a finding that suggests poor or a lack of establishment on the archipelago. North American breeding Bobolinks, which regularly migrate through Galapagos, revealed lineage matches between *Plasmodium* spp. found in Bobolinks with Galapagos lineages B and C. Although we cannot conclusively say that Bobolinks are responsible for introducing 2 of the 4 known *Plasmodium* lineages to Galapagos, our approach and findings emphasize that conservation biologists should consider all possible routes by which parasites and pathogens might arrive, even in isolated systems such as the Galapagos Archipelago.

In contrast to the lack of evidence for establishment of lineages B, C, and D in Galapagos, lineage A infections occurred in more than one species at multiple locations and across multiple years, which suggests lineage A is established and is being transmitted regularly in the archipelago. Although none of the species we tested appear to be competent hosts for lineage A, this parasite may be transmitted via mosquito vector from the currently unknown reservoir host in which gametocytes develop to these alternative hosts and DNA from early stages of parasite development (like those shown in Fig. 1) or sporozoites from an infected mosquito bite (Valkiunas et al. 2009) may be amplified by PCR.

Haemosporidian parasites are thought to be similarly detectable by blood smear microscopy and PCR during all stages of development in the blood (Valkiunas et al. 2008). However, a lack of gametocytes in birds testing positive for haemosporidian parasites by PCR has been reported in captive birds (Ferrell et al. 2007; Donovan et al. 2008; Olias et al. 2011). One potential explanation for this discrepancy is abortive parasite development, caused by incompatibilities between host and parasite (Valkiunas 2011). This could occur when a parasite is introduced to a new host in which it is not adapted to complete its life cycle or when spillover from the definitive host occurs due to indiscriminate biting by the vector (Ferrell et al. 2007; Olias et al. 2011).

Blood stages of haemosporidians may never develop if sporozoites inoculated by the vector into the blood are unable to invade host tissues, tissue merogony ensues but infections result in host mortality before maturation of tissue stages, or the host survives tissue merogony and produces merozoites but the parasite development in the erythrocytic stages is severely impeded. Such cases of abortive development have been reported in avian haemosporidian parasites but remain insufficiently investigated in wildlife (e.g., Ferrell et al. 2007; Olias et al. 2011; Valkiunas 2011). Sporozoites or DNA from extracellular meront remnants or a few viable posttissue merogony intracellular parasites in the blood (Fig. 1) may remain in circulation and be a template for DNA amplification by PCR. But these stages are very difficult to detect in blood smears due to light parasitemia and are easily overlooked. Therefore, PCR detection of parasite DNA in blood samples does not confirm that the parasite is completing development and producing gametocytes in the PCR-positive host.

Abortive development of haemosporidian parasites has been described in captive bird populations, when a host species is brought to a new location where it is exposed to local parasites and vectors (Ferrell et al. 2007; Donovan et al. 2008; Olias et al. 2011). In the case of Galapagos *Plasmodium* spp., several key players in this system may be considered introduced. There are 3 mosquito species in Galapagos: a native, brackish-water mosquito (*Aedes taeniorhynchus*), which is estimated to have colonized the archipelago 200,000 years ago (Bataille et al. 2009), an introduced mosquito (*Culex quinquefaciatus*), known to be a competent *Plasmodium* vector elsewhere and well established by the early 2000s (Whiteman et al. 2005), and *Aedes aegypti*, which was introduced in the 1990s and is thought to be less ornithophilic than the other 2 mosquito species (Bataille et al. 2009). It is also possible that the unknown avian species acting as the reservoir for lineage A in the islands is introduced. There are 2 nondomesticated introduced bird species in Galapagos, Smooth-Billed Ani (*Crotophaga ani*) and Cattle Egret (*Bubulcus ibis*). *Plasmodium* spp. have been reported in Cattle Egrets in Central and South America (Bennett et al. 1982; Bishop & Bennett 1992), but lineages of the parasites remain unidentified. The recent ancestors of these introduced birds were likely in contact with *Plasmodium* parasites transmitted on the continent, and they may be suitable hosts for lineage A, which would allow the parasite to complete its life cycle, infect a local competent vector, and be transmitted to endemic bird species that are apparent dead-end hosts for the parasite. This possibility is currently under investigation.

