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Colonization History and Origin of the Galapagos Flycatcher (*Myiarchus magnirostris*) and its parasites

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COLONIZATION HISTORY AND ORIGIN OF THE GALÁPAGOS FLYCATCHER (*MYIARCHUS MAGNIROSTRIS*) AND ITS PARASITES

by

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Dissertation Abstract

The Galápagos flycatcher (*Myiarchus magnirostris*) is an endemic species to the Galápagos Islands, Ecuador, and is among the least studied Galápagos terrestrial birds. In this work I unveiled the origin and colonization history of the Galápagos flycatcher, and also the origin of the parasites that are currently found in/on this bird species. To determine the origin of the Galápagos flycatcher, I rebuilt the phylogeny of the *Myiarchus* genus using cytb and ND2, and applied a Bayesian approach to estimate its colonization time. I discovered that the closest living relative of the Galápagos flycatcher is *Myiarchus tyrannulus* (Brown-crested flycatcher) from Central and North America, and these two sister groups diverged approximately 850,000 years ago. To better understand the Galápagos flycatcher evolution in the Galápagos Archipelago, I used seven microsatellites and morphological characters to compare populations from seven islands. Correlation between genetic diversity and island size pointed to drift as an important diversification force. In general, morphological distances across islands were not correlated with pairwise genetic distances, and local adaptation through natural selection may possibly have contributed to that, but drift and phenotypic plasticity could not be excluded as explanations.

To investigate the origin of the Galápagos flycatcher parasites I studied blood parasites, lice, and mites from Galápagos flycatchers (n = 254) and from *M. tyrannulus* (n = 74) in Costa Rica. We found that different parasite species from the Galápagos flycatchers have different origins: five parasite species colonized the Galápagos Islands with the Galápagos flycatchers' ancestors (two louse species and

three mite species), and two parasite species were acquired from the native bird community after the Galápagos flycatchers ancestors arrived to Galápagos (*Haemoproteus* blood parasite and *Brueelia* louse). To investigate why some parasites found on *M. tyrannulus* (*Plasmodium* blood parasite and *Philopterus* louse) did not colonize Galápagos, I looked at immune responses of *M. tyrannulus* from Costa Rica to their parasites. I found no evidence that these parasites are affecting the health of *M. tyrannulus* more negatively than the other parasites and in a manner that would hinder their ability to colonize Galápagos.

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Chapter 1

Understanding the colonization history of the Galápagos flycatcher (*Myiarchus magnirostris*)

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Abstract:

The Galápagos archipelago has never been connected to any continental land masses, so it is of interest to know the colonization and diversification history of its endemic species. We analyzed the phylogenetic placement of the endemic Galápagos flycatcher, *M. magnirostris*, within *Myiarchus* by using the genes ND2 and cytb (1970bp) to compare 16 of the 22 species that comprise this genus. We also analyzed variability in cytb sequences from 154 *M. magnirostris* individuals captured on seven Galápagos islands. Our phylogenetic analyses recovered the two main *Myiarchus* clades that had been described by previous genetic, morphological, and vocal analyses. *M. magnirostris* is monophyletic and its closest living relative is *M. tyrannulus* from Mexico and Central America. The average age for the split node between these two groups was approximately 850,000 years (95% C.I. 630,735-1,087,557). *M. tyrannulus*, *M. nugator*, *M. nuttingi*, *M. sagrae*, and *M. stolidus* are not monophyletic species. Within *M. magnirostris* itself, we found low nucleotide and haplotype diversities (π =0.0009 and h=0.4913, respectively) and a high genetic

structure among populations. We also detected a star-shaped haplotype network and significantly negative values for Tajima's D and Fu's Fs for this species. Our results suggest that *M. magnirostris* originated from a single colonization event and had a recent population expansion in the Galápagos archipelago.

Introduction

Studies of island species, mostly birds, have contributed important insights to the growth of evolutionary science (Grant, 2001), as islands usually contain relatively simple ecosystems in which the effects of different evolutionary processes can be isolated. The evolution of a recently established population on an island is affected by the founder event itself, but genetic drift shapes the diversity and divergence of island populations over time: island species normally present lower genetic variability and higher differentiation among populations than their closely related species on the mainland (Clegg, 2010; e.g. Bollmer et al., 2006). The low genetic diversity and high divergence relative to the ancestral population are counterbalanced by immigration, which brings new alleles into the populations and homogenizes the diversity across populations. Hence the mobility of the species and the geographic distance from the ancestral population to the colonized island influence differentiation rates of colonizing lineages.

The Galápagos Islands have a volcanic origin and are isolated by approximately 1000 kilometers of ocean waters from the nearest mainland in Ecuador (Cox, 1983; Geist, 1996; Jackson, 1993). Thus they present an interesting context

within which to pose questions about the colonization and establishment of species. The arrival of terrestrial vertebrates, including perching birds (passerines), is especially intriguing, as most of the species that naturally colonized the islands are not able to disperse long distances over the sea (Jackson, 1993).

Galápagos flycatchers, *Myiarchus magnirostris* (Gould) (Passeriformes: Suboscines: Tyrannidae) are endemic to the Galápagos, where they inhabit a variety of habitats and altitudes on all main islands except Darwin, Wolf, and Genovesa (Jackson, 1993, Lanyon, 1978). In contrast to other species such as the Galápagos mockingbirds (Darwin, 1845), Darwin's finches (Grant and Grant 2008), and Galápagos hawks (Bollmer et al., 2003, 2005, 2006), these flycatchers show no conspicuous morphological variation within the archipelago (Lanyon, 1978; pers. observation). They are, however, among the most understudied Galápagos terrestrial bird species.

Myiarchus comprises 22 species distributed from southern North America to southern South America, most of which have very similar plumage and vocal repertoires (Lanyon, 1967, 1978). Joseph et al. (2004) proposed a phylogeny for 19 of these species, and found that *Myiarchus* is monophyletic, and that 18 of the 19 species analyzed are divided into two main clades (Clade I and Clade II). The three species that were not included in the phylogeny were *M. magnirostris* from Galápagos, *M. nuttingi* from Central America, and *M. apicalis* from Colombia. Nevertheless, based on the vocal and morphological description by Lanyon (1978), Joseph et al. (2004) predicted that *M. magnirostris* and *M. nuttingi* would belong to Clade I, and *M. apicalis* to Clade II.

The colonization history of the Galápagos flycatchers is unknown; in order to describe it we need to determine their closest continental relatives and to understand their population structure and dynamics. Hence, we were interested in identifying the sister species of the Galápagos flycatchers (*M. magnirostris*), in inferring the date that *Myiarchus* flycatchers first colonized the Galápagos Islands, and knowing from which geographic region(s) they originated. We also wanted to study the relationships between Galápagos flycatcher populations from different islands. This information is essential to assess the evolutionary processes, like drift, local adaptation, and migration, that underlie the speciation of *M. magnirostris* within a recognized temporal and geographical scale.

Studies have concluded that several vertebrates native to Galápagos came via single colonization events from source populations (Parent et al., 2008; e.g. finches - Burns et al., 2002; Sato et al., 2001b; tortoises - Caccone et al., 2002; mockingbirds - Arbogast et al., 2006; hawks - Bollmer et al., 2006; penguins - Baker et al., 2006; cormorants - Kennedy et al., 2009; frigatebirds - Hailer et al., 2010). These examples point to a history of limited successful colonizations and reinforce the characterization of Galápagos as extremely isolated. Therefore, we hypothesize that Galápagos was successfully colonized only once by *Myiarchus* flycatchers, after which the population grew and expanded its distribution through the archipelago. As a result, we expect that *M. magnirostris* is a monophyletic species with detectable evidence of demographic expansion.

In order to determine the closest phylogenetic lineage to *Myiarchus magnirostris*, we explored one nuclear and three mitochondrial regions to reconstruct

the phylogeny of the *Myiarchus* species in Clade I from Joseph et al. (2004). We also used mitochondrial cytochrome b sequences from *M. magnirostris* individuals captured on different islands to describe their genetic diversity and population structure.

Materials and Methods

Sampling and DNA extraction

We reconstructed a partial phylogeny from the genus *Myiarchus*, including all twelve species from "Clade I" to which *M. magnirostris* belong and four species from "Clade II" (Joseph et al., 2004; see Table 1 for included species). We used blood samples from *M. magnirostris* and *M. tyrannulus*; samples from other species were obtained from the DNA collection of Dr. Robert Ricklefs at University of Missouri - St. Louis or through tissue loans from the University of Kansas Natural History Museum (KUNHM). *M. swainsoni* sequences were extracted from GenBank. Additionally, we used sequences from *Tyrannus melancholicus* and *Empidonax minimus* from GenBank as outgroups (accession numbers are in Table 1). We chose these outgroups because they were the species most closely related to *Myiarchus* (see Tello et al. 2009) that had overlapping gene sequences available on GenBank.

Because *M. tyrannulus* had been previously described as the closest relative of the Galápagos flycatcher (Joseph et. al, 2004, Lanyon, 1978), samples from this species represented different recognized subspecies (Fitzpatrick, 2004; Lanyon, 1960, 1978): *M. t. cooperi* from eastern Mexico (n=4); *M. t. brachyurus* from Costa Rica (n=4); *M. t. cooperi* X *brachyurus* from El Salvador (n=2); and *M. t. tyrannulus* from

Venezuela (n=4), Guyana (n=1), Brazil (n=2), and Paraguay (n=2). We used samples from five *M. magnirostris* (Galápagos flycatchers) collected on different islands and between one and six individuals from the other species.

To study the population genetics of *M. magnirostris* we used samples from154 individuals captured during July and August, from 2007 to 2009, on seven islands from the Galápagos Archipelago: Española, Floreana, Isabela, San Cristóbal, Santa Cruz, Santa Fé, and Santiago (Fig. 1A). Island sample sizes varied from 11 to 29 individuals (Table 2). Blood samples were collected from the brachial vein with heparinized capillary tubes and stored in lysis buffer (Longmire et al., 1988) until DNA extraction. All Galápagos flycatcher samples (blood and DNA) are stored in the Parker lab, at the University of Missouri – St. Louis.

Total genomic DNA was extracted from blood or tissue samples using a modified phenol-chloroform protocol (Sambrook et al., 1989), with a final dialysis step in TAE for DNA ultra-purification. The dialysis step was used to increase the quality, purity, and yield of DNA, allowing it to be preserved for many years.

DNA amplification and sequencing

For inferring phylogenetic relationships among species of *Myiarchus*, we studied four DNA regions: subunits 8 and 6 of ATPase (ATPase 8_6), cytochrome b (cytb), and subunit 2 of NADH dehydrogenase (ND2) from the mitochondrial genome, and intron 7 from the nuclear gene beta-fibrinogen (BF7). For studying *M. magnirostris* populations, we used cytb sequences only.

For amplification of ATPase 8_6, 10 to 40 ng of genomic DNA were used in a 20µl reaction with 0.5 U of Biolase^{TM} Red DNA Polymerase (Bioline), 1X NH_4 Reaction Buffer (Bioline), 40 µM of each dNTP, 0.5 µM of each primer, and 1 mM of $MgCl_2$. Amplification programs started at 94°C for 2 min, followed by 36 cycles of 94°C for 45 sec, 60°C for 50 sec, 72°C for 45 sec, with a final extension step at 72°C for 5 min. For amplification of

cytb and ND2, 10 to 40 ng of genomic DNA were used in 15µl volume reactions with 0.35 U of Biolase^{TM} Red DNA Polymerase (Bioline), 1X NH_4 Reaction Buffer (Bioline), 25 µM of each dNTP, 0.3 µM of each primer, and 1 – 2.5 mM of $MgCl_2$. BF7 amplifications were also carried out in 15 µl volumes, but with 45 µM of each dNTP, 0.5 µM of each primer, and 1 mM of $MgCl_2$. Amplification cycling protocols consisted of an initial denaturation step at 94°C for 2 min, followed by 36 cycles of 94°C for 30 sec, specific annealing temperatures (Table 3) for 45 sec, 72°C for 2 min, and a final extension step at 72°C for 10 min. Negative control tubes, in which no template DNA was added, were used in all amplification runs. All primers and their annealing temperatures (Ta) are listed in Table 3.

Amplified DNA fragments were detected on a gel star©-stained 1.0% agarose gel in TBE. Single band PCR products were purified with Exonuclease and Antarctic Phosphatase (New England BioLabs Inc.): one unit of each enzyme was eluted into 2.6 µl of water and added to 10µl of amplicon, then incubated for 30 min at 37°C and 15 min at 60°C. Purified PCR products were cycle sequenced using Big DYE Terminator Kit (Applied Biosystems), according to manufacturer's instructions, with 35 cycles at 95°C for 25 s, 50°C for 15 s and 60°C for 4 min. Sequencing products

were cleaned using ethanol precipitation with NaOAc and NaOH, and run in an ABI 2000 automatic sequencer (Applied Biosystems). DNA fragments from all samples were sequenced in both directions using the amplification primers and also with internal primers previously published or designed for this study (Table 3). We designed the internal sequencing primers for ATPase 8_6 and cytb based on our first *M. magnirostris* sequences and on GenBank sequences from *Myiarchus* and other Tyrannidae species.

Construction of phylogenetic trees

We used SeqManII v. 4 (1989–1999, DNASTAR, Inc.) to analyze sequence traces and create contigs. Sequences were aligned using Clustal W with default parameters as implemented in MEGA v. 4.0 (Tamura et al., 2007) and for all mitochondrial sequences, we confirmed the absence of double peaks in the electropherograms, and the absence of insertions, deletions, or stop codons in the alignments. Sequence characteristics and divergence estimates were calculated in DnaSP v. 5.10 (Librado and Rozas, 2009) and MEGA v. 4.0 (Tamura et al., 2007); distances were based on the Tamura-Nei substitution model.

Using the sequences obtained from nuclear DNA (intron BF7), we calculated haplotype phases in DNAsp for each sample and used the different haplotypes to generate phylogenetic hypotheses. We ran a Maximum Parsimony tree in MEGA v. 4.0 and tested the robustness of its topology with 500 bootstrap replicates. We also constructed a Maximum Likelihood best tree using GARLI v. 1.0 (Zwickl, 2006).

Maximum Parsimony (MP) and Maximum Likelihood (ML) analyses were

conducted separately for the mitochondrial genes cytb and ND2 and also using concatenated sequences from both genes (three datasets). MP tree searches were performed in Paup v. 4.0b (Swofford, 1998) using a heuristic search with tree-bisection-reconnection (TBR) branch swapping and 1000 random stepwise addition of samples. MP reconstructions were tested with 500 bootstrap replications.

The best fitted evolutionary model was chosen for each mitochondrial dataset through jmodeltest (Posada, 2008) applying the corrected Akaike Information Criterion (AICc). We used AICc because our sample size, which approximates the number of characters in the alignment, was small compared to the number of parameters K (Posada, 2009). Maximum Likelihood trees were computed in GARLI v. 1.0 (Zwickl, 2006). We started each analysis with a random tree, fixed the nucleotide substitution model (GTR) and the among-site rate variation parameters (proportion of invariable sites, alpha for gamma distribution, and number of rate categories), but let GARLI estimate base frequencies, and used default values for Genetic Algorithm and automatic run termination. The robustness of ML phylogeny reconstructions was tested with 100 bootstraps using RAxML v. 7.2.6 (Stamatakis et al., 2005) through CIPRES Science Gateway v. 3.0 (Miller et al., 2009; http://www.phylo.org/sub_sections/portal/). For both MP and ML we constructed consensus trees using Mesquite (Maddison and Maddison, 2010), applying the 50% majority consensus rule.

Bayesian inferences of phylogenetic relationships were conducted in MrBayes v. 3.1.2 (Ronquist and Huelsenbeck, 2003). Analyses were performed as two independent runs using MCMC searches with 10 million generations, each run with

four parallel chains (one cold and the three incrementally heated). GTR+I+G was used and other model parameters were estimated by the software. Trees were sampled every 100 generations, for a total of 100,000 trees per run; trees from the first 2.5 million generations were discarded (burn in of 25,000 trees).

Estimates for the arrival date of *Myiarchus* in Galápagos

Because there are no fossil records for Tyrannidae, our date estimates were based on molecular evolution rates calculated for other bird taxa. First, assuming that sequence evolution has happened in an "approximately clock-like manner" for most bird extant lineages, we applied the substitution rate of 2.07% per million years (Weir and Schluter, 2008) to calculate the time that the *M. magnirostris* lineage split from its continental sister lineage based on the net DNA divergence between these two lineages. We used Tamura-Nei distance to compute this divergence.

However, a maximum likelihood ratio test in Mega v. 5 (Tamura et al., 2011) rejected the null hypothesis of equal evolutionary rates among lineages, for both cytb and ND2 sequence datasets. Therefore, we applied a Bayesian relaxed uncorrelated clock, as implemented in BEAST v. 1.6.1 (Drummond and Rambaut, 2007) to estimate the age of the split node between these two *Myiarchus* lineages. We allowed the substitution rate to vary following a normal distribution, using 2.07% per million years as the mean rate, and its associated standard deviation (± 0.20%) as proposed by Weir and Schluter (2008) for cytb sequences. We used this rate for both genes, but also performed simulations letting BEAST estimate the substitution rate for ND2 only and for both genes, running analyses for each gene separately and also concatenated.

For this we used the evolutionary models found through jmodeltest applying the AICc for each dataset and assigned a prior of Yule lineage birth speciation process. BEAST analyses were run for up to 300 million generations and chain convergence was checked in TRACER v. 1.5.

The resulting standard deviations for the molecular clock using Bayesian analyses were never close to 1, so the hypothesis of evolutionary rate homogeneity among lineages was not rejected by BEAST. Thus we also ran an analysis with the concatenated dataset in BEAST using a strict molecular clock with the rate of 2.07% per million years (Weir and Schluter, 2008) for 10 million generations.

Population analyses

We calculated the haplotypes of *M. magnirostris* with DnaSP v. 5.10 (Librado and Rozas, 2009) and used Network v. 4.5 (fluxus-engineering.com; Bandelt et al., 1999) to construct a median joining network. We treated each island as a different population and used Arlequin v. 3.11 (Excoffier et al., 2005) and DnaSP to calculate multiple genetic diversity and differentiation indices for populations. We applied the hierarchical Analysis of Molecular Variance (AMOVA; Excoffier et al., 1992) to test the level of genetic differentiation among populations based on Φ_{st} values. Φ_{st} is an analogue of the Wright's fixation index (F_{st}) that takes into account the number of mutations between molecular haplotypes (Excoffier et al., 2005). We also calculated Fu's Fs and Tajima's D using Arlequin for each population separately and for all *M. magnirostris* populations together. A deviation from neutrality indicated by significant negative Fu's Fs and Tajima's D values suggests population demographic

expansion. According to our results from jmodeltest (Posada, 2008) using the AICc criteria, we applied the substitution model HKY (Hasegawa-Kishino-Yano; Hasegawa et al., 1985) where possible or the Tamura (1992) substitution model when using softwares in which the option HKY was not available.

Results

Myiarchus phylogeny

Sequence characteristics

In order to find the closest phylogenetic lineage to *M. magnirostris* we attempted to produce sequences from the mitochondrial genes ATPase 8_6 for this species and to compare them with the ATPase 8_6 sequences published by Joseph et al. (2004) for the other *Myiarchus* species. Our sequences from *M. magnirostris* and *M. nugator*, however, were evidently not from the mitochondrial genes we sought, but probably from nuclear DNA, because: (1) the sequence traces (electropherograms) presented several positions with good quality double peaks; (2) *M. magnirostris* and *M. nugator* sequences presented deletions and stop codons in the 842bp sequence alignment we generated including *Myiarchus* spp. sequences from GenBank; (3) in the phylogenetic trees produced using this alignment all *M. magnirostris* and *M. nugator* samples formed a clade sister to "Clade I" (Joseph et al., 2004), but never imbedded within "Clade I". This outcome can be observed when part of the mitochondrial DNA is incorporated into the nucleus (*numts* - Sorenson and Quinn, 1998). The amplification of *numts* instead of the target mitochondrial DNA has been documented as a common problem in bird studies, especially when working

with DNA extracted from blood samples (Sorenson and Quinn, 1998).

We generated an alignment of 791 bp for sequences of the nuclear region BF7 obtained from 25 samples that represented 11 species from Clade I. BF7 sequences are deposited on GenBank under the accession numbers JN835378 to JN835402. The haplotype phases for these sequences represented 21 different haplotypes. Total nucleotide diversity considering these haplotypes was very low (π =0.0065 using Tamura Nei distances), and pairwise differences between haplotypes varied from 0.13% to 1.54%. Most species lacked autopomorphies and the phylogenetic trees showed no support for the relationships among *Myiarchus* species. Therefore no more sequences from this DNA region were pursued and those obtained were not included in further phylogenetic analyses.

Because our amplification and sequencing results from ATPase 8_6 were unreliable and the BF7 intron was uninformative, we only used cytb and ND2 to study the *Myiarchus* species relationships. For these two mitochondrial genes, we obtained sequences from all species, generating alignments of 975bp for cytb from 56 samples (with 209 parsimoniously informative positions), 1035bp for ND2 (with 253 parsimoniously informative positions) from 53 samples, and 2010bp for concatenated genes (with 462 parsimoniously informative positions) from the total of 61 samples. Insertions, deletions, or stop codons were not found in these alignments. Among the *Myiarchus* samples only, total nucleotide diversities using Tamura-Nei (TN) model were 0.04763 for 54 cytb sequences, 0.04844 for 51 ND2 sequences, and 0.04475 for 46 concatenated sequences.

The highest interspecific TN distances were between *M. panamensis* and *M.*

tyrannulus from Mexico (11.06% with cytb only), between *M. panamensis* and *M. oberi* (10.76% with ND2 only), and between *M. panamensis* and *M. tyrannulus* from Venezuela (10.53% with concatenated genes). The lowest pairwise distances were between *M. nugator* and *M. tyrannulus* from Venezuela (0.10% with cytb, 0.19 with ND2, 0.15% with both genes) and between *M. sagrae* and *M. stolidus* (0.10% for cytb and both genes, and 0% for ND2).

Species phylogenetic relationships

Results from Maximum Parsimony, Maximum Likelihood, and Bayesian analyses were consistent, as most of the clades with high support values were the same in all phylogenetic hypotheses obtained (Figs. 2 and 3). As previously described (Joseph et al., 2004), we recovered two well supported main clades within *Myiarchus*: the 12 species expected to belong to Clade I were grouped together and the other four *Myiarchus* species (*M. barbirostris*, *M. panamensis*, *M. swainsoni*, and *M. tuberculifer*) formed Clade II (Table 1, Fig. 2). Mean TN distance within Clade I was 0.03401 and within Clade II was 0.03814, and mean divergence between them was 0.08802 +- 0.00509. Tamura-Nei divergence values between species pairs within Clade I varied from a maximum of 7.25% for the pair *M. validus-M. nuttingi* and a minimum of 0.2% for the pairs *M. nugator-M. tyrannulus* and *M. sagrae-M. stolidus*.

M. magnirostris is represented as a monophyletic lineage, sister to a group formed by *M. tyrannulus* samples from Central America and Mexico (hereafter MtyCAM). *M. tyrannulus* from South America (hereafter MtySA) formed another group together with *M. nugator*, the Grenada flycatcher, and this *M.nugator*-MtySA

group is sister to the group that is formed by *M. magnirostris*-MtyCAM (Figs. 2 and 3). *M. nugator* samples formed a clade but with poor support. In fact, the smallest genetic distances calculated between species pairs were detected between *M. nugator* and MtyrSA. In a similar way, the small genetic distances found between *M. stolidus* and *M. sagrae* are reflected in the fact that these two species are not sorted into separate lineages in our phylogenetic trees. In addition, we found that *M. nuttingi* belongs to Clade I, but the samples from this species only formed a well-supported monophyletic group in Maximum Parsimony analyses.

Time estimates

The net genetic distance (TN) between *M. magnirostris* and MtyCAM using the concatenated dataset was estimated as 1.44%. This was computed using only the five *M. magnirostris* that were used in the phylogenetic analyses and the ten MtyCAM samples that formed its sister clade. Applying the 2.07% divergence rate per million years (Weir and Schlutter, 2008), we estimated that these two groups have been separated on average for 697,584 years, with a standard error of 132,850 years (564,734 – 830,434 years).

