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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 (1970 bp) 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 ($\pi = 0.0009$ 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.
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1. 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 km 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

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species analyzed are divided into two main clades (Clades I and 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.

2. Materials and methods

2.1. 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.

2.2. 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 20 μl reaction with 0.5 U of Biolase™ Red DNA Polymerase (Bioline), 1× 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 s, 60 °C for 50 s, 72 °C for 45 s, with a final extension step at 72 °C for 5 min. For amplification of cytb and ND2, 10–40 ng of genomic DNA were used in 15 μl volume reactions with 0.35 U of Biolase™ Red DNA Polymerase (Bioline), 1× 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 s, specific annealing temperatures (Table 3) for 45 s, 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 10 μl 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.

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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 number	Locality	Accession number	
			CytB	ND2
Clade 1				
Myiarchus antillarum	RicklefsLab-GF103	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-ESI 123	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 Paranagua, Falcón	JQ004327	JQ004382
Myiarchus tyrannulus	Ricklefs Lab-MYTY 32	Venezuela: Península de Paranagua, 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: Rondónia	DQ294532	DQ294576
Empidonax minimus	GenBank		AY143197	AY030125

2.3. 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.

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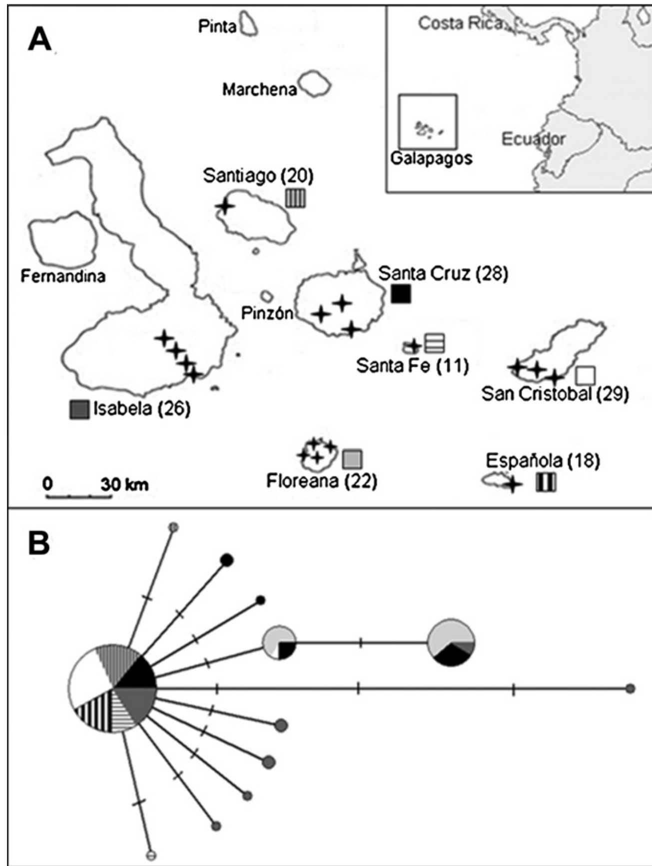

Fig. 1. (A) Map of the Galápagos archipelago, showing its position in relation to Ecuador and Costa Rica. The number of samples from each island used in this study is listed in parenthesis and the regions where they were collected are marked by stars. The squares adjacent to each island represent the pattern used to represent those islands in the haplotype network (Fig. 1B). (B) Median-joining haplotype network generated from cytb sequences (907 bp) 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.

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).

2.4. 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

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	−1.8846 **	−3.0922 *	0.4156
Floreana	22	2	0.4849	0.00053	0.4849	1.3343	1.3923	0.4427
Española	18	1	0.0000	0.00000	0.0000	0.0000	n/a	0.4691
Santa Cruz	28	5	0.6561	0.00118	1.0741	0.1176	−0.5199	0.4100
Santiago	20	2	0.1000	0.00011	0.1000	−1.1644	−0.8793	0.4636
Santa Fé	11	2	0.1818	0.00020	0.1818	−1.1285	−0.4099	0.4596
San Cristóbal	29	2	0.0690	0.00008	0.0690	−1.1492	−1.1835 **	0.4653
Total	154	12	0.4913	0.00087	0.7906	−1.6807 *	−7.1175 **	0.4434

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

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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 (°C)	Reference
ATPase8_6	CO2GQL (A/S)	GGA CAA TGC TCA GAA ATC TGC GG	60	Seutin and Bermingham (unpubl.)[*]
	CO3HMH (A/S)	CAT GGG CTG GGG TCR ACT ATG TG	60	Seutin and Bermingham (unpubl.)[*]
	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	Kocher et al. (1989)
	H16065 (A/S)	GTC TTC AGT TTT TGG TTT ACA AGA C	53	Edwards and 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	Sorenson (2003)
	H6313 (A/S)	ACT CTT RTT TAA GGC TTT GAA GGC	60	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	Brumfield and Edwards (2007)
	FIB-B17L (A/S)	TCC CCA GTA GTA TCT GCC ATT AGG GTT	61	Brumfield and Edwards (2007)
	BF7intF (S)	TTG TAA AGT ACA TAA CTG AGC		Brumfield and Edwards (2007)
	BF7intR (S)	GTG CTC AGT TAT GTA CTT TAC AA		Brumfield and Edwards (2007)

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

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.

2.5. 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 analog 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.