Our results can be used to describe *Plasmodium* parasite transmission zones within the archipelago with greater precision. Previously, the only evidence for geographic location of transmission was location of infected penguins, which move frequently (Nims et al. 2008). Although there is evidence of some movement of Yellow Warblers (Chavez et al. 2012), finches (Petren et al. 2005), and, to a lesser extent, mockingbirds (Hoeck et al. 2010) throughout the archipelago, a concentration of multiple individuals infected with the same lineage at one site is evidence for local transmission.

The majority of lineage A sequences were recovered from birds sampled on southern Isabela near Puerto Villamil. Lineage A was also detected in 2 individuals on Santa Cruz in separate years. Both of these sites are near permanent human populations and therefore near fresh water, which is required by 2 of the potential vectors for development. Lineage C was detected in 3 finches on Floreana (another island with fresh water), sampled within 2 days of one another and nowhere else in the archipelago. This is one case where we found a 100% match with *Plasmodium* parasite sequence from North American breeding Bobolinks. Similarly, lineage B, found in 2 Yellow Warblers at different sites (Fernandina and Isabela), matched a *Plasmodium* sequence amplified from Bobolink samples. Both lineages were found at 1 site or 2 proximate sites and only once. Both of these parasites could be from migratory Bobolinks arriving in Galapagos and infecting local vectors that transmit the parasite to local birds. These lineages may not have established due to very small numbers of infected individuals or to possible incompatibilities among host, parasite, and vector.

Migratory birds can be instrumental in expansion of parasite populations (e.g., Ogden et al. 2008; Cornuault et al. 2012; Jenkins et al. 2012). In Galapagos there is evidence for multiple colonizations by *Haemoproteus* parasites infecting doves (Santiago-Alarcon et al. 2010); however, some of these lineages may have been introduced when Rock Doves (*Columba livia*) were purposefully introduced to the archipelago (P. Parker et al., unpublished data). *Plasmodium* spp. have been reported in Bobolinks at several sites both in North and South America (Bennett et al. 1982). Like some widespread, migratory passerine birds (Black-throated Blue Warblers [*Setophaga caerulescens*] [Fallon et al. 2006]) but not others (Common Yellowthroat [*Geothlypis trichas*] [Pagenkopp et al. 2008]), Bobolinks show no geographic partitioning of *Plasmodium* lineages, so we could not deduce which populations may be stopping in Galapagos and leaving parasites behind. On the basis of sequences in GenBank, lineage B has several matches from around the world (e.g., Uruguay: DQ838998 [Beadell et al. 2006]; New Zealand: HM579783 [Cloutier et al. 2011]; United States: DQ659548 [Beadell et al. 2006] and with several from Central America, e.g., JN819349 and JN819336 [H. Archer et al. unpublished data], GQ395688 [Outlaw & Ricklefs 2010]). Lineage C has 2 identical matches in GenBank, both from North American locations (JN792148 [Dodge et al. 2013] and AY172846 [Schrenzel et al. 2003]).

Oceanic archipelagos such as the Galapagos Islands are typically considered isolated, with low species diversity due to infrequent colonization events. Human activity, especially increased tourism, has a large effect on probability of introductions of invasive plants and insects (Gardener & Grenier 2011). However, the possibility of natural colonization, especially of parasites transported by migratory birds, should not be underestimated. Two of 4 distinct *Plasmodium* lineages detected in Galapagos endemic birds matched sequences amplified from North American breeding Bobolinks. Although this is not confirmation that Bobolinks are responsible for introducing these lineages, the evidence points to higher potential exposure to avian pathogens than one may have thought. Future work should focus on recently introduced birds to continue the search for the reservoirs of *Plasmodium* parasites in Galapagos, sampling of migratory shorebirds known to pass through Galapagos, and if possible, the sampling of Bobolinks in Galapagos from October through December, when regular sightings have been

reported (Pettingill 1983). Additionally, practical measures, such as limiting standing fresh water necessary for *C. quinquefaciatus* development, may reduce the likelihood of transmission of haemosporidian parasites arriving with migrants.

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