Phylogeography of the Vermilion Flycatcher species complex: multiple speciation events, shifts in migratory behavior, and an apparent extinction of a Galápagos-endemic bird species

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Abstract

The Vermilion Flycatcher (*Pyrocephalus rubinus*) is a widespread species found in North and South America and the Galápagos. Its 12 recognized subspecies vary in degree of geographic isolation, phenotypic distinctness, and migratory status. Some authors suggest that Galápagos subspecies *nanus* and *dubius* constitute one or more separate species. Observational reports of distinct differences in song also suggest separate species status for the austral migrant subspecies *rubinus*. To evaluate geographical patterns of diversification and taxonomic limits within this species complex, we carried out a molecular phylogenetic analysis encompassing 10 subspecies and three outgroup taxa using mitochondrial (ND2, Cyt *b*) and nuclear loci (ODC introns 6 through 7, FGB intron 5). We used samples of preserved tissues from museum collections as well as toe pad samples from museum skins. Galápagos and continental clades were recovered as sister groups, with initial divergence at ~1 mya. Within the continental clade, North and South American populations were sister groups. Three geographically distinct clades were recovered within South America. We detected no genetic differences between two broadly intergrading North American subspecies, *mexicanus* and *flammeus*, suggesting they should not be recognized as separate taxa. Four western South American subspecies were also indistinguishable on the basis of loci that we sampled, but occur in a region with patchy habitat, and may represent recently isolated populations. The austral migrant subspecies, *rubinus*, comprised a monophyletic mitochondrial clade and had many unique nuclear DNA alleles. In combination with its distinct song, exclusive song recognition behavior, different phenology, and an isolated breeding range, our data suggests that this taxon represents a separate species from other continental populations. Mitochondrial and nuclear genetic

data, morphology, and behavior suggest that Galápagos forms should be elevated to two full species corresponding to the two currently recognized subspecies, *nanus* and *dubius*. The population of *dubius* is presumed to be extinct, and thus would represent the first documented extinction of a Galápagos-endemic bird species. Two strongly supported mitochondrial clades divide Galápagos subspecies *nanus* in a geographic pattern that conflicts with previous hypotheses that were based on plumage color. Several populations of *nanus* have recently become extinct or are in serious decline. Urgent conservation measures should seek to preserve the deep mitochondrial DNA diversity within *nanus*, and further work should explore whether additional forms should be recognized within *nanus*. Ancestral states analysis based on our phylogeny revealed that the most recent common ancestor of extant Vermilion Flycatcher populations was migratory, and that migratory behavior was lost more often than gained within *Pyrocephalus* and close relatives, as has been shown to be the case within Tyrannidae as a whole.

Keywords: Galápagos; Island colonization; Neotropical biogeography; Phylogenetics; *Pyrocephalus rubinus*; Tyrannidae

1. Introduction

Accurate assessments of biodiversity are important for conservation and scientific research (Dirzo and Raven, 2003; Pratt, 2010; Sangster, 2000). It is often assumed that biodiversity is well understood for commonly studied groups such as birds and mammals (Scheffers et al., 2012). However, various studies and authors have challenged this notion (e.g. Lim, 2012; Pratt, 2010; Sangster, 2000; Zink, 2004). Genetic studies have uncovered hidden biodiversity where morphological studies alone have failed to do so (e.g. López et al., 2015; Saitoh et al., 2015; Toews and Irwin, 2008), even within well-studied species complexes (e.g. Sætre et al., 2001; Salzburger et al., 2002). Exploring hidden biodiversity within the Neotropical region is of particular importance, given that Neotropical taxa historically have received less research attention than northern temperate taxa (Chek et al., 2003; Tobias et al., 2012), and given high levels of endemism in the region (Stattersfield et al., 1998), as well as emerging conservation concerns (e.g. Brooks et al., 2002; Coe et al., 2013; Lees et al., 2016).

Certain features of species-complexes can increase the likelihood of identifying cryptic biodiversity within them. Previous work in birds suggests that island populations present unappreciated taxonomic diversity (Andersen et al., 2014; Johnson and Clayton, 2000; Pratt, 2010; Saitoh et al., 2015). Previous studies also suggest that new species may be uncovered where differences in song have been observed (Alström and Ranft, 2003; Benkman et al., 2009; Irwin et al., 2001; Päckert et al., 2004; Toews and Irwin, 2008). Thus, bird species that include island populations or discrete intra-specific differences in

vocalizations are good candidates for detecting unexpected levels of genetic differentiation or reproductive isolation.