The estimates of average time for the *M. magnirostris*-MtyCAM node using the Bayesian relaxed clock approach implemented in BEAST were given as 836,000 years. However, we never achieved acceptable ESS (effective sample size) values (above 200) for the prior and posterior probabilities, even after 300 million generations, using all three datasets. Nevertheless, using the strict clock we obtained high ESS values for all the parameters, and the average age for the split node between

MtyCAM and *M. magnirostris* was estimated as 849,916 years, with a 95% confidence interval of 630,735 – 1,087,557 years, which encompasses the estimates derived from the DNA divergence between MtyCAM and *M. magnirostris* and from the relaxed clock Bayesian approach (Fig. 3).

M. magnirostris population genetics

We obtained an alignment with 907bp of the cytb gene from 154 samples distributed in seven islands/populations. We identified 12 haplotypes with 13 polymorphic sites, from which only one was parsimoniously informative and the other 12 were singletons. Total genetic diversity among all *M. magnirostris* and also within each population was very low, as indicated by nucleotide (π=0.00087) and haplotype (h=0.4913) diversity values in Table 2. The haplotype network (Fig. 1B) shows that one single DNA haplotype is the most common on all islands, and that nine haplotypes, not very divergent from this one, are rare and found on single islands.

The population with highest genetic diversity was Santa Cruz, followed by Isabela. On Española, only one haplotype was identified out of 18 samples, and this population presented the lowest genetic variation. The analysis of molecular variance (AMOVA) showed that there is high genetic structure among populations from different islands (Φ_{st} = 0.4434). Individual population Φ_{st} values represent their weight on the estimate of the global Φ_{st} from AMOVA and show that populations contributed evenly to the global Φ_{st} (Table 2). The populations from Floreana and Santa Cruz were the only ones significantly different from all the other populations,

but Floreana presented the highest significant pairwise Φ_{st} values. Pairwise Φ_{st} values for all population pairs are listed in Table 4.

Tajima's D and Fu's Fs neutrality tests obtained significant and highly negative values for *M. magnirostris* when considered as one single population, indicating that this species has experienced recent demographic expansion, as expected after a colonization event. When these tests were made for island populations separately, only Isabela presented significant negative values of Tajima's D and Fu's Fs, and San Cristóbal had a significant negative value for Fu's Fs only.

Discussion

Myiarchus magnirostris colonization event

The phylogenetic relationships among *Myiarchus* species we obtained here were consistent with the ones suggested by Joseph et al. (2004). Also, the finding that the closest living relatives of *M. magnirostris* are in *M. tyrannulus* is consistent with the conclusions from previous studies of *Myiarchus* (Joseph et al., 2004; Lanyon, 1978). Monophyly of *M. magnirostris* supports the null hypothesis that the Galápagos Islands were colonized only once by *Myiarchus* birds from a single geographic region. Despite the fact that Ecuador is the closest continental land to the Galápagos Islands, our results suggest that the ancestral population of *M. magnirostris* lived in southwestern Central America. In South America, *M. tyrannulus* occurs only to the east of the Andes and the species' distribution does not include Ecuador. A comparable pattern was described for the Galápagos mockingbirds, where their closest living relatives are currently found in North America, northern South America

and the Caribbean, rather than in Ecuador (Arbogast et al., 2006). Similarly, most of the species identified as the closest living relatives of the Galápagos finches inhabit the Caribbean islands (Burns et al., 2002).

The sister clade to *M. magnirostris* is a monophyletic group of *M. tyrannulus* samples that were collected within the ranges of *M. t. cooperi* (eastern Mexico) and *M. t. brachyurus* (Pacific slope of Central America). *Myiarchus tyrannulus* has an extensive distribution along the Americas and is a "partially migratory" species, but migration movements are not resolved (Fitzpatrick, 2004). Colonization of new areas is more likely to occur in species with large distribution areas and migratory capacity. *M. tyrannulus* populations from the northern hemisphere migrate to the southern part of their distribution ranges during winter. The colonization of Galápagos by *Myiarchus* flycatchers could reasonably have taken place when birds from Central America (MtyCAM) deviated from their migratory route, possibly pushed by the strong northeast trade winds.

Here we propose that the colonization event that initiated the speciation process of *M. magnirostris* in Galápagos happened less than a million years ago (Fig. 3). This estimate suggests that Galápagos flycatchers have inhabited the islands for a shorter time than both the mockingbirds, whose ancestors arrived between 1.6 to 5.5 million years ago (Arbogast et al., 2006), and the finches, which diverged from their continental ancestors around 2 to 3 million years ago (Grant, 1994; Sato et al., 2001a, b). This more recent colonization time for *M. magnirostris* might explain why these birds do not present conspicuous differences in morphology and vocalizations among island populations, in comparison to the remarkable diversification of the finches and

the mockingbirds on the islands. On the other hand, it was proposed that the Galápagos hawks' ancestors arrived on the archipelago much more recently (less than 300,000 years ago) and morphological and genetic differences among populations from different islands are already notable (Bollmer et al., 2003, 2005, 2006). Studies of morphological and vocal data to compare *M. magnirostris* populations from different islands have never been done. In fact not much attention has been paid to this endemic bird species since its taxonomic revision by Lanyon (1978), and further studies are necessary for a more comprehensive understanding of its speciation process.

M. magnirostris population genetics

For *M. magnirostris*, we found that the same DNA haplotype is most common on populations from all islands (except Floreana) and a few haplotypes very similar to this one are specific to each island (Fig. 1B). This haplotype frequency distribution represents the expected outcome for a species after colonization of a new environment followed by demographic and geographical expansion (Fu, 1997).

The oldest above-water islands from Galápagos, San Cristóbal, Española, and Santa Fé, are estimated to be approximately three million years old, and are located in the eastern part of the archipelago (Geist, 1996; White et al., 1993). Among the main islands, the westernmost Isabela and Fernandina rose out of the ocean less than 400,000 years ago (Geist, 1996; White et al., 1993). When the ancestors of *M. magnirostris* arrived in the Galápagos all the other main islands were already suitable for colonization. A more recent colonization by *M. magnirostris* from previously

colonized islands might explain why only the population from Isabela presented significantly negative values for both tests of recent population expansion (Tajima's D and Fu's Fs). On the other hand, the population from San Cristóbal also presented a significantly negative value of Fu's Fs, indicating that a recent population expansion could have also happened on one of the oldest islands.

Genetic diversity (π) within islands varied from 0 in Española to 0.0012 in Santa Cruz (Table 2), and was not correlated with island area (Spearman's rho = 0.571; p = 0.2) or the number of birds sampled on each island (Spearman's rho = 0.321; p = 0.5). Bird abundance was not systematically measured, but this species seemed to be very common on most of the islands visited, with the exception of Española and Santa Fé.

AMOVA detected strong genetic structure among populations (Φ_{st}=0.443), indicating a deficit of admixture between birds from different islands. This estimate, however, is not appropriate to characterize current gene flow among islands. Current gene flow could be elucidated by genetic markers with a faster evolutionary rate, such as microsatellites, which can reveal more recent demographic events. The populations from Floreana and Santa Cruz presented significant Φ_{st} values against all the other populations, but the high total Φ_{st} value does not seem to be biased by these populations, as the population Φ_{st} values show that each population represents approximately the same weight on the estimate of the total Φ_{st}.

Other considerations about the *Myiarchus* phylogeny

In our phylogeny, *M. nuttingi* was represented by two independent lineages,

one with samples from Costa Rica (id numbers CR6, CR13, and CR15), and another from specimens collected in San Salvador (KUNHM collection - id numbers 9314, 9281, and 9288; Figs. 2 and 3). Three subspecies are currently recognized for *M. nuttingi* (Lanyon, 1961), and the lineages found here might represent two of them, *M. n. flavidior* in El Salvador and *M. n. nuttingi* in northwestern Costa Rica, where both races co-occur (Lanyon, 1961). We did not find support for the monophyly of *M. nuttingi*, so taxonomic revision, delimitation of contact zones, and studies of genetic introgression between races of *M. nuttingi* would be important for the confirmation of their status as subspecies. Based on morphological and vocal characters, *M. nuttingi* has been considered closely related to *M. cinerascens* (see Lanyon, 1961), but we found that these two species are not sisters. Instead, the closest relative of *M. cinerascens* is *M. crinitus* from the southeastern US.

M. tyrannulus sequences formed a clade with those of *M. magnirostris* and *M. nugator*, showing that the species currently defined as *M. tyrannulus* is paraphyletic. Playback experiments made with *M. magnirostris* revealed that this species responded to the vocalizations of *M. tyrannulus* and *M. nugator*, but not to other *Myiarchus* species (Lanyon, 1978), confirming that the three species are closely related. In fact, *M. nugator* might represent such a recent colonization of St. Vincent and Grenada that its reciprocal monophyly was not confirmed in the phylogenies presented here and from Joseph et al (2004); it shares genetic lineages with *M. tyrannulus* populations from northern South America (Venezuela and Guyana).

M. sagrae and *M. stolidus* are not reciprocally monophyletic, even though they show no overlap in their distributions; the first is found in the Bahamas, Cuba,

and Grand Cayman Islands, and the second inhabits Jamaica and Hispaniola. This indicates that geographical (and consequently reproductive) isolation resulted in morphological and vocal differentiation faster than in genetic lineage sorting. It seems that differences in plumage and vocalizations among *Myiarchus* species are more easily detectable than differences in DNA molecules (also see Joseph et al., 2004).

Taxonomic revisions are not in the scope of this work, but we suggest that a revision of geographic races of *M. tyrannulus* and *M. nuttingi* is necessary for a more comprehensive classification that is consistent with these emerging patterns.

Conclusions

This work represents one more estimate for the arrival time of a different evolutionary lineage to the Galápagos Islands. The study of the colonization history of one more Galápagos species will help in the reconstruction of the Galápagos ecosystem history and evolution of species interactions, which *per se* affected their own speciation process. The estimate of time for the arrival of *M. magnirostris*' ancestors to the Galápagos, together with the identification of its sister clade, and also the first assessment of its population genetic structure proposed in this work, sets up the framework for understanding the speciation process of this species within a temporal and spatial context.

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Table 1 Samples included in the *Myiarchus* phylogeny showing the respective collection reference numbers available, the original sampling sites, and the accession numbers for the sequences used. Species are ordered by clade number (Joseph et al., 2004; Fig.2).

Species	Collection reference	Locality	Accession number	
			Cyt B	ND2
CLADE I				
Myiarchus antillarum	Ricklefs Lab - GF 103	Puerto Rico: Guanica Forest	JQ004294	JQ004347
Myiarchus antillarum	Ricklefs Lab - GF2 242	Puerto Rico: Guanica Forest	JQ004295	---
Myiarchus antillarum	Ricklefs Lab - UPR 36	Puerto Rico: UPR Agricultural Experiment Station, Lajas	JQ004296	---
Myiarchus cinerascens	KUNHM 11988	USA: Morton, Kansas	JQ004297	JQ004348
Myiarchus cinerascens	KUNHM 11990	USA: Morton, Kansas	JQ004298	JQ004349
Myiarchus crinitus	Ricklefs Lab - M 81	USA: Upper Delta Wildlife Management Area, Alabama	JQ004299	JQ004350
Myiarchus magnirostris	Parker Lab - ES1008	Ecuador: Santa Cruz, Galápagos	JQ004300	JQ004351
Myiarchus magnirostris	Parker Lab - ES1025	Ecuador: Santiago, Galápagos	JQ004301	JQ004352
Myiarchus magnirostris	Parker Lab - ES1049	Ecuador: Santa Fé, Galápagos	JQ004302	JQ004353
Myiarchus magnirostris	Parker Lab - ES1077	Ecuador: Floreana, Galápagos	JQ004303	JQ004354
Myiarchus magnirostris	Parker Lab - ES1123	Ecuador: Isabela, Galápagos	JQ004304	JQ004355
Myiarchus nugator	Ricklefs Lab - GD 122	Grenada, Lesser Antilles	JQ004305	JQ004356
Myiarchus nugator	Ricklefs Lab - GD 157	Grenada, Lesser Antilles	JQ004306	JQ004357
Myiarchus nugator	Ricklefs Lab - SV 82	St. Vincent, Lesser Antilles	JQ004307	JQ004358
Myiarchus nugator	Ricklefs Lab - SV 278	St. Vincent, Lesser Antilles	JQ004308	JQ004359
Myiarchus nuttingi	KUNHM 9281	El Salvador: Zacatecoluca, La Paz	---	JQ004360
Myiarchus nuttingi	KUNHM 9288	El Salvador: Zacatecoluca, La Paz	JQ004309	JQ004361
Myiarchus nuttingi	KUNHM 9314	El Salvador: Zacatecoluca, La Paz	---	JQ004362
Myiarchus nuttingi	Parker Lab - CR6	Costa Rica: Palo Verde, Guanacaste	---	JQ004363
Myiarchus nuttingi	Parker Lab - CR13	Costa Rica: Palo Verde, Guanacaste	---	JQ004364
Myiarchus nuttingi	Parker Lab - CR15	Costa Rica: Palo Verde, Guanacaste	---	JQ004365
Myiarchus oberi	Ricklefs Lab - SL 125	Santa Lucia, Lesser Antilles	JQ004310	JQ004366
Myiarchus sagrae	Ricklefs Lab - C 156	Grand Cayman Island	JQ004311	---
Myiarchus sagrae	Ricklefs Lab - C 228	Grand Cayman Island	JQ004312	JQ004367
Myiarchus sagrae	Ricklefs Lab - ELE-064	The Bahamas: Eleuthera	JQ004313	JQ004368
Myiarchus stolidus	Ricklefs Lab - DR2-240	Dominican Republic: Sierra de Bahoruco National Park	JQ004314	JQ004369
Myiarchus stolidus	Ricklefs Lab - DR2-252	Dominican Republic: Sierra de Bahoruco National Park	JQ004315	JQ004370
Myiarchus stolidus	Ricklefs Lab - J 173	Jamaica	JQ004316	JQ004371
Myiarchus tyrannulus	Ricklefs Lab - MEX 423	Mexico: Campeche, Yucatan Peninsula	JQ004317	JQ004372
Myiarchus tyrannulus	Ricklefs Lab - MEX 682	Mexico: Campeche, Yucatan Peninsula	JQ004318	JQ004373
Myiarchus tyrannulus	KUNHM 186	Paraguay: Concepción	JQ004319	JQ004374
Myiarchus tyrannulus	KUNHM 2094	Mexico: Campeche, Yucatan Peninsula	JQ004320	JQ004375
Myiarchus tyrannulus	KUNHM 2112	Mexico: Campeche, Yucatan Peninsula	JQ004321	JQ004376
Myiarchus tyrannulus	KUNHM 3063	Paraguay: Concepción	JQ004322	JQ004377
Myiarchus tyrannulus	KUNHM 5693	Guyana	JQ004323	JQ004378
Myiarchus tyrannulus	KUNHM 9511	El Salvador: Zacatecoluca, La Paz	JQ004324	JQ004379
Myiarchus tyrannulus	KUNHM 9512	El Salvador: Zacatecoluca, La Paz	JQ004325	JQ004380
Myiarchus tyrannulus	Ricklefs Lab - MYTY 04	Venezuela: Península de Araya, Sucre	JQ004326	JQ004381
Myiarchus tyrannulus	Ricklefs Lab - MYTY 12	Venezuela: Península de Paranaguá, Falcón	JQ004327	JQ004382
Myiarchus tyrannulus	Ricklefs Lab - MYTY 32	Venezuela: Península de Paranaguá, Falcón	JQ004328	JQ004383
Myiarchus tyrannulus	Ricklefs Lab - MYTY 37	Venezuela: El Indio, Isla Margarita	JQ004329	JQ004384
Myiarchus tyrannulus	Ricklefs Lab - BR2	Brazil: Cáceres, Mato Grosso	JQ004330	---
Myiarchus tyrannulus	Ricklefs Lab - BR3	Brazil: Cáceres, Mato Grosso	JQ004331	---
Myiarchus tyrannulus	Parker Lab - CR1	Costa Rica: Palo Verde, Guanacaste	JQ004332	JQ004385
Myiarchus tyrannulus	Parker Lab - CR25	Costa Rica: Santa Rosa, Guanacaste	JQ004333	JQ004386
Myiarchus tyrannulus	Parker Lab - CR63	Costa Rica: Santa Rosa, Guanacaste	JQ004334	JQ004387
Myiarchus tyrannulus	Parker Lab - CR66	Costa Rica: El Hacha, Guanacaste	JQ004335	JQ004388
Myiarchus validus	Ricklefs Lab - J 361	Jamaica	JQ004336	JQ004389
Myiarchus validus	Ricklefs Lab - J 390	Jamaica	JQ004337	JQ004390
Myiarchus validus	Ricklefs Lab - J 613	Jamaica	JQ004338	---
Myiarchus validus	Ricklefs Lab - J 674	Jamaica	JQ004339	---
Myiarchus yucatanensis	KUNHM 2095	Mexico: Campeche, Yucatan Peninsula	JQ004340	JQ004391
Myiarchus yucatanensis	KUNHM 2096	Mexico: Campeche, Yucatan Peninsula	JQ004341	JQ004392
CLADE II				
Myiarchus barbirostris	Ricklefs Lab - J 758	Jamaica	JQ004342	JQ004393
Myiarchus panamensis	Ricklefs Lab - GAM04 314	Panama: Gamboa	JQ004343	---
Myiarchus panamensis	Ricklefs Lab - PAN 19	Panama: Barro Colorado Island	JQ004344	JQ004394
Myiarchus tuberculifer	Ricklefs Lab - GAM04 131	Panama: Gamboa	JQ004345	JQ004395
Myiarchus tuberculifer	Ricklefs Lab - MEX 659	Mexico: Campeche, Yucatan Peninsula	JQ004346	JQ004396
Myiarchus swainsoni	GenBank	Brazil: Amapá	DQ294512	DQ294556
OUTGROUP				
Tyrannus melancholicus	GenBank	Brazil: Rondônia	DQ294532	DQ294576
Empidonax minimus	GenBank		AY143197	AY030125

Table 2 Populations of *Myiarchus magnirostris* from seven islands with their genetic diversity and tests of neutrality. N = number of samples analyzed; H = haplotype number; *h* = haplotype diversity; π = nucleotide diversity; *k* = average number of nucleotide differences; D = Tajima's D value; Fs = Fu's Fs value. Significant negative values for D and Fs are indicative of population expansion.

Island	N	H	*h*	π	*k*	D	Fs	Φ_{st}
Isabela	26	7	0.5723	0.00108	0.9754	-1.885**	-3.092*	0.4156
Floreana	22	2	0.4849	0.00053	0.4849	1.334	1.392	0.4427
Española	18	1	0.0000	0.00000	0.0000	0.000	n/a	0.4691
Santa Cruz	28	5	0.6561	0.00118	1.0741	0.118	-0.520	0.4100
Santiago	20	2	0.1000	0.00011	0.1000	-1.164	-0.879	0.4636
Santa Fé	11	2	0.1818	0.00020	0.1818	-1.129	-0.410	0.4596
San Cristóbal	29	2	0.0690	0.00008	0. 0690	-1.149	-1.184**	0.4653
TOTAL	154	12	0.4913	0.00087	0.7906	-1.681*	-7.118**	0.4434

* $p < 0.05$; ** $p < 0.01$.

Table 3 Primers used for amplification and sequencing of the DNA regions included in this study. A = used for amplification; S = used in sequencing; Ta = PCR annealing temperature.

DNA region	Primer Name	Primer Sequence	Ta	Reference
ATPase8_6	CO2GQL (A/S)	GGA CAA TGC TCA GAA ATC TGC GG	60°C	Seutin & Bermingham*
	CO3HMH (A/S)	CAT GGG CTG GGG TCR ACT ATG TG	60°C	Seutin & Bermingham *
	ATPase_297F (S)	CAA CTC CGA TTC TTC CAT CTA ATC AC		this study
	ATPase_514R (S)	CTA GTG CAA TTG AGG GTT GGT TTC		this study
CytB	L14841 (A/S)	CCA TCC AAC ATC TCA GCA TGA TGA AA	53°C	Kocher et al (1989)
	H16065 (A/S)	GTC TTC AGT TTT TGG TTT ACA AGA C	53°C	Edwards & Wilson (1990)
	intR Myiarchus (S)	GTT TCG TGT AGA AAT GTA AGG TGG		this study
	intF Myiarchus (S)	ACA CTC ACC CGA TTC TTT GCC		this study
ND2	L5216 (A/S)	GGC CCA TAC CCC GRA AAT G	60°C	Sorenson 2003
	H6313 (A/S)	ACT CTT RTT TAA GGC TTT GAA GGC	60°C	Sorenson 2003
	L5758 (S)	GGN GGN TGA ATR GGN YTN AAY CAR AC		Sorenson 2003
	H5766 (S)	RGA KGA GAA RGC YAG GAT YTT KCG		Sorenson 2003
BF7	FIB-B17U (A/S)	GGA GAA AAC AGG ACA ATG ACA ATT CAC	61°C	Brumfield & Edwards (2007)
	FIB-B17L (A/S)	TCC CCA GTA GTA TCT GCC ATT AGG GTT	61°C	Brumfield & Edwards (2007)
	BF7intF (S)	TTG TAA AGT ACA TAA CTG AGC		Brumfield & Edwards (2007)
	BF7intR (S)	GTG CTC AGT TAT GTA CTT TAC AA		Brumfield & Edwards (2007)

* http://www.stri.si.edu/sites/bermingham/research/primers/index.html

Table 4 Pairwise Φ_{st} values between populations of *M. magnirostris* from seven Galápagos Islands. Significant pairwise values ($p < 0.05$) are in bold.

	Santa Cruz	Santiago	Santa Fé	Floreana	Isabela	S.Cristóbal	Española
Santa Cruz	0.0000						
Santiago	**0.2021**	0.0000					
Santa Fé	**0.1544**	0.0134	0.0000				
Floreana	**0.3964**	**0.8220**	**0.7820**	0.0000			
Isabela	**0.0856**	0.0115	-0.0127	**0.6054**	0.0000		
S. Cristóbal	**0.2140**	0.0030	0.0323	**0.8431**	0.0169	0.0000	
Española	**0.2014**	-0.0054	0.0472	**0.8387**	0.0060	-0.0176	0.0000

Figure Titles

Fig. 1: **(A)** Map of the Galápagos archipelago, showing its position in relation to Ecuador and Costa Rica. The number of samples from each island used in this study is listed in parenthesis and the regions where they were collected are marked by stars. The squares adjacent to each island represent the pattern used to represent those islands in the haplotype network (Fig.1B).

(B) Median-joining haplotype network generated from cytb sequences (907bp) of 154 Galápagos flycatchers (*Myiarchus magnirostris*). Each circle represents a different haplotype and circle sizes or slices are proportional to the number of individuals with the same haplotype. Number of nucleotide substitutions between haplotypes is represented by the number of dashes and the length of lines between circles. Shades and patterns represent different islands: dark grey = Isabela; light grey = Floreana; thick black and white stripes = Española; black = Santa Cruz; light grey with black stripes = Santiago; white with black stripes = Santa Fé; and white = San Cristóbal.

Fig. 2: Best tree (cladogram) obtained with Maximum Likelihood in Garli for 16 species of *Myiarchus* (n=61) using concatenated sequences from ND2 (1035 bp) and cytb (935bp). Numbers on nodes represent ML bootstrap values (RaxML - 100bs)/ MP bootstrap values (Paup – 500bs)/ Bayesian posterior probabilities (Mr.Bayes – 10million generations). Dashes represent nodes not present in the considered analysis. Sequences from *Empidonax minimus* and *Tyrannus melancholicus* were extracted from GenBank and used as outgroups. Clades I and II represent those described by

Joseph et al. (2004). Boxes delineate *M. magnirostris*, *M. tyrannulus* from Central America and Mexico (MtyCAM), and *M. tyrannulus* from South America (MtySA).

Fig. 3: Bayesian condensed phylogram with posterior probabilities and average node ages obtained using a strict molecular clock (2.07%/MY) in BEAST with concatenated sequences from ND2 (1035 bp) and cytb (935bp). Individuals/branch tips from each lineage were condensed within triangles. CAM = Central America and Mexico; SA = South America.