3. Results

3.1. Myiarchus phylogeny

3.1.1. 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 842 bp 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 975 bp for cytb from 56 samples (with 209 parsimoniously informative positions), 1035 bp for ND2 (with 253 parsimoniously informative positions) from 53 samples, and 2010 bp 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).

3.1.2. 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.

3.1.3. 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 Schluter, 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).

3.2. magnirostris population genetics

We obtained an alignment with 907 bp 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 ($\pi = 0.00087$) 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 ($\Phi_{st} = 0.4434$). 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.

4. Discussion

4.1. 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

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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 (935 bp). Numbers on nodes represent ML bootstrap values (RaxML - 100bs)/ MP bootstrap values (Paup – 500bs)/Bayesian posterior probabilities (Mr. Bayes – 10 million 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).

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

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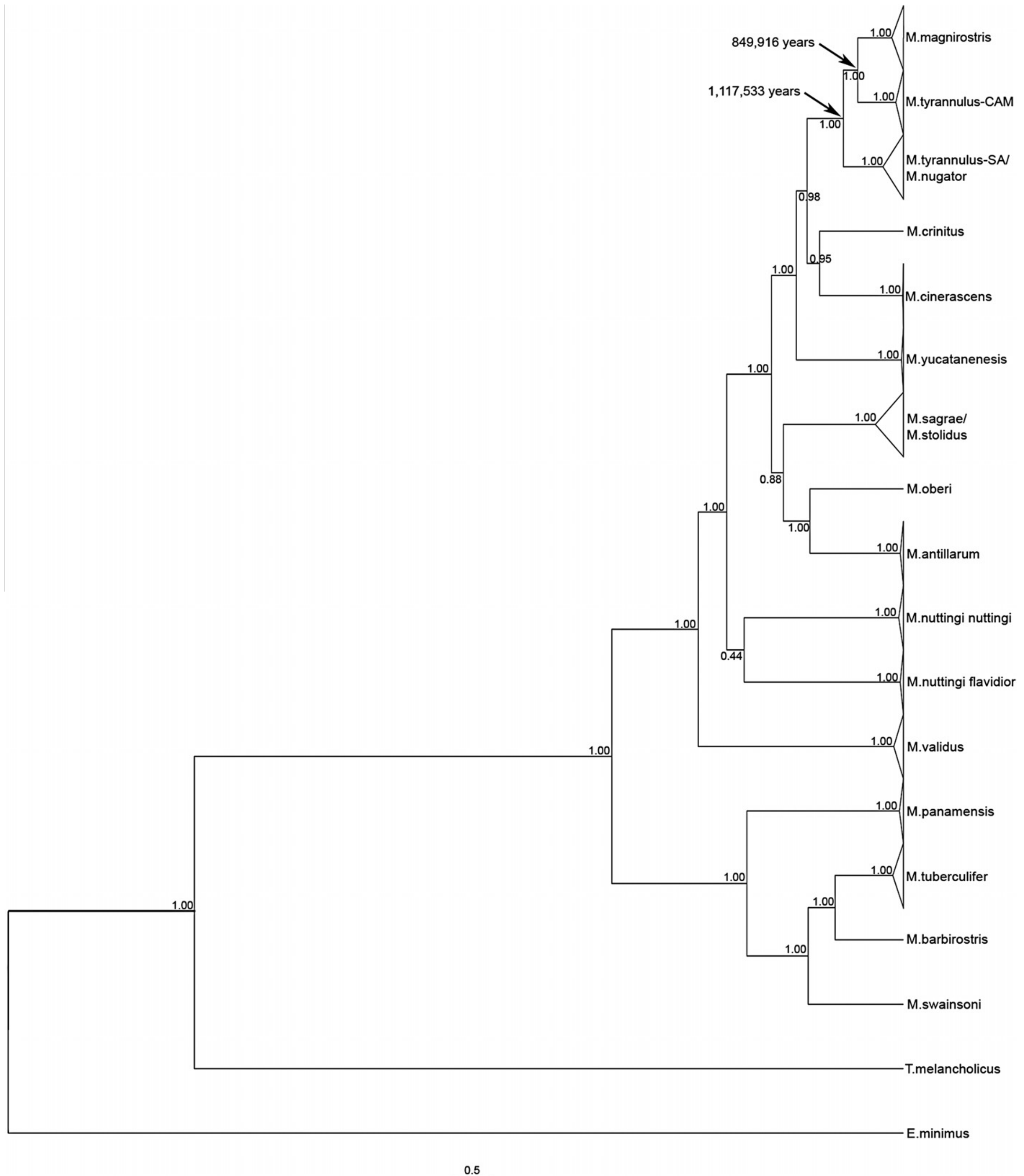

Fig. 3. Bayesian condensed phylogram with posterior probabilities and average node ages obtained using a strict molecular clock (2.07%/MY) in BEAST with concatenated sequences from ND2 (1035 bp) and cytb (935 bp). Individuals/branch tips from each lineage were condensed within triangles. CAM = Central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

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.

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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

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 and 5.5 million years ago (Arbogast et al., 2006), and the finches, which diverged from their continental ancestors around 2–3 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, 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.

4.2. 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 ($\Phi_{st} = 0.443$), 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}.

4.3. 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

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M. nuttingi is necessary for a more comprehensive classification that is consistent with these emerging patterns.

4.4. 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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