The Vermilion Flycatcher, *Pyrocephalus rubinus* (Passeriformes: Tyrannidae), presents a useful model for exploring hidden biodiversity within a well-known, largely Neotropical bird species. Due to its wide distribution, brightly colored males, and conspicuous habits and vocalizations (Farnsworth and Lebbin, 2004; Fjeldså and Krabbe, 1990), the Vermilion Flycatcher has featured prominently in human culture (e.g. d'Orbigny, 1835) and has caught the attention of scientists as far back as 1743 (Buffon, 1778; Carmi, 2013). It has also increasingly been of interest to researchers studying evolutionary questions ranging from environmental influences on molt (Butler, 2013) to song variability (Ríos-Chelén et al., 2012a, 2012b, 2005; Ríos-Chelén and Garcia, 2007; Rivera-Cáceres et al., 2011) to melanism (Schmitt et al., 2016; van Grouw and Nolasco, 2012). Yet, taxonomic limits within the Vermilion Flycatcher are not fully understood. The Vermilion Flycatcher has an unusual distribution for a passerine: breeding populations occur in the tropics and temperate regions of both North and South America, with migratory and non-migratory populations on both continents (Fig. 1). In addition, the Vermilion Flycatcher includes populations that could potentially represent reproductively isolated lineages, including island forms in the Galápagos archipelago and a population of austral migrants in southern South America that has been noted for a distinct song (Jaramillo, unpublished data) (Fig. 1). A molecular phylogenetic study of the Vermilion Flycatcher would help gauge to what extent these and other populations have been isolated and have differentiated from each other. It would also help assess the merits of the current intra-specific taxonomy of the Vermilion Flycatcher,

which reflect subspecies designations erected by various authors over an extended period of time using different criteria.

The taxonomic history of the Vermilion Flycatcher is complex. Due to its wide geographic distribution (Fig. 1), morphological variability, and the occurrence of a dark morph in southern coastal Peru (Cory and Hellmayr, 1927; Zimmer, 1941), there has been confusion and disagreement in the taxonomic literature over the number of forms of the Vermilion Flycatcher and their geographic delimitations (Carmi, 2013). Modern treatments have considered the Vermilion Flycatcher to be a single, polytypic species in a monotypic genus, although authors have disagreed over the number of subspecies. Most recent authors (Clements et al., 2014; Dickinson, 2003; Dickinson and Christidis, 2014; Farnsworth and Lebbin, 2004; Gill and Donsker, 2015; Traylor, 1979) recognize at least 12 subspecies in six distinct geographical areas, as follows (Fig. 1):

- In North America, three contiguously distributed subspecies: *P. r. flammeus* van Rossem, 1934, *P. r. mexicanus* Sclater, 1859 and *P. r. blatteus* Bangs, 1911.
- In the pine savanna of northeastern Nicaragua and eastern Honduras, one subspecies: *P. r. pinicola* Howell, 1965.
- In northern South America, one subspecies: *P. r. saturatus* Berlepsch and Hartert, 1902.
- In western South America, four contiguously distributed subspecies: *P. r. piurae* Zimmer, 1941, *P. r. ardens* Zimmer, 1941, *P. r. obscurus* Gould, 1839 and *P. r. cocachacrae* Zimmer, 1941.

- A subspecies that breeds in southern South America and winters north into the Amazon basin: *P. r. rubinus* (Boddaert, 1783).
- Two subspecies inhabiting the Galápagos Islands: *P. r. dubius* Gould, 1839 from San Cristóbal Island, and *P. r. nanus* Gould, 1838 from most of the rest of the archipelago.

Many authors also list a 13th subspecies, *P. r. major* Pelzeln, 1871 from southeastern Peru (Clements, 2007; Clements et al., 2014; Dickinson, 2003; Gill and Donsker, 2015; Traylor, 1979). However, *P. r. major* is often called into question, as very few specimens with locality data have been assigned to it and breeding records are lacking (Clements, 2007; Cory and Hellmayr, 1927; Dickinson and Christidis, 2014; Farnsworth and Lebbin, 2004; Traylor, 1979; Zimmer, 1941). Ellison et al. (2009) recognize only 11 subspecies, combining *P. r. piurae* and *P. r. obscurus*.

Previous taxonomic treatments of the Vermilion Flycatcher have relied on plumage color and pattern, size, morphological characters, geographic ranges, and migratory status. In several cases, the criteria used to diagnose forms have been subtle. When Zimmer split subspecies *obscurus* of the western coast of South America into four subspecies (Zimmer, 1941), he noted that differences among these forms were somewhat obscured by variation due to season and age. He also noted multiple individuals atypical of their localities or of intermediate character states, and he left gaps between the ranges of several subspecies, thus rendering unclear the taxonomic designation of birds occurring in those gaps.

Likewise, Bangs and van Rossem separated the North American subspecies *blatteus* and *flammeus* from subspecies *mexicanus* based largely on geographic gradients in dimensions, color, and streaking (Bangs, 1911; Ridgway, 1907; van Rossem, 1934). Van Rossem's

description of *flammeus* in particular seems to imply a broad zone of intergradation between subspecies *flammeus* and *mexicanus*.