Figure 1

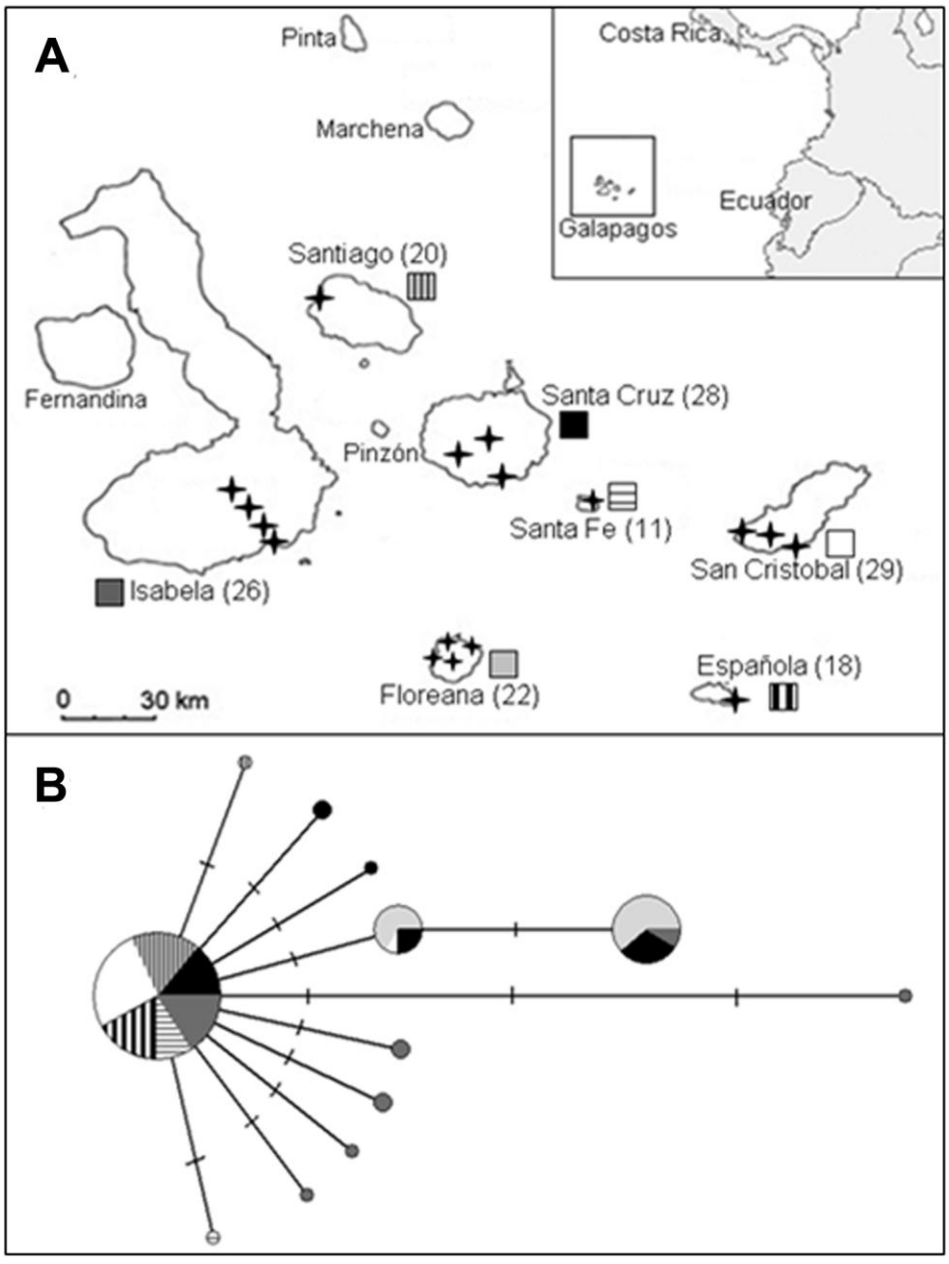

A
Pinta
Marchena
Costa Rica
Galapagos
Ecuador
Santiago (20)
Fernandina
Santa Cruz (28)
Pinzón
Santa Fe (11)
San Cristobal (29)
Isabela (26)
Floreana (22)
Española (18)
0 30 km
B

Figure 2

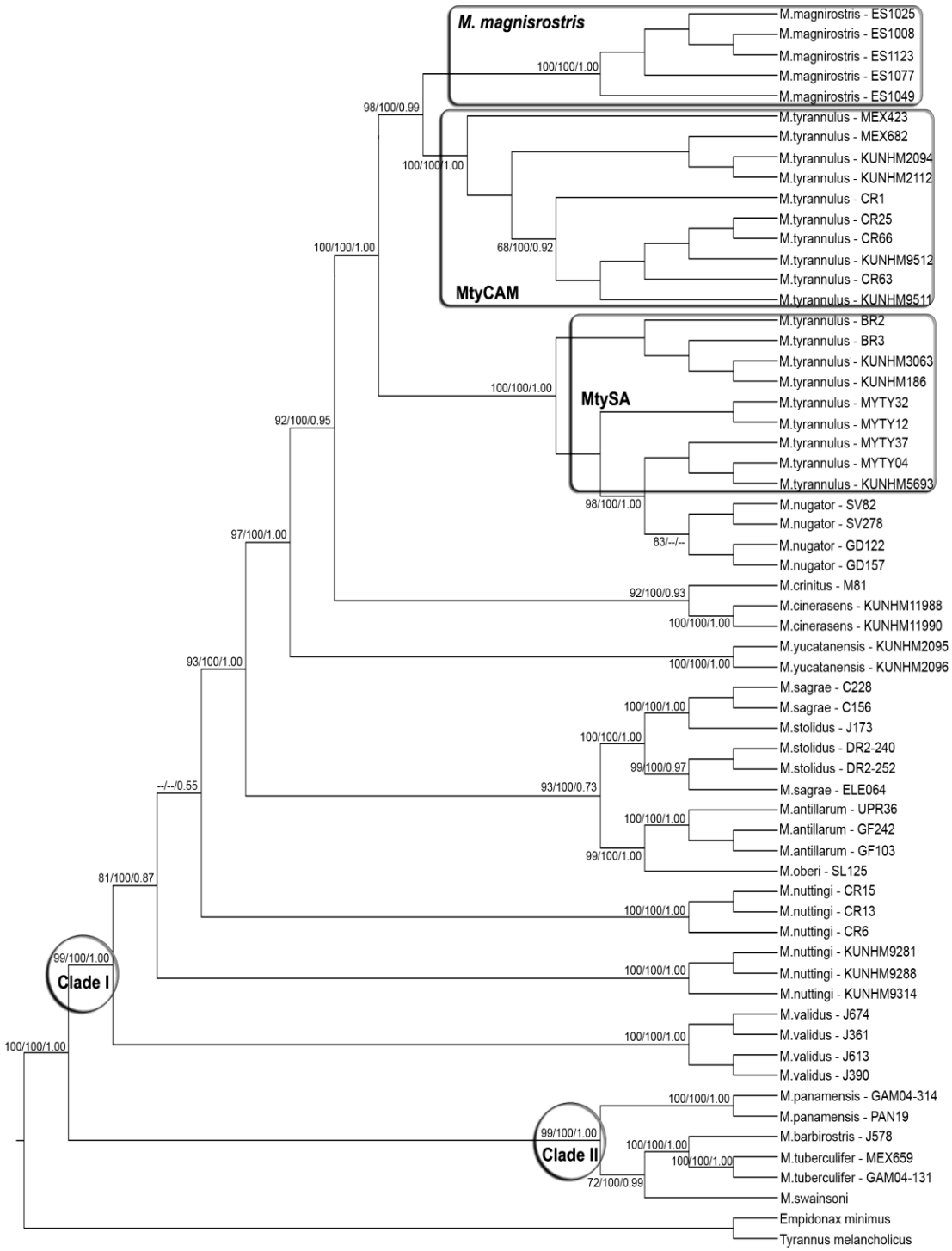
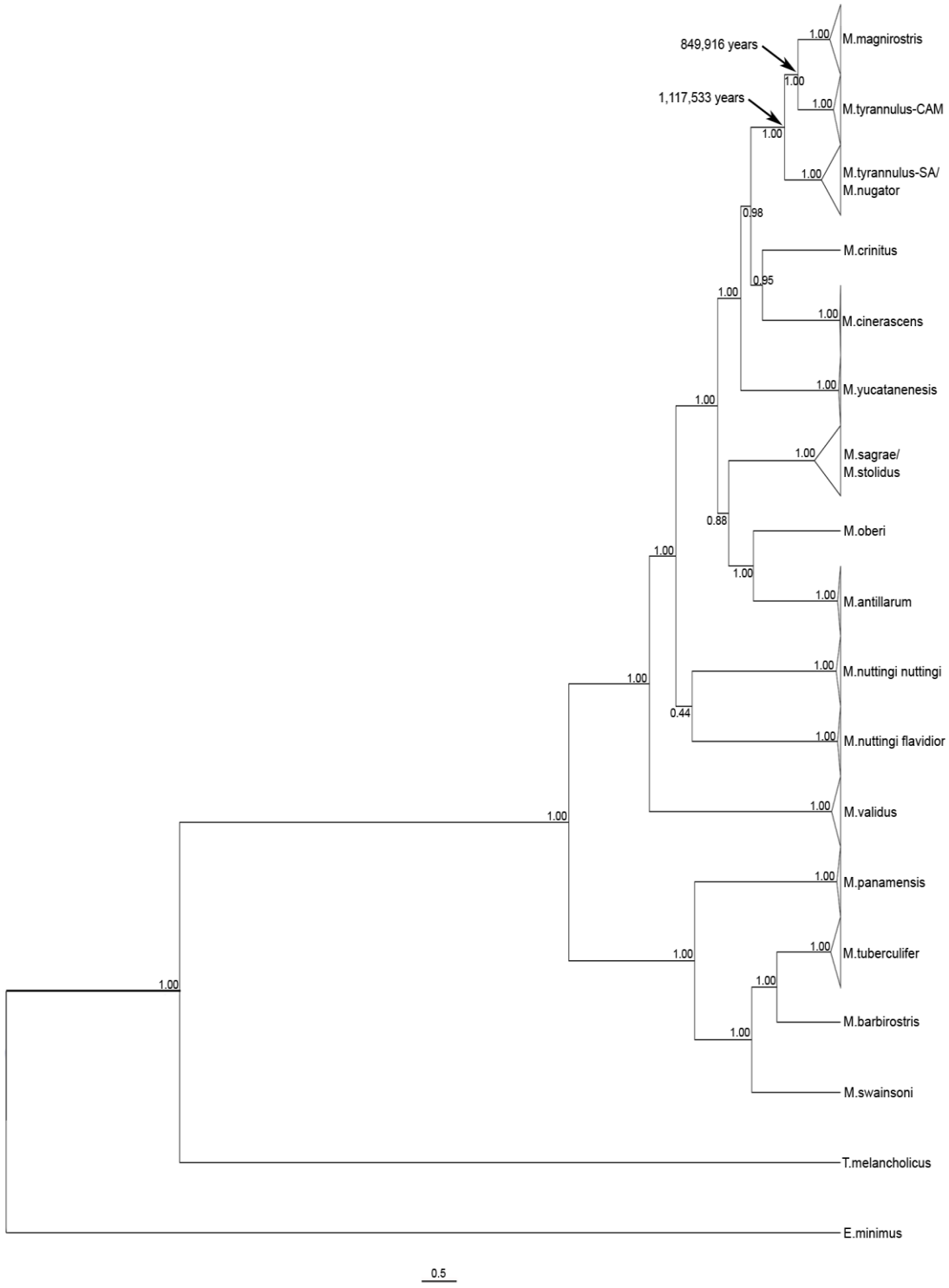

M. magnisrostris
M.magnirostris - ES1025
M.magnirostris - ES1008
M.magnirostris - ES1123
M.magnirostris - ES1077
M.magnirostris - ES1049
MtyCAM
M.tyrannulus - MEX423
M.tyrannulus - MEX682
M.tyrannulus - KUNHM2094
M.tyrannulus - KUNHM2112
M.tyrannulus - CR1
M.tyrannulus - CR25
M.tyrannulus - CR66
M.tyrannulus - KUNHM9512
M.tyrannulus - CR63
M.tyrannulus - KUNHM9511
MtySA
M.tyrannulus - BR2
M.tyrannulus - BR3
M.tyrannulus - KUNHM3063
M.tyrannulus - KUNHM186
M.tyrannulus - MYTY32
M.tyrannulus - MYTY12
M.tyrannulus - MYTY37
M.tyrannulus - MYTY04
M.tyrannulus - KUNHM5693
M.nugator - SV82
M.nugator - SV278
M.nugator - GD122
M.nugator - GD157
M.crinitus - M81
M.cinerasens - KUNHM11988
M.cinerasens - KUNHM11990
M.yucatanensis - KUNHM2095
M.yucatanensis - KUNHM2096
M.sagrae - C228
M.sagrae - C156
M.stolidus - J173
M.stolidus - DR2-240
M.stolidus - DR2-252
M.sagrae - ELE064
M.antillarum - UPR36
M.antillarum - GF242
M.antillarum - GF103
M.oberi - SL125
M.nuttingi - CR15
M.nuttingi - CR13
M.nuttingi - CR6
M.nuttingi - KUNHM9281
M.nuttingi - KUNHM9288
M.nuttingi - KUNHM9314
M.validus - J674
M.validus - J361
M.validus - J613
M.validus - J390
M.panamensis - GAM04-314
M.panamensis - PAN19
M.barbirostris - J578
M.tuberculifer - MEX659
M.tuberculifer - GAM04-131
M.swainsoni
Empidonax minimus
Tyrannus melancholicus
Clade I
Clade II
98/100/0.99
100/100/1.00
100/100/1.00
68/100/0.92
100/100/1.00
92/100/0.95
98/100/1.00
83/--/--
97/100/1.00
92/100/0.93
100/100/1.00
93/100/1.00
100/100/1.00
100/100/1.00
99/100/0.97
--/--/0.55
93/100/0.73
100/100/1.00
99/100/1.00
81/100/0.87
100/100/1.00
99/100/1.00
100/100/1.00
100/100/1.00
100/100/1.00
100/100/1.00
99/100/1.00
100/100/1.00
100/100/1.00
72/100/0.99

Figure 3


849,916 years
1,117,533 years
M.magnirostris
M.tyrannulus-CAM
M.tyrannulus-SA/
M.nugator
M.crinitus
M.cinerascens
M.yucatanenesis
M.sagrae/
M.stolidus
M.oberi
M.antillarum
M.nuttingi nuttingi
M.nuttingi flavidior
M.validus
M.panamensis
M.tuberculifer
M.barbirostris
M.swainsoni
T.melancholicus
E.minimus
0.5

Chapter 2

Molecular and morphological variation in the Galápagos flycatcher (*Myiarchus magnirostris*)

Abstract

Myiarchus magnirostris (Passeriformes: Tyrannidae) is an endemic species that inhabits most of the Galápagos Islands, Ecuador. Here, to better understand the evolution of *Myiarchus magnirostris*, we used neutral polymorphic molecular markers and variable morphological characters to compare birds sampled from seven of the Galápagos Islands. Genetic diversity within islands was strongly correlated with island size, supporting our hypothesis that drift is important in the distribution of the genetic diversity of *M. magnirostris*. We detected significant population structure (F_{st} = 0.0945; $p < 0.0001$); nevertheless, the correlation between genetic and geographic distances among islands was not significant, suggesting that isolation by distance may not be driving their differentiation. Our samples were grouped into four Bayesian genetic clusters representing birds from: (1) Española, (2) San Cristóbal, (3) Floreana and Santa Cruz, and (4) Santiago, Santa Fé, and Isabela. Using morphological data, we detected significant differences between males and females and also between islands. While Floreana, Santa Cruz, Santiago, and Isabela are not morphologically differentiated, morphological differentiation was observed for Santa Fé, even in the presence of gene flow, and for Española and San Cristóbal, which are more isolated genetically. Local adaptation and genetic drift cannot be disentangled

as contributing evolutionary forces for the differentiation of these two islands after cessation of gene flow. In general, morphological distances across islands were not correlated with pairwise genetic distances, and local adaptation through natural selection may possibly have contributed to that, but drift and phenotypic plasticity could not be excluded as explanations.

Introduction

Volcanic island ecosystems represent the ideal setting for testing evolutionary hypotheses: they are isolated, have limited and clearly defined landmasses, comprise less ecological complexity than continental areas, and the different islands of an archipelago work as natural replicates (see Valle & Parker 2012). Isolation and limited distribution determine demographic delimitations for species and populations and lead to stochastic drift and reduced gene flow, which results in endemic species with low genetic diversity and high differentiation (Clegg 2010; Frankham et al. 2002).

Species differentiation and diversification on islands have been commonly attributed to the phenomenon of adaptive radiation and divergent selection, like the Darwin finches in Galápagos, the *Anolis* lizards in the Caribbean Islands, the honeycreepers and the silverswords in Hawaii, and the *Nesotes* beetles in the Canary Islands (Emerson, 2002). This differentiation can be accentuated due to fluctuations of the environment (Grant & Grant, 2002) or changes in species composition of the local community, which can show faster turnover rates on smaller islands (Price

2008; e.g. Grant & Grant, 2008). Even though selection and local adaptation play an important role in population differentiation, it has been shown that morphological differentiation between islands can also be generated by founder effects, at least in short term evolution (Kolbe et al. 2012).

Additionally, reduced migration contributes to this differentiation; the combination of drift and highly restricted gene flow, for example, is thought to have generated most of the remarkable distinctiveness in body size and behavior among island populations of the Galápagos hawk (*Buteo galapagoensis*) (Bollmer et al. 2003, 2005).Conversely, gene flow among populations usually counteracts the effects of drift and selection, reducing rates of genetic differentiation and speciation (Price 2008). However, morphological differences can arise even in the presence of gene flow due to local adaptation (e.g. Petren et al. 2005).

Population genetic studies generate important information for prioritizing species conservation strategies. First, it is widely accepted that genetic variability is important for the long term survival of populations and species, since low genetic variation could restrict the emergence of future evolutionary adaptations (Milligan et al. 1994). Further, the evaluation of gene flow rates between populations allows us to estimate movement of individuals and population connectivity, which are valuable for understanding and controlling pathogen and parasite transmission in a host species and in the community with which it interacts (Parker et al. 2006; Santiago-Alarcon 2006). Species endemic to islands may be very susceptible to the arrival of new pathogens because they have small population sizes and might have lost their resistance to diseases, because of the remarkable colonization bottleneck, through

genetic drift, or due to the absence of selective pressure by parasites (Frankham 1998; Milligan et al. 1994).

The Galápagos Islands are a unique ecosystem, preserved close to its pristine conditions, and with an important legacy for the theory of evolution. There has been much information generated about diversification and population genetics of many endemic species of Galápagos plants and animals, but the Galápagos flycatcher (*Myiarchus magnirostris*; Passeriformes: Tyrannidae) is one of the least studied terrestrial bird species. They are very common birds on most of the main islands (Jackson 1993), but they do not present recognized differences in morphology and vocalizations among island populations (Lanyon 1978), in contrast to the remarkable diversification of the Darwin's finches (Grant & Grant 2008) or the Galápagos mockingbirds (Arbogast et al. 2006). Recently we discovered that the maximum colonization age for the Galápagos flycatcher was approximately 850,000 years ago (Sari & Parker 2012), more recent than the arrival of these two other groups of endemic birds (1.6 to 5.5 MY for mockingbirds (Arbogast et al. 2006); 2 to 3 MY for finches (Sato et al. 2001)). Therefore, the speciation process and differentiation of the Galápagos flycatcher might be still incipient and it is our goal to understand its evolution.

Here, we used highly polymorphic molecular markers to assess the genetic diversity of *M. magnirostris* within and among seven of the Galápagos Islands. We hypothesized that limited population size erodes genetic diversity through drift, and predicted that molecular diversity increases with island area. We also hypothesized that gene flow is restricted by geography, and hence, genetic distances between

islands should be positively correlated with their geographical distances.

To better understand the evolution of *M. magnirostris*, we also complemented our population genetic study with an evaluation of the morphological variation within and across islands. We hypothesized that the lack of correspondence between molecular and morphological variation may indicate that local adaption through selection could be counterbalancing migration and drift. While this approach is potentially important for our understanding of the interaction among different microevolutionary processes underlying the diversification of *M. magnirostris*, we recognize that disentangling these processes is challenging, especially if the variation in morphological characteristics is not genetically inherited.

Methods

Data collection

During the months of July and August from 2007, 2008, and 2009 we captured 229 Galápagos flycatchers (*M. magnirostris*) on seven of the Galápagos Islands: Santa Cruz, Santiago, Santa Fé, Floreana, Española, Isabela, and San Cristóbal. We used playbacks of songs and calls that we recorded from *M. magnirostris* on Galápagos to attract the birds to mistnets. Blood samples were collected from all birds using heparinized capillary tubes after puncturing the brachial vein and were stored in lysis buffer (Longmire et al. 1988) until DNA extraction.

In the field, we took seven morphological measurements: body weight measured to the nearest 0.1 g using a 50 g spring scale (Pesola® Baar, Switzerland); right wing length (unflattened) to the nearest 0.5 mm; right tarsus length to the

nearest 0.1 mm, as the distance between the bent intertarsal joint and the center of the foot; bill width to the nearest 0.1 mm at the nares; exposed bill culmen (bill length) to the nearest 0.1 mm; tail length to the nearest 0.5 mm, measured from the base of the uropygeal gland; and total bird length to the nearest 1 mm, measured with the bird lying on a ruler as the distance from the tip of the bill to the end of the tail.

Population genetics using microsatellites

Laboratory analyses

We used 137 samples of *M. magnirostris* from seven islands (Table 1). Total genomic DNA was extracted from samples stored in a lysis buffer (Longmire et al. 1988) using a standard phenol-chloroform protocol, followed by a final dialysis step in 1X TNE_2 (10 mM Tris, 10 mM NaCl, 2 mM EDTA) for DNA ultra-purification. Microsatellite primers specifically designed for *Myiarchus* were not available, so to find informative microsatellite loci that could be used in this study, we tested primers that had been previously reported to amplify polymorphic loci in related species (from the families Tyrannidae and Pipridae). We tested primers designed for: *Mionectes striaticollis* (11 loci; Bardeleben & Gray 2005), *Empidonax minimus* (5 loci; Tarof et al. 2001), *Sayornis phoebe* (12 loci; Beheler et al. 2007; Watson et al. 2002), *Chiroxiphia linearis* (2 loci; McDonald & Potts 1994), *Chiroxiphia lanceolata* (3 loci; Duval & Nutt 2005), *Manacus manacus* (6 loci; Piertney *et al.* 2002), and *Manacus* spp. (6 loci; Yuri et al. 2009; R. Brumfield pers.comm.). From the 26 loci that were successfully amplified, only seven presented polymorphism for *M. magnirostris* and were selected for this work: LTMR8, Man 3, Man 6, Lan22,

EMIC23, AAGG-209, AAAG-33 (Table S1).

For amplification of loci LTMR8, Man 3, Man 6, and Lan22 we used fluorescently labeled forward primers. For amplification of loci EMIC23, AAGG-209, and AAAG-33, we applied a 5' CAG tag to one of the primers (the one with lower melting temperature; see Croshaw et al. 2005) and a 5'GTTT tail to the other (Brownstein et al., 1996). Detailed PCR protocols are in supplemental material. Fragment sizes were determined by an ABI 2000 automatic sequencer (Applied Biosystems) using 0.5 μl of size standard GENESCAN LIZ (500) in a 20 μl reaction with 1-3 μl amplicon and 18.5-16.5 μl HiDi. Individual genotypes were manually scored using Genemapper v.4.01 software (Applied Biosystems, Life Technologies, Carlsbad, CA). Approximately 10% of the samples were repeated across loci to confirm genotype assignments, and one-third of all homozygotes were re-run to ensure we were not incorrectly assigning genotypes due to allelic dropout.

Statistical analyses

We used MICRO-CHECKER v. 2.2.3 (van Oosterhout et al, 2004) to detect typographic errors and also to check our genotypes for null alleles (non-amplified alleles) and scoring errors due to stuttering and large allele drop-out (short allele dominance). All birds sampled from one island were considered to be from the same population. Linkage disequilibrium between loci was tested for each island (population) in Arlequin v. 3.11 (Excoffier et al. 2005) using ln likelihood ratio G-tests (10,000 permutations), and also in GenePop using the Markov chain method (100 batches, 10,000 steps and 1,000 burn-in per batch). Deviations from Hardy-

Weinberg equilibria were tested for each locus within populations in Arlequin v. 3.11 and also in GenePop using 1,000,000 Markov chain steps. Diversity measures (within and across populations) were estimated with Arlequin v. 3.11 and FSTAT v. 2.9.3.2 (Goudet 2002). We used R v.2.13 to run simple regressions between the natural logarithm of island area and genetic diversity measures within islands (number of alleles and heterozygosity) to test for evidence of drift in different population sizes.

Population genetic structure was measured by Analysis of Molecular Variance (AMOVA; Excoffier et al. 1992) and also by pair-wise F_{st} values between populations using Arlequin v 3.11. Significance of pair-wise F_{st} values was calculated using sequential Bonferroni correction. To test whether samples collected in different localities on one island represented different subpopulations, we also performed a three-level AMOVA, in which we assessed the distribution of genetic variation within sampling localities (n = 136 birds), among sampling locations within islands (n = 19), and among islands (n = 7). Migration rates between islands were calculated as the proportion of immigrants received per island from each island, as implemented in the software BayesAss v. 1.3. (Wilson & Rannala 2003), using the default parameters.

To estimate the number of genetic groups/populations that our samples represent, we used STRUCTURE v.2.3.3 (Pritchard et al. 2000), which performs genotype clustering through a Bayesian approach. The optimum number of genetic clusters (genetic groups; k) was determined using mean values of log likelihood, L(K), and the ΔK statistics by Evanno et al. (2005). We executed analyses using the admixture model with correlated allele frequencies, one with no prior information and the other using islands as our prior population information (LOCPRIOR). For each

analysis we performed five runs per k (k = 1 through k = 10) with a burn-in of 200,000 cycles followed by 800,000 additional cycles. When using the correlated allele frequencies model, however, the presence of a very divergent population might influence the inference of the number of clusters, and, as recommended in the STRUCTURE v 2.3 documentation, this population should be removed from the analyses for the model to achieve better results. Therefore, after evaluating the results from our first two analyses, we excluded samples from the most differentiated population (Española) and repeated both analyses, using the same parameters. *A posteriori*, we estimated AMOVA and pair-wise F_{st} values between the clusters defined by STRUCTURE using Arlequin v. 3.11.

In order to test whether genetic distances among populations were correlated with geographic distances among islands (isolation by distance) we performed Mantel tests as implemented in IBDWS (Jensen *et al*. 2005) using 10,000 permutations. As a measure of genetic distances we used pair-wise F_{st} values from Arlequin. Geographic distances among islands were obtained from the GPS coordinates of the main sampling locations on each island; when samples were obtained from multiple locations per island, we used the coordinates from locations where most samples were obtained. We used Geographic Distance Matrix Generator v. 1.2.3 (Ersts, online) to generate pair-wise geographic distances from the geographic coordinates using spherical functions.

Molecular sexing and morphological analyses

We were interested in evaluating the morphological variation among *M.*

magnirostris populations from different islands. However, significant morphological variation between sexes could generate noise in our analyses. Since *M. magnirostris* is sexually monomorphic, we used a PCR based method to identify sex of sampled individuals, as described by Fridolfsson & Ellegren (1999). Details about this protocol can be found in the supplemental material.

All statistical analyses on morphological data were performed in SPSS v.19.0.0, in R v.2.13 using the package "vegan" (Oksanen et al. 2011), or in both, unless otherwise cited. We ran t-tests using all seven morphological measurements as variables to test for differences between males and females. To explore the differences in morphology among islands, we used MANOVA, considering each sex separately and all individuals together. We also ran ANOVAs to identify which variables varied significantly across islands. We applied a multivariate approach using Principal Component Analyses (PCA) to evaluate the spatial distribution of the morphological variation between sexes and also among islands. PCAs were based on a correlation matrix, and components were not rotated. We discarded individuals with too much missing morphological data from our PCAs and our final dataset had 214 individuals from seven islands (Fig.1). Because the first three Principal Components obtained from these analyses (PC1, PC2, and PC3) represent the overall body size and shape of the birds (see Table S3 for PC loadings), we also used PC1, PC2, and PC3 as variables to compare islands using ANOVA and post hoc pairwise Tukey tests.

In order to test for an association between genetic distances and morphological divergence among islands, we used Mantel tests through IBDWS

(Jensen *et al*. 2005). We used pair-wise F_{st} values as a measure of genetic distances between islands (Table 3). To estimate the relative magnitude of morphological divergence between islands, we first calculated the center of the distribution for each island in the multivariate measurement space using the first two principal components (centroids). Then, we calculated the Euclidean and the Mahalanobis distances among these centroids, which were used in the Mantel tests. We also performed the same analyses using the clusters defined by STRUCTURE as the genetic/morphological groups.

We tested for morphological differentiation between birds captured in different habitats/altitudes to explore the possibility of ecological divergence within islands. The larger islands from Galápagos have an altitudinal gradient in which the lowlands (sea level to 200 m) are composed by arid and coastal vegetation and the highlands (above 200 m) are mostly covered by moist forest (see Kleindorfer et al. 2006). Because the sexes were morphologically distinct (see "Morphology" in results section below) and the number of females captured was much smaller than the number of males (n = 67), we did these altitudinal comparisons using data only from males (n = 147). We sampled birds on lowlands from all seven islands, but birds from highlands were only sampled on Santa Cruz ($n_L = 20$; $n_H = 8$), Floreana ($n_L = 14$; $n_H = 10$), Isabela ($n_L = 25$; $n_H = 3$), and San Cristóbal ($n_L = 25$; $n_H = 4$), where n_L = the number of lowland birds and n_H = the number of highland birds. We compared birds from highlands and lowlands across islands ($n_L = 122$; $n_H = 25$) and within each island using t-tests.

Results

Population genetics

Excess of homozygotes was found for the locus AAGG-209 only in the Floreana population, as indicated by MICRO-CHECKER and by deviation from Hardy-Weinberg equilibrium tests performed with Arlequin. Therefore, we present only the results from analyses excluding the AAGG-209 genotypes from Floreana. We did not exclude the locus AAGG-209 from our analyses because no evidence of null alleles or deviation of HW for this locus was found in the other populations. There was no evidence of deviation from HW equilibrium in any other population for any other locus. We also did not find any evidence for global heterozygote excess or deficit. All the genetic diversity patterns obtained in this study were the same whether or not we excluded the data from locus AAGG-209 for Floreana.

Linkage disequilibrium was detected between the loci LTMR8 and Lan22 for Santiago and Española only. We did not discard these data from our analyses, however, because if these loci were actually physically linked, we would detect evidence of linkage in all our populations.

Overall, we detected 81 alleles for *M. magnirostris*, and the number of alleles per locus varied from 2 to 17 (mean = 5.45). The number of alleles per island varied from 30 in Floreana (over 6 loci after excluding 4 alleles detected in the locus AAGG-209) to 45 in Santa Cruz over 7 loci (Table 1). A total of 24 private alleles were found: 12 from Española, zero for Santa Fé, and two or three for the other five islands. Expected heterozygosity (h; calculated as Nei's unbiased genetic diversity)

within populations for each locus ranged widely, from 0 to 0.903, with a mean of 0.585.

Total expected heterozygosity (Ht) estimated across all loci was 0.659, and average expected heterozygosity per population was somewhat similar, ranging from 0.499 in Santa Fé to 0.655 in Santa Cruz (Table 1). We found that heterozygosity was significantly related to island area ($r^2 = 0.82$, $F_{1, 5} = 28.5$, $p = 0.003$) and mean number of alleles was marginally related to area ($r^2 = 0.48$, $F_{1, 5} = 6.53$, $p = 0.051$).

The AMOVA revealed significant population structure, in which 9.45% of the *M. magnirostris* genetic variation is found among islands ($F_{st} = 0.0945$; $p < 0.0001$), but no variation among localities within islands was detected ($F_{sc} = -0.0102$; $p = 0.868$). We also found significant pair-wise F_{st} values between all island comparisons, except between Santiago and Isabela (Table 2); the strongest differentiation values were between Española and all other islands and the smallest values were between Isabela and all other islands except Española. The Mantel correlation between genetic (pairwise F_{st} values) and geographic distances was not significant and we rejected the model of isolation by distance.

Unidirectional migration rates were calculated for all islands and can be found in Figure 1 and Table S2. The most isolated islands are San Cristóbal and Española, which have the lowest immigration rates (0.0177 and 0.0173, respectively) and minimal emigration rates to all other islands. Santiago also has a very low immigration rate (0.0175), but it sends many emigrants, contributing to high immigration rates in Santa Cruz (0.1905 from Santiago), Santa Fé (0.2359 from Santiago), and Isabela (0.2498 from Santiago) (Fig.1). The other island with high

immigration rate is Floreana, whose immigrants are mostly from Santa Cruz. Besides the high immigration rate Santa Fé receives from Santiago, it also receives lower immigration rates (around 0.014) from most of the other islands.

Using the method from Evanno et al. (2005), the Bayesian clustering analyses performed in STRUCTURE (whether using LOCPRIOR or not) revealed that the individuals from all islands were divided into two clusters (k=2), one cluster only with birds from Española (n=20), and another with birds from the other six islands (n=117). However, the maximum likelihood values for k, the probability assignments, and the graphs produced by STRUCTURE indicated that k = 4 (without LOCPRIOR) or k = 5 (with LOCPRIOR as islands) could also represent an appropriate number of genotype clusters. When the individuals from the most divergent population, Española, were removed from the analyses as suggested in the STRUCTURE v.2.3 documentation, three clusters (k = 3) were clearly revealed when applying the Evanno et al. (2005) method, whether using LOCPRIOR or not. Therefore, we recognize that our samples were divided in four clusters: the Española population, the San Cristóbal population, one cluster with individuals from Floreana and Santa Cruz, and one cluster with individuals from Santiago, Santa Fé, and Isabela. Among these latter five island-populations, birds from Santiago had the highest clustering assignment probabilities. Interestingly, the F_{st} obtained among these four clusters was 0.0979 ($p <$ 0.0001), very similar to the value obtained among the island populations, indicating that the differences among the STRUCTURE clusters are driving the F_{st} value among populations and not the islands themselves.

Morphology

We found that of the 229 individuals captured, 158 were males and 71 were females. T-tests showed that males are significantly larger than females for the variables weight (t_{228} = 2.413; p = 0.017), wing length (t_{228} = 2.398; p = 0.017), and tail length (t_{223} = 2.055; p = 0.041), but there were no significant differences between sexes considering total length (t_{228} = 0.990; p = 0.323), bill width (t_{228} = -0.878; p = 0.381), bill length (t_{228} = 0.479; p = 0.633), and tarsus (t_{228} = 0.575; p = 0.566). The differences between males (n = 127; with no missing data) and females (n = 53; with no missing data) were also detected by the segregation of each sex distribution in the multivariate measurement space (PC1 = 46.94%; PC2 = 23.51%; Fig. 3).

MANOVA and ANOVAs were significant across islands considering males and females together or separately for all the variables (see statistics in Table S3). In the Principal Component Analysis (PCA) when considering only males (n = 127), the first three components explained 80.39% of the variation among the males (PC1 = 43.32%; PC2 = 22.39%; PC3 = 14.69%; see Table S3 for PC loadings). The distribution of samples (points) in the multivariate measurement space shows that Española and San Cristóbal are separated from other islands in PC2 (Fig. 4A), and Santa Fé is separated in PC3 (Fig. 4B). Samples from other islands were not spatially segregated. This pattern of morphological differentiation across islands is consistent but less evident when considering both sexes together. Considering only females, however, this pattern is not so clear, probably due to the much smaller number of females captured per island. Hence the results we report here regard the analyses using only males. ANOVAs comparing overall body size and shape determined by

the PCs were significant (PC1: $F_{6,127} = 29.46$, $p < 0.0001$; PC2: $F_{6,127} = 23.66$, $p < 0.0001$; PC3: $F_{6,127} = 26.78$, $p < 0.0001$). *Post hoc* pairwise comparisons of means for these PCs supported our interpretation of the distribution of samples in the multivariate measurement space (PCA), showing that for PC1, San Cristóbal is statistically different from all the islands, except from Santa Fé; for PC2, Española is significantly different from all the other islands, and San Cristóbal is different from all islands but Isabela; for PC3, Santa Fé is significantly different from all the other islands (Fig. 5).

Mantel tests correlating the morphological centroid distances (Euclidean or Mahalanobis) with F_{st} values between islands were not significant, meaning that genetic distances between islands are not correlated with their morphological distances. The Mantel test between the pairwise F_{st} values and the morphological distances among the four groups defined by STRUCTURE was also not significant.

The t-tests across all islands showed that highland males have significantly larger body weight ($t_{144} = -3.126$; $p = 0.002$) than males from lowlands. The comparisons between highland and lowland males within each island varied greatly: in Santa Cruz, highland males have significantly larger bill width ($t_{23.8} = -2.652$; $p = 0.014$); in San Cristóbal, highland males have significantly larger total length ($t_{25} = -2.508$; $p = 0.019$); in Isabela, highland males have significantly larger wing length ($t_{26} = -2.631$; $p = 0.014$), but smaller total length ($t_{25} = 2.563$; $p = 0.017$); and in Floreana, males from highlands have significantly smaller bill width ($t_{20.56} = 3.008$; $p = 0.007$), bill length ($t_{22} = 2.261$; $p = 0.034$), and tarsus length ($t_{22} = 2.326$; $p = 0.030$) than males from lowlands. Therefore, morphological differences between highland and

lowland birds within islands were evident, but did not show the same pattern for each island.

Discussion

Intrapopulation genetic diversity

Heterozygosity and number of alleles were very high, similar to the highest values reported for Darwin finches (Petren et al. 2005) or the Galápagos Dove (*Zenaida galapagoensis* (Santiago-Alarcon et al. 2006), but higher than the values obtained for other Galápagos terrestrial birds, like the mockingbirds (*Mimus* spp.; Hoeck et al. 2010) and the yellow warbler (*Dendroica petechia aureola*; Chaves et al. 2012). Additionally, diversity (mean number of alleles and heterozygosity) was comparable across islands, but the smallest islands, Santa Fé, Española, and Floreana had the lowest heterozygosities while the highest diversities were found in the largest islands, Isabela and Santa Cruz. The Galápagos flycatchers are found inhabiting a wide variety of habitats and altitudes on the sampled islands (Jackson, 1993; Lanyon, 1978); therefore island area can be used as a good proxy for population size (e.g. Hoeck et al., 2010; Petren et al. 2005). Smaller populations may have reduced genetic diversity as a result of stronger genetic drift, and the strong correlation we detected between heterozygosity and island area is good evidence that genetic drift is playing an important role in the evolution of the Galápagos flycatchers. Genetic drift was also found to be significant in the distribution of genetic diversity of warbler finches (*Certhidea* spp.; Petren et al. 2005), Galápagos mockingbirds (Hoeck et al. 2010) and Galápagos hawks (*Buteo galapagoensis*; Bollmer et al. 2005, 2006).

Distribution of the genetic diversity across islands

Pairwise F_{st} comparisons were significant between all pairs of islands, and Española had the highest pairwise F_{st} values. Its genetic distinctiveness was also revealed by its lowest migration rates among all islands and its separation as an independent genetic cluster by STRUCTURE. San Cristóbal also forms a distinct genetic cluster in STRUCTURE and experiences migration rates as low as Española's. The genetic distinctiveness of these two islands results from a balance between drift, which reduces genetic diversity and randomly changes allele frequencies through time, and migration, which homogenizes allele frequencies among island populations through gene flow (Clegg 2010; e.g. Hoeck et al. 2010). Because both Española and San Cristóbal have comparable and very low levels of gene exchange with the other islands, stronger drift experienced on Española due to its rather smaller size, results in its higher genetic distinctiveness.

Further, Española and San Cristóbal are located at the most southeastern part of the Archipelago, and could be considered peripheral populations of *M. magnirostris*. Highest differentiation of peripheral populations was detected in Galápagos for the Darwin finches (Petren et al. 2005) and the mockingbirds (Hoeck et al. 2010). These outcomes were consistent with the model of isolation by distance in these two bird groups, in which gene flow is limited by geographical isolation. We did not find a significant correlation between genetic distance (F_{st}) and geographical distances between islands, rejecting the model of isolation by distance for the Galápagos flycatchers. Alternatively, this might indicate that any distance is enough to cause their genetic differentiation once the birds have arrived on an island.

Floreana had the second largest pairwise F_{st} values against all islands but Santa Cruz, from which it also receives a high immigration rate. Together, Santa Cruz and Floreana compose the third STRUCTURE genetic cluster, and the fourth cluster is formed by birds from the islands of Santiago, Santa Fé, and Isabela. Santa Cruz has the population of birds with the lowest probabilities of assignment to one genetic cluster (Fig. 2; Santa Cruz has two color blocks about the same size/probability), sharing a number of alleles with the fourth cluster, probably because of the high immigration rate from Santiago.

Interestingly, the highest immigration rate to Santa Fé is also from Santiago but not from Santa Cruz, which is the geographically closest island to Santa Fé, positioned between Santiago and Santa Fé (Fig. 1). Santa Fé is the only island with immigration rates higher than 1% from all the other islands. In spite of that, this island has the lowest genetic diversity, working like a sink population. This is the smallest island in which we sampled birds, and genetic drift is probably strong enough to overcome the gene flow experienced by this population and reduce its genetic variability, but not enough to cause genetic differentiation of this island.

Genetic clustering of the central islands and distinctiveness of San Cristóbal and Española is a pattern that was also detected for other terrestrial birds from Galápagos, such as the mockingbirds (Hoeck et al. 2010; Stefka et al. 2011) and the yellow warbler (Chaves et al. 2012; Española island not sampled).

Migration in flycatchers is predominantly in a southward direction. This direction is opposite to that of the strongest wind currents, which are from the south-southeast towards north-northeast, suggesting that wind currents are not important for

the movement of the Galápagos flycatchers. Other Galápagos bird species present higher migration rates from south to north, implying that they are using these currents for dispersal (e.g. Levin and Parker in prep – Nazca booby (*Sula granti*); Chaves et al. 2012 – yellow warbler). On the other hand, the Galápagos Dove presents no migration pattern among islands, and its independence of the wind currents was attributed to its strong flight capabilities (Santiago-Alarcon et al. 2006), which could also be the case for the Galápagos flycatchers, since they probably have a good ability to fly to neighboring islands in search of resources independent of wind currents.

Patterns of diversity with mitochondrial DNA and microsatellites

The genetic diversity previously reported for *M. magnirostris* using mitochondrial DNA (mtDNA; Sari & Parker 2012) was somewhat consistent with the microsatellite data: Santa Cruz and Isabela have the highest genetic diversity while Española has the lowest. However, no significant correlation was found between genetic diversity and island size using mtDNA. Regarding population differentiation (F_{st}), only Santa Cruz and Floreana were significantly different from the other islands using mtDNA, while all but one pairwise F_{st} comparisons were significant using microsatellites. A consistent pattern was that Floreana and Santa Cruz belong to the same Bayesian genetic cluster using microsatellites and also share mtDNA haplotypes that are not present in most of the other islands (Sari & Parker 2012). However, the general pattern observed was a substantially larger differentiation among islands using microsatellites than using mtDNA. Population analyses using microsatellites, however, are expected to show different patterns than analyses using mtDNA;

microsatellites have a much faster mutation rate and are better for evaluating more recent demographic events, such as current migration rates, while mtDNA retains more information about historical demographic events (e.g. Chaves et al. 2012; Stefka et al. 2011; Levin & Parker in prep).

Morphological variation and genetic diversity

The morphological characters we used in this study reflect overall body size and shape, and also bill size and shape of the Galápagos flycatchers. Therefore, we expect that the fitness of these birds is influenced by these characteristics, which comprise variation upon which selection will act, given this variation is genetically inherited. In contrast, microsatellites are neutral loci that are not under selection and are, in turn, good tools for estimating the outcomes of drift and gene flow. The comparison between the variation in morphology and in neutral loci should then allow us to identify the role of different microevolutionary processes on the evolution of a species. We did not detect a significant correlation between genetic distances and morphological distances between islands, supporting the hypothesis that the morphological and the neutral characters used are evolving independently. This same pattern has been observed for several other species in Galápagos, like the Galápagos Dove (Santiago-Alarcon 2006), the mockingbirds (Hoeck et al. 2010, Stefka et al. 2011), and the yellow warbler (Chaves et al. 2012).

Significant morphological distinctiveness was detected for Santa Fé, Española, and San Cristóbal. Española and San Cristóbal are also genetically distinct and show very low gene exchange with all the other islands, so local adaptation and

genetic drift cannot be disentangled as contributing evolutionary forces for the morphological differentiation of flycatcher populations on these two islands after cessation of gene flow. The population of *Geospiza conirostris* from Española is also sufficiently differentiated in morphology, behavior, and genes from other islands' populations to deserve its own species status (Petren et al. 2005). Similarly, the Galápagos hawks present remarkable morphological, behavioral, and genetic differences among populations from nine islands, the most extreme case again being Española, but the differentiation of this species is mostly attributed to genetic drift (Bollmer et al. 2003, 2006).

Differently, Santa Fé is not genetically differentiated, and its morphological differentiation has risen even in the presence of substantial immigration of birds from all the other islands. This differentiation could possibly indicate local adaptation to different environmental conditions or a distinct community composition of Santa Fé. Petren et al. (2005) supported the hypothesis of diversification with gene flow for the Darwin's finches, in which low levels of gene flow can provide enough genetic variability to small, "drift-prone" populations, upon which natural selection can act and start the differentiation process. Using the microsatellite data, however, we detected that drift has strong influence on the genetic diversity of Santa Fé; in a comparable way, the morphological differentiation of the small island of Santa Fé could have resulted from a random fixation of different morphological characters. Santiago-Alarcon et al. (2006) also found significant morphological differences between doves from Santa Fé and those from Santa Cruz or Española, even in the presence of high gene flow rates.

Price (2008) reviewed several studies of bird diversification on islands and he observed that smaller islands have morphological variation and differentiation more often than larger islands. This could be explained by the random and more frequent fluctuation in the species composition of smaller islands, resulting in changes in the selective pressures (Price 2008). Interestingly, two of the islands where the Galápagos flycatchers are morphologically distinct, Santa Fé and Española, are the smallest islands we analyzed (Table 2). Conversely, Galápagos flycatchers from larger islands as Santa Cruz, Isabela, and Santiago, share alleles and morphological features, showing no evidence of local adaptation on each of these islands.

We also detected significant morphological differences between Galápagos flycatchers from the highlands and from the lowlands even in the presence of gene flow, since no genetic variation among localities within islands was observed. These different altitudinal zones have very distinct environmental characteristics and species composition, which could support our hypothesis that the morphological characters analyzed here may be involved in local adaptation. However, the morphological differences between highland and lowland birds within islands did not show the same pattern for each island. Chaves et al. (2012) used the same characters to compare yellow warblers captured on four Galápagos Islands (Santa Cruz, Santiago, San Cristóbal, and Isabela) and found no differentiation either among or within islands (between highlands and lowlands), suggesting no evidence of local adaptation (or adaptive divergence) for the yellow warbler.

Our interpretation that local adaptation through natural selection could contribute to the morphological differentiation detected is based on the assumption

that the variation of the studied morphological characters are genetically inherited. It is possible however that this assumption is not realistic. This way, the absence of correlation between genetic and morphological distances that we detected instead could have risen as a consequence of phenotypic plasticity of all or some of the morphological characters studied.

Conclusions

The process of speciation and diversification of populations has been studied for different species endemic to Galápagos, and each of them shows a different story. Some taxa that have colonized the islands earlier in time experienced extensive speciation, like the Darwin finches (Sato et al. 2001) or the Giant tortoises (Caccone et al. 2002), but also remarkable differentiation among islands occurred in the Galápagos Hawks, which have a much more recent colonization date (Bolmer et al. 2006). The Galápagos flycatcher colonized the Galápagos at maximum about 850,000 years ago (Sari & Parker 2012), and we found that during this time, some morphological and genetic differentiation can be found in some islands. Our comparison between molecular and morphological data allowed us to better understand the speciation process of this species and to point to different evolutionary forces that are possibly contributing to this process in different islands. While it is clear that genetic drift has had a major impact on the current distribution of genetic variation, further studies and experiments would be necessary to prove the genetic inheritance of the morphological characters studied before we are able to disentangle the impact of genetic drift and natural selection acting upon morphological variation.

In spite of that, our estimate of population genetic structure and migration rates are a significant contribution to the knowledge about how to manage and conserve the Galápagos Islands ecosystem, since disease transmission can only be understood and controlled if the movement of infected hosts is recognized

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Table 1 Measures of genetic diversity for Galápagos flycatchers from seven islands. n= number of samples used in the population genetic analyses; N_a = number of alleles; h = Nei's expected heterozygosity (unbiased gene diversity); Ht = total expected heterozygozity. Island areas used in regression analyses against genetic diversity are presented under island names.

	Santa Cruz 986 km^2		Santiago 585 km^2		Santa Fé 24 km^2		Floreana 173 km^2		Española 60 km^2		Isabela 4640 km^2		San Cristóbal 558 km^2		Total	
	N = 21		n = 20		n = 11		n = 21		n = 20		n = 22		n = 22		n = 137	
Locus	Na	h	Na	H	Na	h	Na	h	Na	h	Na	h	Na	h	Na	Ht
LTMR8	9	0.707	10	0.767	7	0.764	2	0.368	3	0.466	9	0.821	7	0.721	17	0.777
Man 3	10	0.862	6	0.793	6	0.841	7	0.745	11	0.895	8	0.845	9	0.790	17	0.878
Man 6	9	0.826	9	0.866	9	0.891	9	0.769	14	0.903	8	0.842	9	0.854	21	0.900
Lan22	2	0.512	2	0.329	2	0.255	2	0.498	1	0.000	2	0.426	2	0.275	2	0.372
EMIC23	2	0.286	4	0.422	2	0.091	3	0.219	2	0.434	3	0.308	3	0.504	5	0.348
AAGG-209	7	0.738	7	0.811	4	0.259	4[§]	0.601[§]	5	0.511	6	0.711	6	0.730	9	0.772
AAAG-33	6	0.655	3	0.395	3	0.391	7	0.781	3	0.576	5	0.486	3	0.368	10	0.565
All Loci	45		41		33		34		38		41		39		81	0.659
Mean	6.43	0.655	5.86	0.627	4.71	0.499	4.86	0.567	5.43	0.541	5.86	0.634	5.57	0.605	5.45	0.578

[§]Discarded from population genetic structure analyses

Table 2 Pair-wise F_{ST} values between island populations of Galápagos flycatchers estimated from microsatellites with Arlequin 3.1.

	Santa Cruz	Santiago	Santa Fé	Floreana	Española	Isabela	S.Cristóbal
Santa Cruz	0.0000						
Santiago	0.0777	0.0000					
Santa Fé	0.0964	0.0265	0.0000				
Floreana	0.0286	0.1375	0.1649	0.0000			
Espanola	0.1480	0.1778	0.1775	0.1677	0.0000		
Isabela	0.0280	0.0083^{NS}	0.0489	0.0718	0.1208	0.0000	
S. Cristóbal	0.0869	0.0829	0.1076	0.1082	0.1056	0.0372	0.0000

NS: non-significant at the 0.05 level after sequential Bonferroni correction

Figure Titles

Figure 1 Map with the main islands from the Galápagos Archipelago (Darwin and Wolf islands not shown), number of samples used per island, and unidirectional migration rates. Islands sampled were Isabela, Santiago, Santa Cruz, Floreana, Santa Fé, Española, and San Cristóbal and the respective number of samples that were included in the morphological analyses are in parenthesis (total = 214 samples). Black arrows indicate high migration rates, from 0.1905 to 0.2832, also indicated by bold numbers. Gray arrows indicate low migration rates from 0.0113 to 0.0431, and rates smaller than 0.0087 are not presented.

Figure 2 Bayesian clusters (k=4) resulted from STRUCTURE analyses with data obtained from 137 Galápagos flycatchers using seven microsatellite loci. Each box is an island and colors represent the four clusters. Each vertical bar represents an individual bird and the proportion of each color in the bar represents the probability of assignment of this individual to each cluster (probabilities are in the y axis). Numbers below island names indicate the cluster to which it was assigned.

Figure 3 Multivariate distribution of the morphological variation (PCA) of 214 Galápagos flycatcher from seven Galápagos Islands. Each point is an individual, black circles represent females (n = 67) and white diamonds represent males (n = 147).

Figure 4 Multivariate distribution of the morphological variation (PCA) of Galápagos flycatcher males from seven Galápagos Islands. Each point is an individual and each color represents the island where it was captured and measured. The morphological distinctiveness of Española (black circles) and San Cristóbal (stars) can be seen when plotting PC1 against PC2 (A), and the distinctiveness of Santa Fé (gray circles) when PC1 is plotted against PC3 (B).

Figure 5 Means of PC1, PC2, and PC3 for Galápagos flycatcher males from each island plotted with 95% confidence interval bars. San Cristóbal is distinct from all islands but Santa Fé for PC1; Española is different from all islands for PC2; and Santa Fé is distinct from all islands for PC3. Letters above bars represent the different statistical groups.

Figure 1

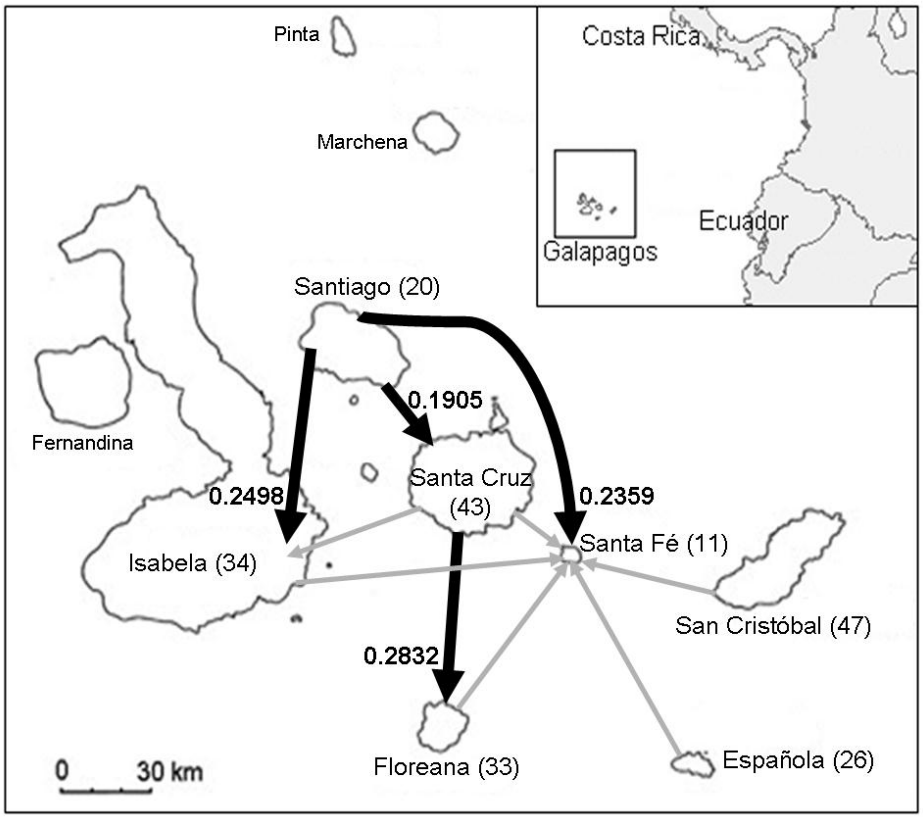

Pinta
Marchena
Costa Rica
Ecuador
Galapagos
Santiago (20)
0.1905
Fernandina
Santa Cruz
(43)
0.2498
0.2359
Santa Fé (11)
Isabela (34)
San Cristóbal (47)
0.2832
0
30 km
Floreana (33)
Española (26)

Figure 2

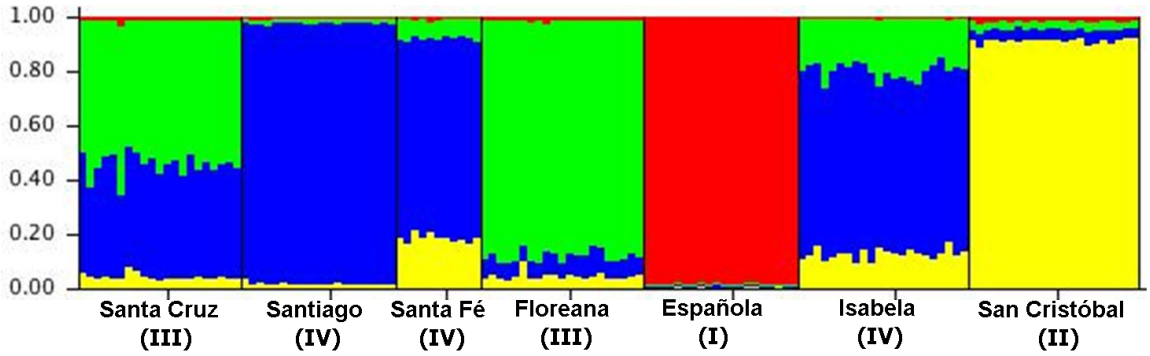

1.00
0.80
0.60
0.40
0.20
0.00
Santa Cruz (III)
Santiago (IV)
Santa Fé (IV)
Floreana (III)
Española (I)
Isabela (IV)
San Cristóbal (II)

Figure 3

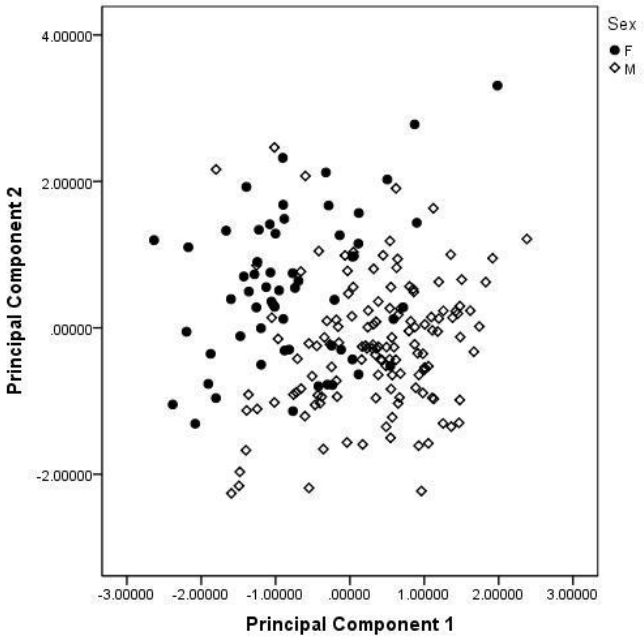

Sex
F
M
4.00000
2.00000
.00000
-2.00000
Principal Component 2
-3.00000
-2.00000
-1.00000
.00000
1.00000
2.00000
3.00000
Principal Component 1

Figure 4

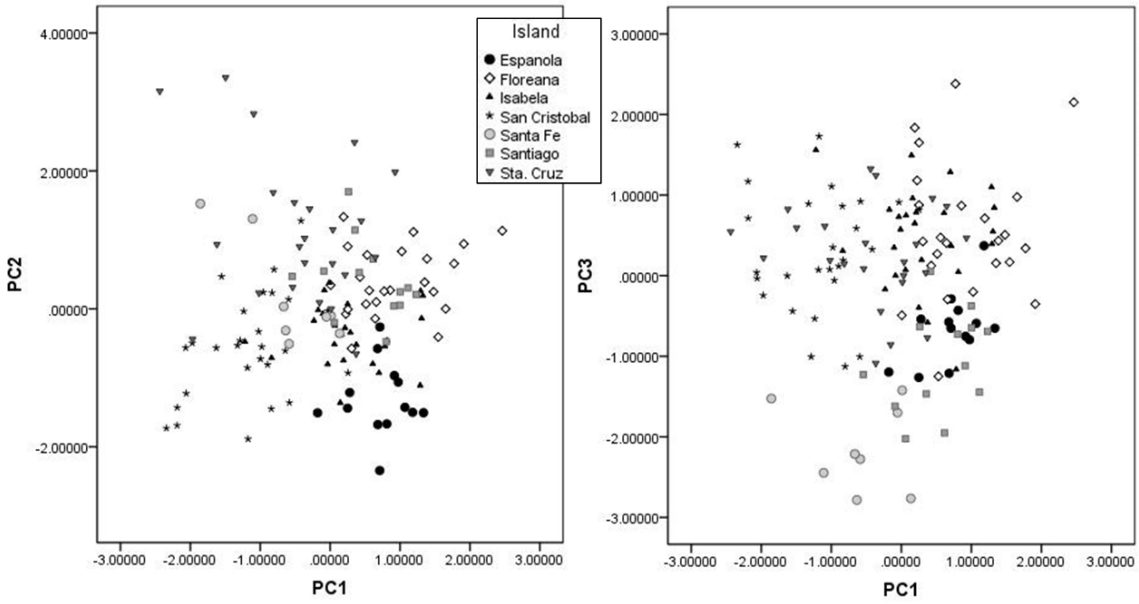
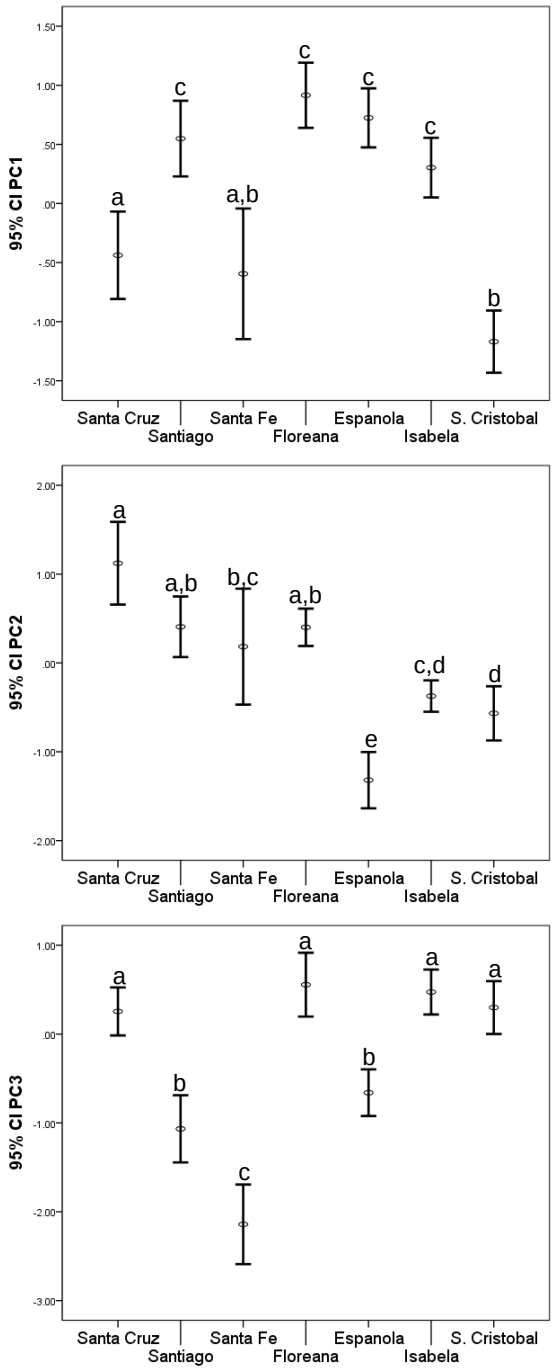

Island
Espanola
Floreana
Isabela
San Cristobal
Santa Fe
Santiago
Sta. Cruz
PC2
PC1
PC3
PC1

Figure 5


95% CI PC1
1.50
1.00
.50
.00
-.50
-1.00
-1.50
a
c
a,b
c
c
c
b
Santa Cruz
Santiago
Santa Fe
Floreana
Espanola
Isabela
S. Cristobal
95% CI PC2
2.00
1.00
.00
-1.00
-2.00
a
a,b
b,c
a,b
e
c,d
d
95% CI PC3
1.00
.00
-1.00
-2.00
-3.00
a
b
c
a
b
a
a

Supplemental material (Chapter 2)

Methods

Polymorphic microsatellite loci amplification

For the amplification of loci LTMR8, Man 3, Man 6, and Lan22 we used fluorescently labeled forward primers. The PCR reactions for amplifying LTMR8, Man 3, and Man 6 were made separately for each locus in a total volume of 13 µl, with 0.2 µl Biolase^{TM} Red DNA Polymerase (Bioline; 1 unit per reaction), 2.0 µl of 10X Biolase^{TM} buffer, 2 µl of dNTP (ProMega^{TM}) at 1 mM each (final concentration 0.15 mM of each dNTP), 1 µl of $MgCl_2$ at 50 mM (final concentration 3.85 mM), 0.5 µl of each primer (forward and reverse) at 10 µM (final concentration 0.385 µM each primer), and 0.5 µl of DMSO (di-methyl sulphate). The PCR reaction for amplifying the locus Lan22 was the same, except that only 0.5 µl of $MgCl_2$ was used (final concentration 1.92 mM). Amplification program started with a 94°C denaturation step for 2.5 min, and it was followed by 35 cycles with 30 sec at 94°C, 30 sec at specific annealing temperatures (see Ta in table S1), and 30 sec at 72°C, followed by a 72°C final extension step for 5 min.

For the amplification of EMIC23, AAGG-209, and AAAG-33, the 5'CAG tag was applied to the primer with lower melting temperatures, so in the forward primer for the EMIC23 locus and in the reverse primers for the loci AAGG-209 and AAAG-33. The 5'GTTT tail was applied to the other primer from each pair. The PCR reactions were made separately for each locus in a total volume of 12.5 µl, with 0.2 µl JumpStart^{TM} Taq DNA Polymerase (Sigma-Aldrich), 1.25 µl of 10X JumpStart^{TM} buffer, 0.75 µl of dNTP (ProMega) at 2.5 mM each, 1 µl of $MgCl_2$ at 25 mM, 0.125

µl of BSA (ProMega) at 10mg/ml, 0.5 µl of the primer with the 5'GTTT tail (usually the at 10 µM, 0.1 µl of the primer with the 5'CAG tag at 10 µM, and 0.45 µl of the fluorescent labelled tag that attaches to the 5'CAG tag from the primer. In order to enhance the annealing between the 5'CAG tag, the fluorescent labeled tag, and the target DNA, we used a "touch-down" amplification program, which started with a 95°C denaturation step for 10 min, followed by 30 cycles with 20 sec at 95°C, 20 sec at annealing temperatures that dropped 0.5°C per cycle, with the first cycle at 60°C and the 30th cycle at 45.5 °C, and 30 sec at 72°C, followed by 20 cycles with 20 sec at 95°C, 20 sec at 45.5 °C, and 30 sec at 72°C, followed by a 72°C final extension step for 10 min.

Molecular Sexing

To differentiate males and females we followed the molecular protocol proposed by Fridolfsson & Ellegren (1999) with modifications. This protocol is used to amplify a region of the CHD1 intron that shows a constant size difference between CHD1W and CHD1Z. For the Galápagos flycatchers (*Myarchus magnirostris*), one band of approximately 600bp (CHD1Z) was detected on males and two bands were detected on females, one with the same size of the male band (CHD1Z) and one of approximately 450bp (CH1DW).

For amplification of this DNA region, 1 µl of total genomic DNA (10-40 ng/µl) was used in a 10 µl reaction with 0.065 µl of Takara *Ex Taq* DNA Polymerase (0.3125 units), 1.5 µl of 10X *Ex Taq* Buffer without $MgCl_2$, 0.15 µl of Takara dNTP at 2.5 mM each (final concentration 37.5 µM of each dNTP), 0.65 µl of $MgCl_2$ at 25

mM (final concentration 1.625 mM), and 0.5 µl of each primer at 10 µM (2550F and 2718R; final concentration 0.5 µM each primer). Amplification program started with a denaturation step of 2 min at 94°C, and it was followed by 31 cycles of 94°C for 45 sec, 48°C for 45 sec, and 72°C for 45 sec, followed by a final extension step of 10 min at 72°C. Amplified DNA fragments were detected by loading 5 µl of amplicon on a gel star©-stained 2.0% agarose gel in TBE and running the gel at 90V for at least 100 min.

Table S1 Characteristics of the polymorphic loci for Galápagos flycathers used in this work in comparison to the original marker description. Na = number of alleles. Protocol names refer to different DNA polymerase and amplification methods used, as described in the Methods section of the Supplemental Material; Ta = annealing temperature.

	Primer Description				This publication		
Locus	Allele size range	Na	Species	Reference	Protocol used (see methods in Supplemental Material)	Allele size range	Na
LTMR8	140-148	3	*Chiroxiphia linearis*	McDonald & Potts 1994	Bioline; Ta = 52°C	157-199	17
Man 3	236	14	*Manacus manacus*	Piertney *et al.* 2002	Bioline; Ta = 52°C	210-274	17
Man 6	221	11	*Manacus manacus*	Piertney *et al.* 2002	Bioline; Ta = 51°C	200-284	21
Lan22	146-167	7	*Chiroxiphia lanceolata*	Duval & Nutt 2005	Bioline, with less $MgCl_2$; Ta = 54.5°C	152-156	2
EMIC23	298-314	12	*Empidonax minimus*	Tarof et al. 2001	Jumpstart	303-311	5
AAGG-209	285-350	7	*Mionectes striaticollis*	Bardeleben & Gray 2005	Jumpstart	263-297	9
AAAG-33	257-350	14	*Mionectes striaticollis*	Bardeleben & Gray 2005	Jumpstart	265-299	10

Table S2 Estimates of migration rates (proportion of individuals) between island populations of Galápagos flycatchers, derived by BayesAss as the proportion of immigrants received per island from each island. Total immigration into one island is the sum of immigration rates from all other islands.

	Migration from							
	Santa Cruz	Santiago	Santa Fé	Floreana	Española	Isabela	S.Cristóbal	Total Immigration
Migration into								
Santa Cruz	0.7745	0.1905	0.0067	0.0068	0.0061	0.0069	0.0086	0.2255
Santiago	0.0032	0.9825	0.0028	0.0029	0.0028	0.0028	0.0031	0.0175
Santa Fé	0.0137	0.2359	0.7008	0.0118	0.0121	0.0113	0.0144	0.2992
Floreana	0.2832	0.0079	0.0067	0.6817	0.0059	0.0068	0.0078	0.3183
Espanola	0.0029	0.0027	0.0030	0.0027	0.9827	0.0029	0.0031	0.0173
Isabela	0.0431	0.2498	0.0055	0.0060	0.0063	0.6808	0.0085	0.3192
S. Cristóbal	0.0033	0.0037	0.0027	0.0026	0.0028	0.0027	0.9823	0.0177

Means of the posterior distributions of m, the unidirectional migration rate into each population, are shown. Populations from which individuals migrate are listed in the columns, while populations into which they immigrate are listed in the rows. Values along the diagonal are the proportions of individuals from each island that do not migrate each generation.

Table S3 Extraction values (loadings) of each morphological variable for the first three principal components obtained (PC1, PC2, PC3) from the analysis of 127 male Galápagos flycatchers.

Morphological Measurements	Principal Components		
	PC1	PC2	PC3
Bird Weight	.579	.390	.647
Total Length	.706	-.408	.439
Bill Width	.432	.731	-.038
Bill Length	.794	.217	-.203
Wing	.814	-.382	-.240
Tarsus	.683	.471	-.342
Tail	.711	-.614	-.110

Table S4 Statistical tests on morphological data to compare Galápagos flycatchers (n = 214) from seven Galápagos islands. Individuals with missing data were all excluded for the MANOVA or excluded case by case for each ANOVA.

Test	All Birds	Males Only	Females Only
MANOVA – all variables	$F_{6, 187} = 15.392$ $p < 0.0001$	$F_{6, 127} = 13.524$ $p < 0.0001$	$F_{6, 53} = 6.626$ $p < 0.0001$
ANOVA – body weight	$F_{6, 204} = 36.698$ $p < 0.0001$	$F_{6, 139} = 32.776$ $p < 0.0001$	$F_{6, 58} = 11.279$ $p < 0.0001$
ANOVA – total length	$F_{6, 198} = 25.693$ $p < 0.0001$	$F_{6, 134} = 19.679$ $p < 0.0001$	$F_{6, 57} = 11.710$ $p < 0.0001$
ANOVA – bill width	$F_{6, 202} = 25.254$ $p < 0.0001$	$F_{6, 135} = 17.897$ $p < 0.0001$	$F_{6, 60} = 9.271$ $p < 0.0001$
ANOVA – bill length	$F_{6, 205} = 13.131$ $p < 0.0001$	$F_{6, 138} = 10.457$ $p < 0.0001$	$F_{6, 60} = 4.711$ $p < 0.0001$
ANOVA – wing length	$F_{6, 205} = 20.944$ $p < 0.0001$	$F_{6, 140} = 32.069$ $p < 0.0001$	$F_{6, 58} = 13.769$ $p < 0.0001$
ANOVA – tarsus length	$F_{6, 205} = 15.732$ $p < 0.0001$	$F_{6, 139} = 11.884$ $p < 0.0001$	$F_{6, 59} = 5.370$ $p < 0.0001$
ANOVA – tail length	$F_{6, 197} = 18.401$ $p < 0.0001$	$F_{6, 135} = 20.399$ $p < 0.0001$	$F_{6, 55} = 9.408$ $p < 0.0001$
ANOVA - PC1	$F_{6, 187} = 27.906$ $p < 0.0001$	$F_{6, 127} = 29.457$ $p < 0.0001$	$F_{6, 53} = 9.432$ $p < 0.0001$
ANOVA - PC2	$F_{6, 187} = 21.875$ $p < 0.0001$	$F_{6, 127} = 23.661$ $p < 0.0001$	$F_{6, 53} = 7.417$ $p < 0.0001$
ANOVA - PC3	$F_{6, 187} = 34.940$ $p < 0.0001$	$F_{6, 127} = 26.780$ $p < 0.0001$	$F_{6, 53} = 7.493$ $p < 0.0001$

Chapter 3

Tracking the origin of the Galápagos flycatcher lice, haemosporidian parasites, and feather mites

Eloisa H. R. SARI, Hans KLOMPEN, and Patricia G. PARKER

Manuscript Submitted to Journal of Biogeography

ABSTRACT

Aim To discover the origin of the lice, haemosporidian parasites, and feather mites found on/in the Galápagos flycatchers (*Myiarchus magnirostris*), by testing whether they colonized the islands with *M. magnirostris'* ancestors or if they were acquired by *M. magnirostris* after its arrival to Galápagos.

Location Galápagos Islands, Ecuador, and north-western Costa Rica.

Methods We collected lice, feather mites, and blood samples from *M. magnirostris* on seven Galápagos islands (n=254) and from its continental sister species (*M. tyrannulus*; n=74) in Costa Rica, and identified them to species level using traditional taxonomy and DNA sequencing.

Results The blood parasites from the two bird species were different: *M. tyrannulus* had *Plasmodium* only and very few *M. magnirostris* were infected by *Haemoproteus*

multipigmentatus from Galápagos Doves. *M. tyrannulus* was parasitized by three louse species, two of which were also found on *M. magnirostris* (*Ricinus marginatus* and *Menacanthus distinctus*). We also collected one louse specimen from *M. magnirostris* that was identified as *Brueelia interposita*, a species commonly found on finches and yellow warblers from Galápagos, but never recorded for *M. tyrannulus*. The richness of mite species was lower for *M. magnirostris* than for *M. tyrannulus*; all mite species or genera from *M. magnirostris* were also sampled on *M. tyrannulus*, but *M. tyrannulus* had two additional mite species.

Main Conclusions Our results revealed that two louse and three mite species found on *M. magnirostris* likely came to the Archipelago with these birds' colonizing ancestors, but that one louse species and the haemosporidian parasites were acquired from the Galápagos bird community after the arrival of the *M. magnirostris* lineage. We also confirmed that, for closely related hosts, island mite richness was lower than on the continent. This study elucidates the origin of island parasites and other symbionts (mites), which are rarely explored by the field of island biogeography.

Keywords Costa Rica; Feather mites; Galápagos; Haemosporida; Island colonization; *Myiarchus*; Phthiraptera.

INTRODUCTION

Studies of natural colonization of islands by living organisms have contributed greatly to the development of biogeography as a science (*e.g.*, MacArthur & Wilson, 1967; Whittaker, 1998; Losos & Ricklefs, 2010). Discovering the geographical origin of colonists that successfully arrive and become established on isolated islands, often differentiating into new species, is an important part of these studies. Perhaps the best described pattern generated from such studies is that, due to their isolation and limited size, islands present lower species diversity than larger continental areas (MacArthur & Wilson, 1967; Whittaker, 1998). Despite this extensive research, island biogeography of parasites has received minimal attention (Nieberding *et al.*, 2006), and colonization histories of island parasites are mostly unknown.

The study of host-parasite relationships and distributions is important to understand the biogeography of both groups (McDowall, 2000; Lafferty *et al.*, 2010). For instance, associations between different groups of fishes and their metazoan parasites have elucidated the colonization histories of the parasites (*e.g.* Plaisance *et al.*, 2008) and the historical biogeography of the hosts (*e.g.* McDowall, 2000) and that of both the hosts and their parasites (Carney *et al.*, 2000; Choudhury & Dick, 2001). These studies are also important because the pressure by parasites could influence the contraction and reduction of their host species (taxon cycling) in space and time, but more biogeographical work on parasites is still needed (Ricklefs, 2011).

Here we present a novel study about the origin of three taxonomic groups that are found in close association with the endemic Galápagos flycatcher (*Myiarchus magnirostris* Gould; Passeriformes: Suboscines: Tyrannidae): lice, haemosporidian

parasites, and feather mites (we refer to feather mites as symbionts rather than parasites, because there is little evidence they negatively affect their hosts' fitness; see Galván *et al*., 2012).

The Galápagos Islands (Fig. 1) are separated by about 1000 kilometres of open waters from the nearest mainland in Ecuador (Jackson, 1993; Geist, 1996). This archipelago has low species diversity in comparison to other islands or close continental landmasses (Linsley & Usinger, 1966; Jackson, 1993), representing a simpler community with more limited numbers of species interactions. Several lice, blood parasites, and mites have been studied in Galápagos birds (see Table 1). Therefore, these islands present an interesting opportunity to understand interactions between these symbionts and their bird hosts and also their colonization histories. Studying the origin of parasites from an endemic island species is important also because island populations are considered vulnerable to new diseases (Parker *et al.*, 2006; Lindstrom *et al.*, 2009). Island species have small and isolated populations, and might have lost their resistance to pathogens because of the pronounced colonization bottleneck, through genetic drift, or due to the absence of selective pressure by parasites (Frankham, 1998). The introduction of non-native parasites and pathogens to Galápagos is of great concern for the conservation of its unique and intact avifauna (Wikelski *et al.*, 2004; Parker *et al.*, 2006), so it is important to elucidate the colonization histories of the parasites themselves (Lindstrom *et al.*, 2009).

The distribution patterns of hosts and their parasites (and other symbionts) are influenced by both their intrinsic co-evolutionary dynamics, such as co-speciation and host range expansion events, and also by ecological changes and the external

environment (Thompson, 2005; Ricklefs, 2010). When host species colonize new locations, they can lose, transfer, or gain parasites (Lafferty *et al.*, 2010) and other symbionts. We can, therefore, classify the bird symbionts (parasites and others) found on the Galápagos Islands into three groups according to their origin: (A) those that came to the islands with the ancestors of their host species; (B) those that were acquired following colonization from other host species in the native bird community; and (C) those that were introduced to the islands by humans. In order to define the origin of the symbionts found on an endemic species from Galápagos, it is necessary to discover which of these are found on the continental closest relatives of their hosts and to compare them with the parasites and other symbionts found on the native bird community in Galápagos. Here we use this approach to find the origin of the chewing lice (Order Phthiraptera), feather mites (Hyporder Astigmata (Schatz *et al.*, 2011), and blood parasites (Order Haemosporida) from the Galápagos flycatchers.

The Galápagos flycatchers (*M. magnirostris*) are endemic to the Galápagos Archipelago, where they inhabit a variety of habitats and altitudes on most of the islands (Jackson, 1993). Recently, we proposed that the *Myiarchus* colonizers that gave origin to *M. magnirostris* arrived from Central America approximately 850,000 years ago, and their closest living relative is *Myiarchus tyrannulus* from Central and North America (Sari & Parker, 2012). In order to find the origin of the parasites and other symbionts from *M. magnirostris,* we have collected lice, feather mites, and blood samples from this bird species and from *M. tyrannulus* in Costa Rica.

We hypothesize that: (1) the parasites and feather mites that are present in both host species arrived to the Galápagos Archipelago with the ancestors of *M.*

magnirostris; and (2) those that are found on *M. magnirostris* but are not found on *M. tyrannulus* were acquired after *M. magnirostris* arrived to Galápagos, either from another bird species native to Galápagos or were introduced by humans. Furthermore, we hypothesize that the island bird host, *M. magnirostris*, will present lower parasite and mite species richness than the continental species *M. tyrannulus*. This is the first study, to our knowledge, that elucidates the origin of multiple taxonomic groups that live in close association with an island host.

MATERIALS AND METHODS

Collection of samples

We captured 254 Galápagos flycatchers (*M. magnirostris*) on seven Galápagos islands (Fig. 1) and 74 Brown-crested flycatchers (*M. tyrannulus*) in four localities in Costa Rica (Table 2). Birds were attracted to mist-nets using playback songs and were released after collection of samples. Blood samples were collected from all birds using heparinized capillary tubes. A few drops of blood were used to make two or three blood smears and the rest was stored in lysis buffer until DNA extraction. Blood smears were fixed in methanol for three minutes at the end of each sampling day.

Lice and feather mites were sampled via dust ruffling of the birds using 1% pyrethroid insecticide (Flea & tick powder; Zodiac brand). We worked approximately ½ to 1 teaspoon of the powder into birds' feathers and body (including the head), and let it sit for the time biometric measurements were taken, followed by ruffling of the feathers. During ruffling, birds were held over a clean plastic tray to collect dislodged

lice and mites, which were collected from the tray with a forceps using magnifying glasses, and were stored in 95% ethanol. Before dust-ruffling, all birds were visually examined, and lice and mites were opportunistically collected using entomological forceps. A total of 95 *M. magnirostris* and 63 *M. tyrannulus* were dust-ruffled, but the visual exam and opportunistic collection of lice and mites were done for 203 *M. magnirostris* and all 74 *M. tyrannulus* captured. Prevalence values were calculated for each species as the number of birds that carried that each louse or mite species divided by the total number of samples analysed (Table 3; Margolis et al., 1982).

Haemosporidian parasites screening

We used microscopy and PCR techniques to detect the presence of haemosporidian parasites in the blood samples. The blood smears were stained using Giemsa stain as described by Valkiūnas (2005) and inspected for parasites by microscopy for five minutes at 200x magnification, followed by examination of 100 fields at 1,000x magnification.

Genomic DNA was extracted from blood samples as described in Sari & Parker (2012). We used the method of Waldenstrom *et al.* (2004) to detect haemosporidian parasites from the genera *Plasmodium*, *Haemoproteus*, and *Leucocytozoon*, by amplifying the first region (580bp) of the mitochondrial gene cytochrome b (cyt b). All samples were screened twice, using slightly different PCR conditions (see below). In each PCR reaction, both a positive control (*Plasmodium* infected sample) and one or several negative controls (blanks) were used. All samples that amplified parasite DNA amplification only once were retested for

confirmation.

For the first DNA amplification, 1 µl of total genomic DNA was used in a 25µl reaction with 0.625 units of Takara *Ex Taq* DNA Polymerase (0.125 µl), 1X *Ex Taq* Buffer without $MgCl_2$, 0.2 mM of each dNTP, 1.75 mM of $MgCl_2$, and 0.4 µM of each external primer (HaemNF and HaemNR2). Amplification program had 20 cycles of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 45 sec, followed by a final extension step at 72°C for 10 min. 1µl of the amplicon from this reaction was used for a nested reaction with the same reagent concentrations, but using the internal primers HaemF and HaemR2. Amplification program was the same, but repeated for 35 cycles. For the second PCR screening, the PCR programs included a 3 minute denaturation step at 94°C (as in Waldenstrom *et al.*, 2004) and a lower annealing temperature (48°C), in order to enhance the detection of parasites.

Amplified internal DNA fragments (524bp) were detected on a gel star©-stained 2.0% agarose gel in TBE. PCR products were purified with Exonuclease and Antarctic Phosphatase and sequenced using Big DYE Terminator Kit with 30 cycles at 95°C for 20 sec, 50°C for 10 sec and 60°C for 4 min, and run in an ABI 2000 automatic sequencer . DNA fragments from all samples were sequenced in both directions using HaemF and HaemR2 (or HemoR; Perkins & Schall, 2002).

We used SeqManII v.4 (1989–1999, DNASTAR Inc., USA) to analyse sequence traces and create contigs. Sequences were aligned using Clustal W with default parameters as implemented in MEGA v.4.0 (Tamura *et al.*, 2007). Identification of haemosporidian parasite lineages was done by searching GenBank for sequences that were similar to those we obtained, using a megablast search option.

Lice and mites identification and molecular analyses of lice and their hosts

Mites and lice were initially sorted to morphospecies using a dissecting microscope. For species identification, representative specimens of each morphospecies were slide mounted and examined by specialists on each taxonomic group (lice – Dr. Ricardo Palma; mites – H. Klompen; see voucher numbers in Appendix S2) using a compound microscope. After that, we used a dissecting microscope to sort and identify to species a total of 204 feather mites and 2 lice from *M. magnirostris*, and 892 feather mites and 496 lice from *M. tyrannulus.*

We used a molecular approach to compare the one *Brueelia* louse we collected from one *M. magnirostris* to sequences of *Brueelia galapagensis* from Galápagos mockingbirds (*Mimus* spp.; Stefka *et al.*, 2011) available on GenBank and of *Brueelia interposita* from one Galápagos yellow warbler (*Dendroica petechia aureola*) that we sampled opportunistically. DNA was extracted using the voucher method (Cruickshank *et al.* 2001). We amplified and sequenced a fragment of approximately 650bp from the mitochondrial gene cytochrome oxidase c I subunit I (COI) using the primers LCO1490 and HCO2198 (Folmer *et al.*, 1994). PCR reactions were similar to those used for Haemosporida (see above), but including 0.08 mg/mL of BSA. Amplification program started with denaturation at 94°C for 4 min, followed by 35 cycles of 94°C for 60 sec, 40°C for 60 sec, and 72°C for 60 sec, followed by a final extension step at 72°C for 7 min.

We also used this molecular approach to obtain COI sequences from *Ricinus marginatus* lice collected from the two host species, *M. tyrannulus* from Costa Rica and *M. magnirostris.* We wanted to estimate the genetic distance between lice found

on both host species and to compare their distance with the genetic divergence between the bird species. We used DNA from four individual *Ricinus*, three collected from different *M. tyrannulus* and one from *M. magnirostris*.

We also obtained sequences from COI (1550bp) from *M. magnirostris* (n=5) and *M. tyrannulus* from Costa Rica (n=4) following Chaves *et al.* (2008), but with annealing temperature of 61°C and the same PCR reaction as the one we used for the louse COI. Sequence characteristics and genetic distances for lice and birds, between samples collected on Galápagos and in the continent, were calculated using MEGA v. 4, using Tamura-Nei (TN) substitution model. GenBank accession numbers are in Appendix S1.

RESULTS

Haemosporidian parasites

We obtained a 480 bp alignment of cyt b sequences from haemosporidian parasites. We detected a high prevalence of *Plasmodium* sp. in *M. tyrannulus* samples from Costa Rica, but the prevalence detected by microscopy (13.5%; 10/74) was lower than the prevalence detected by PCR (52.7% ; 39/74) (Table 3). *Plasmodium* was not found in any *M. magnirostris* sample, but we detected *Haemoproteus multipigmentatus* in five out of 254 *M. magnirostris* screened by PCR (2% prevalence). No parasites were seen in blood smears from *M. magnirostris*. All DNA sequences (n = 39) from *Plasmodium* obtained from *M. tyrannulus* were identical, and the DNA haplotype found was also described from a variety of bird species around the world (Beadell *et al.*, 2006).

Lice

Total prevalence of lice on *M. tyrannulus* was 66% (49/74) when considering all captured birds and 73% (46/63) when considering only the birds that were dust-ruffled, much higher than the prevalence of lice calculated for *M. magnirostris*, 0.9% (2/206). The lice collected from *M. tyrannulus* belonged to three species: *Ricinus marginatus* (Amblycera: Ricinidae), *Menacanthus distinctus* (Amblycera: Menoponidae), and *Philopterus* sp. (Ischnocera: Philopteridae), the last presenting the highest prevalence: 60.8% (45/74) (Table 3). These three species have been previously recorded from *M. tyrannulus* and other species from the genus *Myiarchus* and the Tyrannidae family (Oniki, 1999; Price *et al.*, 2003).

We collected only two individual lice from *M. magnirostris*, including one *Ricinus marginatus* on Santa Cruz, and one *Brueelia interposita* (Ischnocera: Philopteridae) on San Cristóbal. While the occurrence of *Ricinus marginatus* on *M. magnirostris* was previously described, *Brueelia* has never been recorded for *Myiarchus* flycatchers (Price *et al.*, 2003). In addition to *Ricinus marginatus*, specimens of *Menacanthus distinctus* were also collected from *M. magnirostris* before (R.L. Palma pers. comm. 2011), but we did not find this species in our collections.

Lice and host genetic divergence

Our COI sequences of *Brueelia interposita* (678 bp) from *M. magnirostris* and from the Galápagos yellow warbler were identical, but there were 106 segregating sites between these and sequences of *Brueelia galapagensis* from Galápagos

mockingbirds (18.1% TN genetic distance), confirming our specimens are not *B. galapagensis*.

We found 36 polymorphic sites when comparing *Ricinus* from Galápagos (n=1) and from Costa Rica (n=3) using 608 bp of COI. The net genetic distance between lice from these two regions was estimated as 6.27% (0.0627 ± 0.0099). For the hosts (*M. magnirostris* and *M. tyrannulus*), using 611 bp from the same COI region as sequenced for the lice, we found seven polymorphic sites. The net genetic distance between *M. magnirostris* (n=5) and its sister species *M. tyrannulus* (n=4) was estimated as 0.66% (0.0066 ± 0.0031), almost ten times smaller than the genetic distance between the lice.

Mites

Feather mites were found with a total prevalence of 85.1% (63/74) for *M. tyrannulus* and 11.3% (23/203) for *M. magnirostris*. Five species of feather mites were collected from *M. tyrannulus*: *Trouessartia* sp. (Trouessartiidae), *Nycteridiocaulus* nr. *lamellus* Atyeo 1966, *Tyrannidectes berlai* Mironov 2008, *Amerodectes* sp. (Proctophyllodidae), and one species of Analgidae not further identified (Table 3). Among these, *Trouessartia* sp., *Tyrannidectes berlai*, and *Nycteridiocaulus lamellus* were commonly found, but the other two species were rarely collected.

Three species of mites were collected from *M. magnirostris*: *Trouessartia* sp., *Tyrannidectes berlai*, and *Nycteridiocaulus* sp. (Table 3); and the first two appear to be the same species found on *M. tyrannulus*. Identification of *Trouessartia* to species

level, however, is hampered by the fact that this group has not been revised recently. In the most comprehensive species level keys for the genus (Santana, 1976), the *Trouessartia* from *Myiarchus* keys close to *Trouessartia corolligera* Gaud 1968, from South Pacific starlings (*Aplonis* spp.), but this revision lacks any records of *Trouessartia* species from Tyrannidae. Valim *et al.* (2011) listed *Trouessartia* as associated with Tyrannidae, but we could not find records of specific identification for these *Trouessartia* species.

The third species collected from *M. magnirostris*, *Nycteridiocaulus* sp., does not appear to be identical to the corresponding species in Costa Rica, which is probably *Nycteridiocaulus lamellus*. Identification in this group is largely based on males, and although we obtained a few males from Costa Rica, we have none from Galápagos. However, based on consistent differences in the dorsal ornamentation in females (Fig. 2) we conclude that the specimens from Galápagos are most probably not conspecific with those from *M. tyrannulus* from Costa Rica.

DISCUSSION

We were interested in understanding the origin of the lice, blood parasites, and mites found on the Galápagos flycatchers (*M. Magnirostris*). We found that different parasite species have different origins. The haemosporidian parasites detected in *M. magnirostris* were acquired after its arrival to the islands from the endemic Galápagos doves (*Zenaida galapagoensis*). Two of the louse species from *M. magnirostris* came to the islands with the ancestors of this host and one louse species was acquired from the native bird community. And finally, we found that three species of mites from *M.*

magnirostris probably came with the ancestors of these birds to the Galápagos, but morphological differentiation (and perhaps speciation) was observed in one of these mite species.

We also detected much lower prevalence of parasites and mites for *M. magnirostris* than for *M. tyrannulus* (Table 3). In addition, the total number of commensal (mite) species found on *M. magnirostris* (n=3) was lower than the number of species found on *M. tyrannulus* (n=6), which supports the expected pattern of lower species diversity on islands compared to continental areas (MacArthur & Wilson, 1967; *e.g.* Smith & Carpenter, 2006). These findings might reveal an island syndrome, but could also be related to differences in environmental conditions between Galápagos and Costa Rica, as it has been proposed that birds that live in drier environments have lesser lice than birds in more humid locations (Moyer *et al.*, 2002).

Haemosporidian parasites

The *Haemoproteus multipigmentatus* that we detect in *M. magnirostris* belongs to the subgenus *Haemoproteus* and is found parasitizing Galápagos doves, with very high prevalence and intensity, and also dove species from the New World (Santiago-Alarcon *et al.*, 2010; Valkiūnas *et al.*, 2010). The competent host for this parasite in Galápagos is the fly *Microlynchia galapagoensis* (Hippoboscidae), which is a species associated only with the Galápagos doves in Galápagos (Valkiūnas *et al.*, 2010), and there are no reports of this fly on a flycatcher.

The *Haemoproteus* parasites that are commonly found parasitizing passerine birds elsewhere in the world belong to the subgenus *Parahaemoproteus* (Beadell *et al.*, 2006; Martinsen *et al.*, 2008). No blood parasites have been reported before for Galápagos passerines (e.g. Lindstrom *et al.*, 2009), but parasites from the subgenus *Parahaemoproteus* were detected in five blue-footed boobies (*Sula nebouxii*) in Galápagos (Levin *et al.*, 2011). Parasites from the subgenus *Haemoproteus,* like *H. multipigmentatus,* were thought to be specific to Columbiformes (doves and pigeons; Santiago-Alarcon *et al.*, 2010), but recently Levin *et al.* (2011) have reported the association of this subgenus with frigatebirds and gulls. These parasites, however, have rarely (if ever) been reported for passerines.

Our detection of *H. multipigmentatus* in *M. magnirostris* was only by PCR, never by microscopy. The absence of meronts or gametocytes (reproductive stage of a haemosporidian parasite) in blood smears indicates that these parasites may not be completing their life cycle (Valkiūnas, 2005) in *M. magnirostris*, and that this bird species might not be a competent host. The occurrence of *H. multipigmentatus* in *M. magnirostris* could be the result from a "spill-over", where, in rare cases, a hippoboscid fly that has bitten an infected dove could leave its typical host and inject *Haemoproteus* sporozoites into another bird species. These sporozoites could then be detected by PCR, but not in blood smears (Valkiūnas *et al.*, 2009).

The great majority of our *M. magnirostris* samples were collected during the months of July and August, during the Galápagos dry season, but our five samples of *Haemoproteus* were found among the few birds (n=27) captured during the wet season (February-April). Transmission of blood parasites is expected to be higher

during the wet season because of the increased number of vectors; the higher number of flies available could hence be associated with the "spill-over" of *Haemoproteus multipigmentatus* to *M. magnirostris*. Also, the five birds with *Haemoproteus* were captured on the island of Santa Cruz, in the city of Puerto Ayora, one of the most urbanized areas of the Galápagos Archipelago. We believe that this "spill-over" could also be caused by environmental disturbance, indicating that human activities could be actively changing the species interactions of the Galápagos natural community. Unfortunately our data do not allow testing this hypothesis. Furthermore, Santa Cruz was the island where we collected the most samples (n= 70; Table 1) and our detection of *H. multipigmentatus* in *M. magnirostris* only on that island could therefore be biased.

Lice

The two louse species from both *M. magnirostris* and *M. tyrannulus*, *Ricinus marginatus* and *Menacanthus distinctus*, most probably came to Galápagos with the ancestors of *M. magnirostris*. Many species of chewing lice are found only on a single bird host species (Johnson & Clayton, 2003), but here we have an example of host speciation without the speciation of two species of body louse (Amblycera). The same pattern is observed for the endemic Galápagos hawk (*Buteo galapagoensis*), which shares all louse species with its continental sister species, the Swainson's hawks (*Buteo swainsoni*; Price *et al.*, 2003; Whiteman *et al.*, 2007, 2009). The Galápagos hawk diverged from its sister species only about 180,000 years ago (Bollmer *et al.* 2006), while *M. magnirostris* and *M. tyrannulus* diverged about

850,000 years ago, suggesting that the process of speciation for lice can take much longer that it takes for their hosts, as mentioned by McDowall (2000). Another corroborating example of this pattern is seen for the Galápagos dove, which diverged from its continental sister species about 2 million years ago (Johnson & Clayton, 2000), and they share one species of louse (Johnson & Clayton, 2002; Price *et al.*, 2003).

We revealed that the genetic divergence between *Ricinus* (6.27%) collected from both *Myiarchus* species is approximately ten times larger than the divergence between their host species (0.66%). When Whiteman *et al.* (2009) compared the genetic distance between the head louse *Craspedorrhynchus* sp. found on the Galápagos hawks and on its sister species with the distance between the two host species, they also found a difference of the same magnitude. This trend can be explained by the faster generation time for the lice in comparison to their hosts: each generation of a flycatcher (1 year) corresponds to about six generations of a louse (40-60 days; Johnson & Clayton, 2003). In addition, it is thought that louse mitochondrial DNA has a much faster evolutionary rate than the homologous molecules in birds (Page *et al.*, 1998).

This higher genetic divergence obtained between the lice in comparison to their hosts, paired with the invariable morphology for the lice, might be a result of the differences between the environment that hosts and their parasites experience. The speciation of *M. magnirostris* can be explained by drift and also by natural selection, due to the colonization of a new area with a different environment. While drift was also involved in the genetic differentiation of the lice on these two bird sister species,

the environment for the lice are the feathers and body of their hosts, which have had little to no structural change and therefore do not represent a selective pressure that would invoke morphological changes in the lice. However, we have not explored the morphology of the louse samples we collected, and it is possible that the taxonomy of *Ricinus* needs to be revisited. Based on our findings, we suggest that general taxonomy of island parasites deserves a closer look.

Finally, we collected a *Brueelia interposita* louse from one *M. magnirostris* but not from *M. tyrannulus*. Three *Brueelia* species can be found in Galápagos: *Brueelia galapagensis* on Galápagos mockingbirds, *Brueelia chelydensis* on four Darwin finches, and *Brueelia interposita* on three Darwin finches and Galápagos yellow warblers (Price *et al.,* 2003; e.g. Stefka *et al.,* 2011; R.L. Palma pers. comm. 2011). Because the *Brueelia* from *M. magnirostris* and from the Galápagos yellow warbler are morphologically and genetically identical, we believe that this represents a classical example of a parasite that was acquired by *M. magnirostris* after its arrival to the islands and interaction with the local community.

Brueelia presents high dispersal ability through phoresis (transport), in which it moves to different hosts by attaching to parasitic flies (Diptera: Hippoboscidae; Harbinson & Clayton, 2011; Stefka *et al.*, 2011). Wing louse species like *Brueelia* frequently present evolutionary histories less associated with their hosts, with fewer cospeciation events (Johnson *et al.*, 2002; Harbinson & Clayton, 2011). Stefka *et al.* (2011) studied the phylogeography of Galápagos mockingbirds and three of their ectoparasite species and noted that *Brueelia* had the least population structure, implying that its phoresis on Hippoboscidae flies in Galápagos is substantial. Deem *et*

al. (2011) reported the presence of the Hippoboscidae fly *Ornithoica vicina* on several Galápagos terrestrial birds, including the Yellow warbler (Table 1), but not *M. magnirostris*. The *Brueelia* we collected could have been transported by this Hippoboscidae fly from a warbler to a *M. magnirostris*. This non-specific dispersal of hippoboscid flies is consistent with our finding that a fly-transmitted blood parasite specific to Galápagos doves (*H. multipigmentatus*) was detected in Galápagos flycatchers.

Mites

Two of the five mite species we identified were identical on *M. tyrannulus* and *M. magnirostris*: *Trouesartia* sp. and *Tyrannidectes berlai*. *Tyrannidectes berlai* was described for *M. tyrannulus* from Brazil and it seems to be host-specific to the *Myiarchus* genus (Mironov *et al.*, 2008; Valim & Hernandes, 2010; Valim et al., 2011); its presence in Costa Rica and Galápagos does represent however a significant range extension of this mite. Similarly, *Trouessartia* can be quite host-specific and, even though we could not get to species identification, the specimens from *M. tyrannulus* and *M. magnirostris* are different from the other *Trouessartia* species reported for Galápagos, *Trouessartia geospiza* from the small ground finch (*Geospiza fuliginosa*; OConnor *et al.*, 2005). In addition, *Trouessartia* (n=25) we collected opportunistically from the other Tyrannidae from Galápagos, *Pyrocephalus rubinus* (n=1), are very similar but different from those collected from *Myiarchus*.

It is interesting that, among the three mite genera shared between *M. tyrannulus* and *M. magnirostris*, *Nycteridiocaulus* is the only one in which

morphological differentiation, and perhaps speciation, has occurred after colonization. Genetic studies comparing these three lineages of mites would be insightful to understand their rate of diversification in relation to each other and to their hosts.

Why did some parasites and mites from *M. tyrannulus* not colonize Galápagos?

Lower parasite diversity on islands can result because of the founder effect inherent in the colonization process, in which colonizing hosts may reach islands carrying only a partial subset of their native parasite community (Nieberding *et al.*, 2006; Lafferty *et al.*, 2010). Our results support this idea, since we recorded a few parasites and mites on *M. tyrannulus* in Costa Rica that we could not find in Galápagos. We detected *Plasmodium* sp. in *M. tyrannulus* from Costa Rica with high prevalence (53%), but we did not detect this parasite in any *M. magnirostris*. We can think of three explanations for the absence of *Plasmodium* in *M. magnirostris*. First, the common ancestors of *M. tyrannulus* and *M. magnirostris* were not infected by *Plasmodium* because this parasite only started interacting with the *M. tyrannulus* lineage after its split from the *M. magnirostris* lineage approximately 850,000 years ago. Another possibility is that *Plasmodium* was present in the common ancestors of these *Myiarchus* but the birds that arrived to Galápagos either were not infected or were infected but were not able to successfully colonize the islands. *Plasmodium* can be pathogenic and negatively impact host fitness and survival. The colonization of a new environment is a very stressful event, and birds with higher fitness had higher chances to successfully establish on Galápagos. Finally, *Plasmodium* could have

arrived to Galápagos with *M. magnirostris* ancestors but gone extinct because of the absence of a competent vector in which it could complete its life cycle and be transmitted to other hosts. Although *Plasmodium* has been detected in Galápagos penguins (*Spheniscus mendiculus*; Levin *et al.*, 2009), the responsible vector has not yet been identified. There are three species of mosquitoes in Galápagos that could potentially be vectors for this parasite but none of them was present before 200,000 years ago (Bataille *et al.*, 2009; Whiteman *et al.*, 2005), long after the estimated arrival date for *Myiarchus* flycatchers to Galápagos (Sari & Parker, 2012).

Philopterus was the louse species we found with the highest prevalence (60.8%) on *M. tyrannulus,* but it has never been found on *M. magnirostris* by us or other researchers. Among the three louse species we collected from *M. tyrannulus*, *Philopterus* is probably the one with the most specialization to stay attached to the host's feathers; it belongs to the suborder Ischnocera while the other two louse species belong to the suborder Amblycera, which generally comprises more mobile lice that can leave their host in search of a new one (Johnson & Clayton, 2003). Similarly to our discussion for *Plasmodium,* it is possible that the ectoparasite community of *M. tyrannulus* has changed through time, and *Philopterus* might not have been present on the common ancestors of *M. tyrannulus* and *M. magnirostris* when these two lineages split approximately 850,000 years ago. On the other hand, because lice can have a patchy distribution on their hosts, *Philopterus* could be absent just from the *Myiarchus* individuals that colonized Galápagos by chance only (and they "missed the boat"; see Paterson *et al.* 1999). Another explanation could be associated with the relative damage that *Philopterus* could cause to host feathers. It is

thought that Ischnoceran lice can cause enough damage to the birds' feathers to result in thermoregulatory costs for the birds, and consequently, reduce the fitness of parasitized individuals (Clayton *et al.*, 1999). In this case, the birds that were parasitized by *Philopterus* may not have successfully arrived and established on Galápagos.

For *M. tyrannulus*, we have detected two species of feather mites that we did not find on *M. magnirostris*. These were detected in one or very few *M. tyrannulus*, while the three mite species that were found on both bird species presented a much higher prevalence on *M. tyrannulus*. There is little evidence that feather mites can affect their hosts' fitness (Galván *et al.*, 2012), so probably the two mite species that did not colonize Galápagos were not present in *M. magnirostris* ancestors.

Final conclusions

Our study shows that most of the parasite and commensal species carried by the Galápagos flycatchers (*M. magnirostris*) probably came with the ancestors of these birds to Galápagos, and others have spilled over to flycatchers from other native hosts. We also confirmed that the colonization of a new area by a host and the interactions of this host with the local community can change the host-parasite interactions and the specificity of parasites. We did not note any parasites or feather mites in/on *M. magnirostris* that could be characterized as introduced by humans, but the knowledge about which parasites are native to a host is equally important for the conservation of this host species and also the community with which it interacts. The characterization of the origin from these symbionts is an important piece for our

understanding about the evolutionary history of species interactions in the Galápagos community.

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Table 1 Bird parasites and mites registered for Galápagos that are relevant for this study. Species within taxonomic groups are in alphabetical order.

Taxonomic Group	Parasite species	Hosts in Galápagos	References
	Haemoproteus (Haemoproteus) iwa	Great frigatebird (*Fregata minor*) Magnificent frigatebird (*Fregata magnificens*)	Padilla *et al.*, 2006; Levin *et al.*, 2011
	Haemoproteus (Haemoproteus) jenniae	Swallow tail gull (*Creagrus furcatus*)	Levin *et al.*, in press
	Haemoproteus (Haemoproteus) multipigmentatus	Galápagos dove (*Zenaida galapagoensis*)	Santiago-Alarcon *et al.*, 2010; Valkiūnas *et al.*, 2010
	Haemoproteus (Haemoproteus) sp.	Red-footed booby (Sula sula) Swallow tail gull (*Creagrus furcatus*) Nazca booby (*Sula granti*)	Padilla *et al.*, 2006; Levin *et al.*, 2011
	Haemoproteus (Parahaemoproteus) sp.	Blue-footed booby (*Sula neubouxii*)	Levin *et al.*, 2011
	Plasmodium sp.	Galápagos penguin (*Spheniscus mendiculus*)	Levin *et al.*, 2009
Chewing lice Order Phthiraptera			
	Brueelia chelydensis	Large tree finch (*Camarhynchus psittacula*) Medium ground finch (*Geospiza fortis*) Small ground finch (*Geospiza fuliginosa*) Cactus finch (*Geospiza conirostris*)	Linsley & Usinger, 1966; Price *et al.*, 2003
	Brueelia galapagensis	Galápagos mockingbirds (*Mimus* spp) Small ground finch (*Geospiza fuliginosa*)	Price *et al.*, 2003; Stefka *et al.*, 2011
	Brueelia interposita	Yellow warbler (*Dendroica petechia aureola*)	Linsley & Usinger, 1966; R.L. Palma, pers. comm. 2011
	Colpocephalum turbinatum, *Craspedorrhynchus* sp., *Degeeriella regalis*	Galápagos hawk (*Buteo galapagoensis*)	Price *et al.*, 2003; Whiteman *et al.*, 2007, 2009
	Columbicola macrourae, *Physconelloides galapagensis*	Galápagos dove (*Zenaida galapagoensis*)	Johnson & Clayton, 2002; Price *et al.*, 2003
	Columbicola macrourae, *Physconelloides galapagensis*	Galápagos hawk (*Buteo galapagoensis*) – atypical host/straggler	Whiteman *et al.*, 2004
	Menacanthus distinctus	Galapagos flycatcher (*M. magnirostris*)	R.L. Palma pers. comm. 2011

	Myrsidea darwini	Large tree finch (*Camarhynchus psittacula*) Small ground finch (*Geospiza fuliginosa*) Large ground finch (*Geospiza magnirostris*)	Palma & Price, 2010
	Myrsidea nesomimi	Galápagos mockingbirds (*Mimus* spp.), and registered straggling events to several Darwin finch species	Palma & Price, 2010; Stefka *et al.*, 2011
	Myrsidea ridulosa	Yellow warbler (*Dendroica petechia aureola*)	Palma & Price, 2010
	Philopterus insulicola	Galápagos Vermillion flycatcher (*Pyrocephalus rubinus nanus*)	Linsley & Usinger, 1966; Price *et al.*, 2003
	Ricinus marginatus	Galápagos flycatcher (*Myiarchus magnirostris*)	Price *et al.*, 2003
	Other louse species	Darwin finches	Price *et al.*, 2003
Feather mites Hyporder Astigmata	*Amerodectes atyeoi* *Dermoglyphus* sp. *Mesalgoides geospizae* *Proctophyllodes darwini* *Strelkoviacarus* sp. *Trouessartia geospiza* *Xolalges palmai*	Darwin ground finches (*Geospiza* spp.)	Mironov & Pérez, 2002; OConnor *et al.*, 2005; Lindstrom *et al.*, 2009
	Analges spp. (4 species)	Galápagos mockingbirds (*Mimus* spp.)	Stefka & Smith, pers. comm.; Stefka *et al.*, 2011;
Parasitic flies Order Diptera Family Hippoboscidae	*Microlynchia galapagoensis*	Galápagos dove (*Z. galapagoensis*) Galápagos mockingbird (*Mimus parvulus*)	Valkiūnas *et al.*, 2010; Deem *et al.* 2011
	Ornithoica vicina	Yellow warbler (*Dendroica petechia aureola*) Large tree finch (*Camarhynchus psittacula*) Darwin ground finches (Geospiza spp.) Galápagos mockingbirds (*Mimus* spp.) Short-eared owl (*Asio flammeus*)	Deem *et al*. 2011

Also see Deem *et al.*, 2011 and Parker *et al.*, 2006 for other compilations of Galápagos bird parasites studies.

Table 2 Number of birds per island or locality sampled for haemosporidian parasites screening tests and for collection of lice and mites..

Locality	Haemosporidian screen	Visual exam and/or Dust-ruffle	Dust-ruffle
M. magnirostris			
Galápagos Islands	254	203	94
Española	26	26	11
Floreana	33	33	26
Isabela	39	35	20
San Cristóbal	55	47	6
Santa Cruz	70	35	17
Santa Fé	11	11	9
Santiago	20	16	5
M. tyrannulus			
Costa Rica	74	74	63
Palo Verde	19	19	17
Santa Rosa	37	37	33
Horizontes	2	2	2
El Hacha	16	16	11

Table 3 Prevalence data (number of infected birds/total birds sampled) for blood parasites, lice and feather mites for the two bird species, Galápagos flycatchers (*M. magnirostris*) and Brown-crested flycatchers (*M. tyrannulus*). Numbers for feather mites was calculated based on examination of all samples from Galápagos (n = 203) and 34 samples from Costa Rica.

Parasites	*M. magnirostris*	*M. tyrannulus*
Haemoproteus multipigmentatus	2% (5/254)	0
Plasmodium sp.	0	52.7% (39/74)
Brueelia interposita	0.5% (1/203)	0
Menacanthus distinctus	0*	14.9% (11/74)
Philopterus sp.	0	60.8% (45/74)
Ricinus marginatus	0.5% (1/203)	17.6% (13/74)
Amerodectes sp.	0	2.9% (1/34)
Analgidae	0	8.8% (3/34)
Nycteridiocaulus spp.	0.5% (1/203)	26.5% (9/34)
Trouessartia sp.	7.4% (15/203)	76.5% (26/34)
Tyrannidectes berlai	2.5% (5/203)	73.5% (25/34)

* species registered for *Myiarchus magnirostris* by R.L. Palma (Museum of New Zealand, Wellington, New Zealand).

Figure titles

Figure 1 Map of the Galápagos archipelago with main islands where *M. magnirostris* is distributed. Sampled islands are indicated with stars. The map insertion shows the position of Galápagos relative to Costa Rica, where samples from *M. tyrannulus* were collected, and to continental Ecuador.

Figure 2 Female specimens of the mite *Nycteridiocaulus* (Proctophyllodidae) collected in Costa Rica from *Myiarchus tyrannulus* (left) and in Galápagos from *Myiarchus magnirostris* (right). Between the two host species, morphological differentiation of dorsal ornamentation can be observed for this lineage of mites.

Figure 1

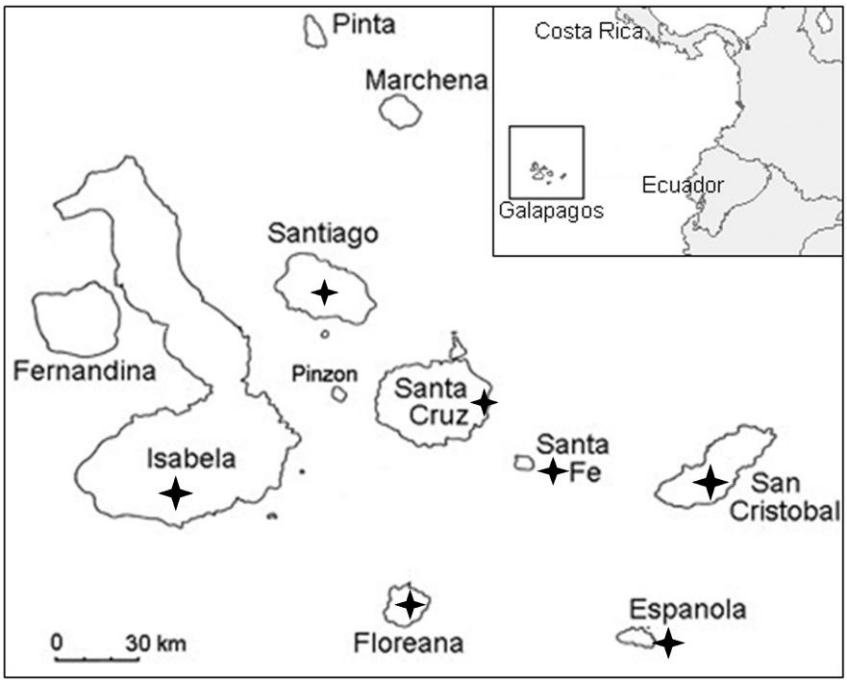
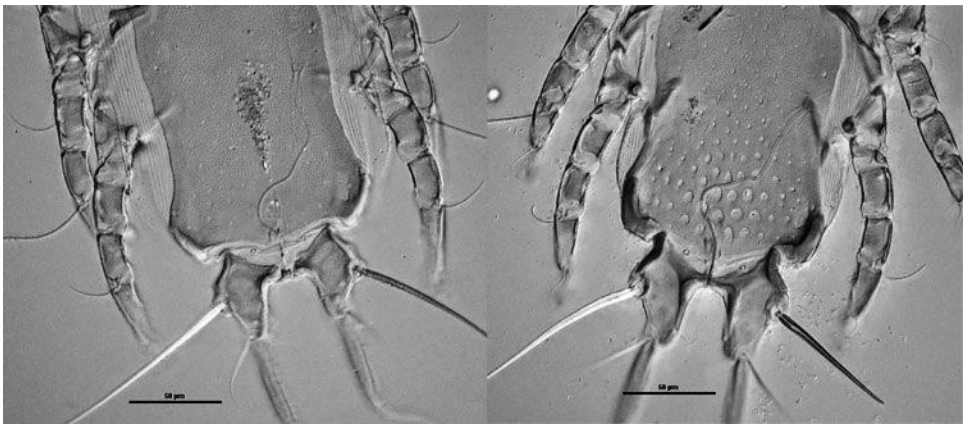


Figure 2



Supplemental information (Chapter 3)

Appendix S1 GenBank accession numbers. Codes after host species are band numbers (for *Myiarchus magnirostris* and *Dendroica petechia aureola*) or personal identification numbers (for *Myiarchus tyrannulus*).

Parasite	Host species and identification	Accession number
Haemoproteus multipigmentatus	*Myiarchus magnirostris* (ES1014) *Myiarchus magnirostris* (ES1059) *Myiarchus magnirostris* (JH1378) *Myiarchus magnirostris* (JH1410) *Myiarchus magnirostris* (JH1593)	
Plasmodium sp.	*Myiarchus tyrannulus* (n=39)	
Brueelia interposita	*Dendroica petechia aureola* (ES1111) *Myiarchus magnirostris* (ES1204)	
Menacanthus distinctus	*Myiarchus tyrannulus* (CR04) *Myiarchus tyrannulus* (CR36)	
Ricinus marginatus	*Myiarchus magnirostris* (ES1065) *Myiarchus tyrannulus* (CR35) *Myiarchus tyrannulus* (CR36) *Myiarchus tyrannulus* (CR51)	
	Bird COI sequences	
	Myiarchus magnirostris (ES1008) *Myiarchus magnirostris* (ES1025) *Myiarchus magnirostris* (ES1049) *Myiarchus magnirostris* (ES1077) *Myiarchus magnirostris* (ES1123) *Myiarchus tyrannulus* (CR1) *Myiarchus tyrannulus* (CR25) *Myiarchus tyrannulus* (CR63) *Myiarchus tyrannulus* (CR66)	

Appendix S2 Voucher numbers for mite and louse species collected from *Myiarchus* flycatchers. Mites are deposited in the Ohio State University Acarology Collection (OSAL) and lice are deposited in the Museum of New Zealand Te Papa Tongarewa (AI).

Taxon	Voucher numbers
Analgidae specimen	OSAL103977 F
Nycteridiocaulus sp.	OSAL103989 F
Nycteridiocaulus cf. *lamellus* Atyeo 1966	OSAL103797 M; OSAL103991 F
Pterodectes sp.	OSAL102661 F
Trouessartia sp.	OSAL103968 M; OSAL103966 F
Tyrannidectes berlai Mironov 2008	OSAL103983 M; OSAL103980 F
Brueelia interposita from *Dendroica petechia aureola*	AI.028425
Brueelia interposita from *Myiarchus magnirostris*	AI.028424
Menacanthus distinctus	AI.028429; AI.028430; AI.028431
Philopterus sp.	AI.028426; AI.028427; AI.028428
Ricinus marginatus from *Myiarchus magnirostris*	AI.028432
Ricinus marginatus from *Myiarchus tyrannulus*	AI.028437; AI.028436; AI.028433

Chapter 4

Do parasites affect the probability of colonization of their hosts? A case study of immune responses to parasitism in *Myiarchus tyrannulus* from Costa Rica

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Abstract

It is not uncommon to see descendant host populations or species that do not have some of the parasites that are strongly associated with their founder or ancestral populations. Parasites can be harmful for their hosts, affecting their health status and fitness. It is then possible that the parasites that do not colonize new areas are the ones that affect the health and fitness of their hosts more negatively, making these individual hosts less likely to be colonizers. Here we tested this hypothesis by analyzing the health condition and immune responses of a group of host birds, *Myiarchus tyrannulus* from Costa Rica, in relation to their feather mites, chiggers, lice, and blood parasites. These birds are the closest relatives to *Myiarchus magnirostris*, the Galápagos flycatchers, and some of the most common parasites found in *M. tyrannulus* from Costa Rica (the louse *Philopterus* and the blood parasite *Plasmodium*) did not successfully colonize the Galápagos Islands. We estimated the health of the birds by measuring their body condition index, packed red blood cell volume, and white blood cell counts and differentials. Different from what we predicted, we did not find that *Philopterus* and *Plasmodium* affected *M. tyrannulus*

health more negatively than the parasites that did successfully colonize Galápagos. We found that chiggers best explained the variation in the largest number of health parameters of their hosts. Our work shows that the interpretation of health parameters is not simple and that most immune responses are specific to the interaction between a particular host and a particular parasite and cannot be easily generalized outside of that context.

Introduction

Parasites are often detrimental for their host's health condition and fitness, but the extent of this cost depends on the type of parasite and the ability of the host to fight against it. The immune system interacts with and is affected by each pathogen it encounters over the lifetime of an individual (Horrocks et al. 2011). Blood parasites, like *Plasmodium*, can elicit immune responses in their hosts and be fatal for some bird species (*e.g.* African black-footed penguins – Graczyk et al. 1994; Hawaiian crows – Massey et al. 1996), but not for others (*e.g.* robins – Ricklefs and Sheldon 2007; passerines – Belo et al. 2011). Ectoparasites can also interact with their hosts in many ways, in some cases eliciting immune responses, as observed for lice that feed on blood and living skin (Johnson & Clayton 2003; *e.g.* Whiteman et al. 2006), in others directly affecting the fitness (Booth et al. 1993) and survival (Brown et al. 1995) of their hosts, or even behaving as "ectosymbionts" or comensalists, with no cost to their hosts, as has been reported for some feather mites (Figuerola 2000; Pap et al. 2005).

The geographical distribution of parasites is associated with the biogeography of their hosts, but parasites from a host ancestral or founder population are often absent on the host descendant populations (Paterson et al. 1999). This pattern could be generated just by chance, since parasites have a patchy distribution ("missed the boat"; see Paterson et al. 1999). When a founder population colonizes a new area, like an isolated archipelago, it is possible to recognize which parasites also successfully colonized the islands with their hosts (Parker & Whiteman 2012), but the rationale behind this probability of colonization is not recognized. The negative effect of parasites on their host fitness, for example, could explain part of this rationale, in which less healthy and less fit birds have a lower probability of colonizing a new area, but this hypothesis has not directly been tested yet. Therefore, it is possible that the classical island biogeography rules could be different for parasites and for their hosts.

Recently we studied the colonization history of *Myiarchus magnirostris*, the Galápagos flycatcher, and we proposed that *Myiarchus tyrannulus* from Central America is its most closely related group (Sari and Parker 2012). We also studied the origin of lice, mites and blood parasites found on *M. magnirostris*, and discovered that most of them are shared with *M. tyrannulus* and were likely also shared by their common ancestor species that colonized the Galápagos Islands (Sari et al., in prep). However, from the thirteen species of parasites studied, two species that parasitize *M. tyrannulus* with a very high prevalence and intensity were not found parasitizing the Galápagos flycatchers: the louse *Philopterus* sp. and the blood parasite *Plasmodium* sp. (Sari et al., in prep). One of the proposed hypotheses was that these two parasites

did not colonize Galápagos because they affect the health of their hosts more negatively than the other parasites that successfully colonized Galápagos with their hosts.

Here we tested this hypothesis by investigating the health condition and immune responses of *M. tyrannulus* to the presence of feather mites, chiggers, lice, and blood parasites. We predicted that birds with *Plasmodium* and/or *Philopterus* (parasites that we have concluded did not accompany the colonizing lineage of flycatchers to Galapagos) should show the following responses: worse body condition, lower packed red blood cell volume, higher leukocyte counts, and higher heterophil to lymphocyte ratio.

Methods

Collection of samples

M. tyrannulus has a large geographical distribution, but we studied a population located in northeastern Costa Rica, which is the southernmost limit for distribution of *M. tyrannulus* in Central America and the most probable source population for the colonization of Galápagos (Sari & Parker 2012; Sari et al., in prep). During the months of May and June of 2010, we captured 74 individuals in four localities in Costa Rica: Palo Verde Biological Station (n = 19), Área de Conservación Guanacaste (ACG) – Parque Nacional Santa Rosa (n = 37), ACG – Estación Experimental Forestal Horizontes (n = 2), and ACG – Sector El Hacha (n = 16). Birds were captured with mist-nets attracted by play-back songs and were released after collection of samples. Blood samples were collected from the brachial

vein using heparinized capillary tubes. Part of the blood was stored into eppendorf tubes with lysis buffer for DNA extraction and a few drops were used to make two or three blood smears. The rest of the blood was kept in capillary tubes, inside a refrigerated cooler while in the field; the tubes were later spun for five minutes using a microhematocrit centrifuge and the packed red blood cell volume (PCV; hematocrit) was read. When more than one capillary tube was available for the same individual bird, the mean PCV was calculated. PCV measures the percentage of the red blood cells in the total blood volume and low PCV values are indicative of acute and chronic infections (see Moreno et al. 1998).

All birds had their morphological measurements taken, including right tarsus length, measured with calipers to the nearest 0.1 mm, as the distance between the bent intertarsal joint and the center of the foot; and body weight measured to the nearest 0.1 g using a 50 g spring scale (Pesola® Baar, Switzerland). Because this species does not have evident sexual dimorphism, we used a PCR based method to identify the sex of sampled individuals, as described by Fridolfsson & Ellegren (1999).

Ectoparasites were sampled via dust ruffling of the birds using 1% pyrethroid insecticide (Flea & tick powder; Zodiac brand) and stored in 95% ethanol, as previously described (Sari et al., in prep).

Parasite identification

We studied four parasite groups: blood parasites (Order Haemosporida), chewing lice (Order Phthiraptera), feather mites (Order Acariformes, Cohort Astigmata), and chiggers (Order Trombidiformes). Blood parasites were identified

through the analyses of blood smears and also by DNA amplification, and ectoparasites were identified to species level by specialist taxonomists, as described by Sari et al. (in prep). Ten species of parasites were included in this study: one Haemosporidian parasite, *Plasmodium* sp.; three lice, *Ricinus marginatus* (Amblycera: Ricinidae), *Menacanthus distinctus* (Amblycera: Menoponidae), and *Philopterus* sp. (Ischnocera: Philopteridae); five feather mites, *Trouessartia* sp. (Trouessartiidae), *Nycteridiocaulus* nr. *lamellus* Atyeo 1966, *Tyrannidectes berlai* Mironov 2008, *Pterodectes* sp. (Proctophyllodidae), and one species of Analgidae not further identified; and one non-identified species of chigger (Trombiculidae).

White blood cell counts and leukocyte differentials

Blood smears were fixed in methanol for three minutes at the end of each sampling day and later stained using Giemsa stain as described by Valkiunas (2005). Utilizing the leukocyte-estimate-from-smear technique (LEFS), ten fields comprising a total of approximately 10,000 erythrocytes were examined for the presence of white blood cells (or leukocytes; thrombocytes not included), under 40X objective with immersion oil (Fudge 2000). The number of white blood cells (WBC) observed was recorded and the total WBC per μl was calculated using the formula: *[raw leukocyte count/ number of high power fields (X40) counted] X 2000* (Fudge 2000). In order to ascertain the percentage of each type of leukocyte found within the specified blood smears, differentials were counted of 100 white blood cells (heterophils, eosinophils, basophils, heterophils, and lymphocytes; thrombocytes not included), under high power (X 100) objective with immersion oil. The blood smears were read in a

consistent pattern and way that assured no cells were counted twice. All leukocyte counts (WBC and differentials) were done by L.R.

The number and proportions of the different white blood cell types are correlated with the health status of individuals. Elevated total white blood cell counts and higher proportions of heterophils and lymphocytes are typical responses to infectious diseases in birds (see Moreno et al. 1998). Another expected response to infection is a higher heterophil to lymphocyte ratio (see Padilla et al. 2006).

Statistical analyses

Prevalence values were calculated for each parasite species as the number of birds that carried that parasite divided by the number of birds analyzed. For the feather mites, however, because their identification and sorting can only be accurately done by a taxonomist, prevalence was calculated for this taxonomic group as a whole, by pooling all mite species together. We used chi-square tests to compare prevalence of each parasite (*Plasmodium*, *Menacanthus*, *Ricinus*, *Philopterus*, feather mites, chiggers) between males and females.

We estimated a body condition index (BCI) of birds as the residuals from a linear regression between tarsus length and body mass, which were normally distributed. BCI was computed for all birds, and BCIs were also computed for males and females separately. In order to test if body condition index (BCI) was correlated with immune responses, we ran linear regressions between BCI and the seven immune parameters: packed red blood cell volume (PCV), white blood cell counts (WBC), heterophils (Het), eosinophils (Eos), lymphocytes (Lym), monocytes (Mon),

and heterophil to lymphocyte ratios (Het/Lym). None of these parameters were normally distributed and they were transformed to be used in parametric tests and models. The transformations used to achieve normality were: cosine of PCV, natural log of WBC, square root of Het, natural log of Eos, arcsine of Lym, square root of Mon, and natural log of Het/Lym. Basophils were excluded from all analyses because they were detected in only 8 individuals and could not be normally transformed. All statistical analyses were performed in R v.2.13.0.

In order to characterize the relationship among the variation in the different leukocyte numbers, we applied a Principal Component Analyses (PCA) using Het, Eos, Lym, and Mon (resulting from the differentials). We then used the first two components of this PCA (PC1 and PC2) as immune parameters.

We used t-tests to compare the means of each health parameter (BCI, PCV, WBC, Het, Eos, Lym, Mon, and Het/Lym) between males and females, and ANOVA's to compare these parameters among localities. We performed t-tests with p-values determined by 10,000 permutations, as implemented in the Deducer package in R, to compare the non-transformed health parameters between parasitized and non-parasitized birds for each parasite.

We ran general linear models to determine the effect that parasites had on health/immune parameters recorded in *M. tyrannulus*. BCI, PCV, WBC, Het, Eos, Mon, and Lym, PC1, and PC2 (see above) were treated as response variables in separate models. We selected explanatory variables in the models by using both forward and backward step-wise selection of models based on AIC values using the 'stepAIC' function in the MASS package in R. The initial list of explanatory variables

included prevalence data for all the parasites (*Plasmodium*, *Menacanthus*, *Ricinus*, *Philopterus*, feather mites, chiggers), host sex, and locality at which collection occurred. We also included interactions among the significant explanatory variables where they occurred. When sex or locality was found to be a significant variable in the best model, another model selection was run for that health parameter for each sex or locality separately.

Results

Prevalence data for each parasite species or taxonomical group included in this study are listed in table 1. All *Plasmodium* DNA sequences obtained from 39 birds were identical. The distribution of *Plasmodium* blood parasites, *Ricinus* lice, feather mites, and chiggers was not different between sexes, but we found that more males are infected with *Menacanthus* than expected ($\chi^2_{(1, 0.05)} = 4.010$; $p = 0.045$), and more females are infected with *Philopterus* than expected ($\chi^2_{(1, 0.05)} = 4.270$; $p = 0.039$) (Table 1).

No significant differences were found between males and females for the health parameters analyzed and means for each health parameter can be found in table 2. Specific significant relationships between each health variable and prevalence of parasites are described in the following sections and listed in table 3.

Body condition index (BCI)

Body condition index was positively related to WBC ($r^2 = 0.045$; $F_{1, 71} = 4.401$; $p = 0.040$), Lym ($r^2 = 0.139$; $F_{1, 71} = 12.62$; $p < 0.001$), and PC1 from the

leukocyte PCA ($r^2 = 0.142$; $F_{1, 71} = 12.9$; $p < 0.001$), and negatively related to Het ($r^2 = 0.134$, $F_{1, 71} = 12.15$; $p < 0.001$) and with Het/Lym ratio ($r^2 = 0.127$, $F_{1, 69} = 11.2$, $p = 0.001$).

In the best model selected, the variation of BCI ($r^2 = 0.101$; $p < 0.001$) was explained by the prevalence of chiggers ($p = 0.014$), mites ($p = 0.007$), the sex of the birds ($p < 0.001$), an interaction between chiggers and sex ($p = 0.039$), and an interaction between mites and sex ($p = 0.023$). A separate model including just males showed that the variation of BCI just in males ($r^2 = 0.336$; $p < 0.001$) was best predicted by chiggers ($p < 0.001$) and the louse *Philopterus* ($p = 0.017$). A separate model for females was not significant.

Birds parasitized by the louse *Philopterus* have significantly lower BCI than birds not parasitized by *Philopterus* ($t = -2.451$; $p = 0.012$). Conversely, birds infected with *Plasmodium* have significantly higher BCI than birds not infected with *Plasmodium* ($t = 2.04$; $p = 0.046$). Among localities, only males had a significantly different BCI ($F_{2, 37} = 5.145$; $p = 0.011$), but this was not observed for females or for both sexes together.

Packed cell volume (PCV)

The variation in the packed red cell volume (PCV) was best explained by the prevalence of chiggers ($r^2 = 0.103$; $p = 0.004$). Moreover PCV is significantly lower in birds infected with chiggers ($t = -2.811$; $p = 0.016$) than those not infected with chiggers.

White blood cell count (WBC)

The variation in the total white blood cell count (WBC) was also best explained by the prevalence of chiggers ($r^2 = 0.040$; $p = 0.048$). In addition WBC was significantly higher in birds infected with chiggers than in birds not parasitized by chiggers ($t = 1.65$; $p = 0.036$). It was also significantly different among localities when considering only males ($F_{2, 37} = 5.186$; $p = 0.010$), but not when considering only females or with both sexes combined.

Leukocyte types

The first axis of the PCA using the different white blood cell types (PC1; Fig.1) explained 69.44% of the variation and it was loaded most strongly by Het (negative score), followed by Eos (negative score) and Lym (positive score), making PC1 a good representation of the Het/Lym ratio. The second axis of this PCA (PC2) explained 22.53% of the variation and it was mostly loaded by Mon (negative score), followed by Het (negative score).

The variation in PC1 and also in the Het/Lym ratio were best explained by locality (PC1 $r^2 = 0.068$; $p = 0.048$; Het/Lym $r^2 = 0.081$; $p = 0.033$), but when separate models were run for each locality, no parasite prevalence or sex explained the variation in PC1 or in Het/Lym ratio. The Het/Lym ratio did not correlate with infection status for any parasite studied.

The variation in lymphocytes (Lym) was also best explained by locality ($r^2 = 0.101$; $p = 0.015$); when separate models were run for each locality, the variation of Lym in Palo Verde was best explained by prevalence of the *Menacanthus* louse ($r^2 =$

0.201; $p = 0.031$). In addition, birds infected with *Plasmodium* have higher Lym than birds not infected with this blood parasite ($t = 2.189$; $p = 0.035$). Conversely, the variation in heterophils (Het) and in eosinophils (Eos) could not be explained by any model, and these leukocytes were not significantly different between infected and non infected birds.

The variation of PC2 ($r^2 = 0.081$; $p = 0.019$) was best explained by prevalence of the *Ricinus* louse ($p = 0.049$) and of *Plasmodium* ($p = 0.040$). *Plasmodium* was also the best explanatory variable for the variation in monocytes (Mon $r^2 = 0.055$; $p = 0.025$), which were significantly lower for birds infected with *Plasmodium* than for birds not infected with *Plasmodium* ($t = -2.026$; $p = 0.048$).

Discussion

We were interested in knowing how different parasites affect their hosts' health and fitness, and especially if the parasites that are found infecting *M. tyrannulus* but not *M. magnirostris* affect their hosts more negatively than the parasites that colonized Galápagos with the ancestors of their contemporary hosts. We used an index of the host body condition and several hematological values that are representative of immune function as health estimators. We found out that some immune parameters (WBC, Het, Lym, Het/Lym ratio) are correlated with *M. tyrannulus* body condition, demonstrating a possible connection between immune responses and fitness in this species. Hence, parasites that affect these immune responses are also likely affecting their host's condition and fitness. The connection between fitness and immune response, however, does not have a simple

interpretation. For example, we observed that BCI is negatively related to the Het/Lym ratio but positively related to WBC, while both of these hematological values are expected to increase in response to infections (see Padilla et al. 2006). However, Salvante (2006) argued that the interpretation of an elevated WBC is ambiguous: it can indicate that the individual has a healthy immune system and is ready to fight an infection or can show that the individual is currently fighting an infection.

Our prediction that birds infected by the louse *Philopterus* and/or the blood parasite *Plasmodium* would present worse health condition than birds not infected or infected by other parasites was not fully supported. *Plasmodium* was the best predictor of monocytes, and together with the louse *Ricinus*, of the PC2 from the leukocyte PCA, which is mostly determined by monocytes. *M. tyrannulus* infected with *Plasmodium* showed significantly fewer monocytes than those uninfected by this parasite. In contrast to our results, birds for which *Plasmodium* infections can be fatal had increased monocytes when they showed *Plasmodium* parasitemias in their blood smears (African black-footed penguins – Graczyk et al. 1994; Hawaiian crows – Massey et al. 1996). In human malaria, monocytes actively phagocytose the merozoite stage of *Plasmodium* and, in association with specific antibodies, they also phagocytose infected erythrocytes (Khusmith et al. 1982). During an acute infection, increased phagocytosis is observed in the infected organs, like liver, spleen, and bone marrow (Khusmith et al. 1982). Most *M. tyrannulus* from which we obtained *Plasmodium* DNA sequences (positives by PCR), did not show high parasitemias in the blood smears, indicative of chronic infections. The reduced number of monocytes

recorded for *M. tyrannulus* infected with *Plasmodium* (positives by PCR) likely does not result from their migration from the peripheral blood to phagocytose parasites in the infected organs, and probably represents lower production of monocytes. This could be offset in part by the larger number of lymphocytes that were observed in *M. tyrannulus* infected with *Plasmodium* in comparison to birds that are not infected with this blood parasite. Elevated lymphocytes have also been described for other bird species when infected with *Plasmodium* (robins – Ricklefs & Sheldon 2007; Hawaiian crows – Massey et al. 1996).

Philopterus, on the other hand, was, together with chiggers, a good predictor of BCI only for male *M. tyrannulus*. The lice that we used in this study belong to two suborders: Ischnocera, which includes *Philopterus*, and Amblycera, which includes *Ricinus* and *Menacanthus*. Ischnoceran lice feed on feathers and dead skin from birds, and Amblyceran lice feed on blood and living skin, sometimes presenting mouthparts modified to suck blood (Johnson & Clayton 2003). Amblycerans can therefore encounter the immune system of their hosts more often than Ischnoceran lice like *Philopterus* (Johnson & Clayton 2003; *e.g*. Whiteman et al. 2006). This could explain our finding that *Philopterus* was not a good predictor of any of the immune parameters, while the presence of *Menacanthus* and *Ricinus* respectively explained the variation of lymphocytes and of the PC2 from the leukocytes PCA. Ischnoceran lice, on the other hand, can cause large amounts of damage to host feathers (Johnson & Clayton 2003; *e.g.* Clayton *et al.*, 1999), sometimes sufficient to result in thermoregulatory costs for the birds, and consequently, reduce the fitness of parasitized individuals (Booth et al. 1993; Clayton *et al.*, 1999). While presence of

Philopterus was not related to any of the immune parameters, we did find that *Philopterus* has a negative direct effect on *M. tyrannulus* BCI. *Menacanthus*, *Ricinus*, *Plasmodium*, and chiggers, on the other hand, did show significant interactions with the immune parameters measured, suggesting possible indirect effects on host BCI.

Differently from what we predicted, our results showed that chiggers are the parasites that explain most variation in the health parameters; they were the best predictors for BCI, PCV, and WBC. The chiggers analyzed in this study were collected directly from large and swollen wounds found on the bird skin, and the immune reaction (inflammation) of the hosts against these parasites was visually evident. Hence, the presence of chiggers could be affecting the health (lower PCV and higher WBC) and fitness of their hosts, and birds carrying these parasites may not have been able to colonize Galápagos. There are no records of chiggers (Trombiculidae) in Galápagos birds (Deem et al. 2011), but this information should be interpreted cautiously because no search for parasites is exhaustive (one would have to check every bird; see Paterson et al. 1991). We have observed small skin cysts on a couple of Galápagos flycatchers (three out of 229 birds handled), with a similar appearance to the wounds found on *M. tyrannulus* (E.H.R. Sari pers. obs.), but no material was collected from these birds, so we cannot assume that they were also caused by chiggers.

Conclusions

In this study we proposed to test an explanation for why some parasites "miss the boat" and are absent from a host founder population in a colonization event (see

Paterson et al. 1999). We hypothesized that the role that these parasites play in the health of their hosts was important in their colonization success, but we did not find consistent support for this hypothesis. The parasites that did not colonize Galápagos (*Philopterus* lice and *Plasmodium*) are not the ones with the largest impact in the health of their hosts. In addition, parasites that have negative effects on their hosts' health (feather mites, *Ricinus* lice) made it to Galápagos, meaning that not only the healthiest birds were successful founders. Chiggers, however, had the strongest effect on the health of their hosts and they probably did not colonize Galápagos, so we believe that our hypothesis deserves further attention and should be further investigated in other species and systems.

Our work shows that the interpretation of health parameters is not simple, and responses that are commonly expected from each health parameter should not be generalized (Salvante 2006). Instead, each response seems to be specific to different types of parasites and to the interaction between them. Therefore, taking these health parameters out of context of their parasites may make interpretation difficult. This should serve as an important caveat for eco-immunologists that parasites cannot be decoupled from immune responses. Furthermore, these health parameters seem to be very species-specific, again meaning that their interpretation should be made cautiously (Smits 2007).

Finally, our work contributes to the fields of conservation medicine and eco-immunology, since the reference values of health parameters and their variation in response to parasites is unknown in most wild populations and species. It is important to identify these reference values (Smits 2007) and to recognize which parasites

directly contribute to the viability and survival of natural populations, so we can understand and predict the consequences of epidemiological events to these and other closely related populations.

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Table 1 Parasite prevalence data of *M. tyrannulus* from Costa Rica for all birds (n = 74), males (n = 40), and females (n = 34).

Parasites	all birds	males	females
Plasmodium sp.	52.7% (39/74)	52.5% (21/40)	52.9% (18/34)
Philopterus sp.	60.8% (45/74)	50% (20/40)*	74% (25/34)*
Menacanthus distinctus	14.9% (11/74)	22.5% (9/40)*	5.9% (2/34)*
Ricinus marginatus	17.6% (13/74)	17.5% (7/40)	17.6% (6/34)
Feather mites	85.1% (63/74)	85% (34/40)	85.3% (29/34)
Chiggers	10.8% (8/74)	7.5% (3/40)	14.7% (5/34)

* prevalence values are significantly different between males and females: more females are infected with *Philopterus* than expected ($\chi^2_{(1, 0.05)} = 4.27$; $p = 0.039$), and more males are infected with *Menacanthus* than expected ($\chi^2_{(1, 0.05)} = 4.01$; $p = 0.045$).

Table 2 Mean health values, tarsus length, and body mass for *Myiarchus tyrannulus* from Costa Rica, calculated from 74 birds. Value ranges are presented in parenthesis. BCI is body condition index, PCV is packed red cells volume (hematocrit), WBC is white blood cell count.

	all birds	males	females
BCI	-2.85×10^{-16} (-5.09 – 5.10)	0.88 ± 1.90 (-1.96 – 5.10)	-1.01 ± 1.41 (-5.09 – 2.99)
PCV (%)	48.25 ± 3.11 (38 – 58)	48.15 ± 3.19 (38 – 54)	48.38 ± 3.06 (43 – 58)
WBC	20.88 ± 19.45 (1 – 113)	16.72 ± 12.87 (2 – 52)	25.65 ± 24.31 (1 – 113)
WBC volume ($x10^3$/ µl)	4.18 ± 3.89 (0.2 – 22.6)	3.34 ± 2.57 (0.4 – 10.4)	5.13 ± 4.86 (0.2 – 22.6)
Heterophils (%)	17.63 ± 14.82 (0 – 65)	16.31 ± 14.97 (0 – 65)	19.15 ± 14.72 (0 – 53)
Eosinophils (%)	8.18 ± 7.09 (0 – 34)	7.15 ± 6.12 (1 – 31)	9.35 ± 8.00 (0 – 34)
Lymphocytes (%)	68.8 ± 19.21 (21 – 98)	71.56 ± 19.15 (21 – 98)	65.68 ± 19.09 (35 – 94)
Monocytes (%)	4.86 ± 4.08 (0 – 16)	4.28 ± 4.35 (0 – 16)	5.53 ± 3.69 (0 – 14)
Basophils (%)	0.51 ± 2.48 (0 – 20)	0.69 ± 3.28 (2 – 20)	0.29 ± 0.97 (0 – 5)
Het/Lym ratio	0.37 ± 0.48 (0.01 – 3.09)	0.36 ± 0.58 (0.01 – 3.10)	0.40 ± 0.37 (0.02 – 1.43)
Tarsus length (mm)	22.55 ± 0.97 (20.0 – 24.4)	22.82 ± 0.98 (20 – 24.4)	22.24 ± 0.87 (27.5 – 34.5)
Body mass (g)	31.76 ± 2.15 (27.5 – 37.4)	32.90 ± 1.92 (29.0 – 37.4)	30.45 ± 1.58 (20.6 – 24.4)

Table 3 Significant correlations between parasites and health parameters from *M. tyrannulus*. M represents the parasite that was a significant predictor for a certain health parameter using a general linear model; + shows that birds parasitized by that specific parasite had a significant larger mean for that health variable than non-parasitized birds; and – shows that birds parasitized by that specific parasite had a significant lower mean for that health variable than non-parasitized birds.

	Plasmodium	*Philopterus*	*Menacanthus*	*Ricinus*	Feather mites	Chiggers
BCI	+	M, –			M	M
PCV						M, –
WBC						M, +
Het						
Eos						
Lym	+		M		+	
Mon	M, –			+	–	
Het/Lym						
PC1						
PC2	M			M, –		

Figure 1 Principal component analysis (PCA) from the proportion of white blood cells: Heterophils, Eosinophils, Lymphocytes, and Monocytes. The size and direction of the arrows indicate the contribution of each variable to PC1 and PC2.