By contrast, the differences used to distinguish several other forms of the Vermilion Flycatcher are pronounced. The two currently recognized Galápagos subspecies are easily distinguished from continental forms based on their smaller size and the buffy, rather than reddish and white, ventral coloration of the females (Gould, 1839). In addition, nearly all classifications have considered *P. r. dubius* of San Cristóbal Island to be distinct from other Galápagos populations based mainly on its smaller size (Gould, 1839; Ridgway, 1894). Although most authors since Hellmayr (Cory and Hellmayr, 1927) have considered all forms of the Vermilion Flycatcher to fall within one species, some authors have suggested that the Galápagos birds may represent one or two separate species (Ellison et al., 2009; Farnsworth and Lebbin, 2004; Steadman, 1986), or as many as five (Ridgway, 1897, 1894).

The conservation status of the Vermilion Flycatcher in the Galápagos Islands is dire. Subspecies *dubius*, endemic to San Cristóbal Island, was last seen in 1987 and is generally presumed to be extinct (Vargas, 1996). Subspecies *nanus*, endemic to much of the rest of the archipelago, has become uncommon to rare on many islands (Wiedenfeld, 2006; B. Fessl, personal communication). Breeding populations on Santa Fé and Rábida Islands were extinct as of 1982 (although they have recently recolonized Rábida; Harris, 1982; Wiedenfeld, 2006; B. Fessl, personal communication), and recent surveys found no birds on Floreana and no more than 20 on Santa Cruz, islands with historically large populations (Merlen, 2013; O'Connor et al., 2010; B. Fessl, personal communication). It is therefore of

urgent interest to know whether the Galápagos Vermilion Flycatchers are distinct enough to warrant recognition as one or more separate species. If it is found that subspecies *dubius* constitutes a distinct species, it would represent the first documented extinction of a Galápagos-endemic species of bird.

Given the uneven taxonomic treatment of the Vermilion Flycatcher, potential species status for the Galápagos and austral migrant subspecies, and serious concerns over the conservation status of Galápagos populations, we sought to test whether the current taxonomy of the genus is consistent with its phylogenetic history. To that end, we present a multi-locus phylogenetic analysis of 10 subspecies (Table 1), including populations from North and South America as well as the Galápagos Islands. We sequenced mitochondrial and nuclear DNA from samples of preserved tissues from museum collections, and supplemented those with DNA sequences from toe pads of museum skins in order to include populations for which no preserved tissues were available.

Finally, we used our results to explore the evolution of migratory behavior in the Vermilion Flycatcher. A recent analysis of ancestral states for a species-level phylogeny of the family Tyrannidae found that migratory behavior was more often lost than gained (Gómez Bahamón, 2014). Here we extend this approach to a phylogeny of subspecies and populations within the Vermilion Flycatcher. Resolving the history of shifts in migratory behavior within the Vermilion Flycatcher can inform our understanding the mechanisms by which it may have diversified.

2. Methods

2.1 Taxon sampling

We sampled 84 individual birds, including 77 specimens of *Pyrocephalus rubinus*, and seven specimens from the closely related outgroup genera *Fluvicola*, *Arundinicola*, and *Gubernetes* (Tello et al., 2009). The *Pyrocephalus rubinus* samples came from the geographic ranges of 10 of the 12 subspecies recognized by Dickinson and Christidis (2014). The number and type of samples used for each subspecies are listed in Table 1. We used museum-archived preserved-tissue samples associated with vouchered specimens for all subspecies for which such samples were available. However, samples of subspecies *mexicanus* from eastern North America and of subspecies *nanus* and *dubius* from the Galápagos were from toe pads from vouchered museum skins housed at the California Academy of Sciences. Specimen localities, collection dates, and museum catalog numbers are listed in Table 2. More detailed specimen information is available in Table S1.

2.2 Molecular work

2.2.1 Extraction

We extracted DNA from preserved-tissue samples using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Valencia, California), following the manufacturer's instructions for purification

of total DNA from animal tissues using spin-columns. Each batch of extractions included a negative control. Extracts were stored at -20°C.

We extracted DNA from toe pad samples in a dedicated “ancient DNA” laboratory using a previously described phenol-chloroform procedure (Carmi, 2013; Dumbacher and Fleischer, 2001). We followed recommended precautions to minimize the risk and maximize detection of potential contamination (Mulligan, 2005; Wandeler et al., 2007; Willerslev and Cooper, 2005), including keeping the lab free of any contact with amplified DNA, bleaching lab surfaces, UV-irradiating all equipment and solutions, using filtered pipet tips and single-use plastics, and wearing protective gear. Extractions were carried out in small batches limited to five samples and one negative control, and an effort was made to handle samples from the same locality on separate days.

2.2.2 Selection of genetic loci for amplification

In order to maximize detection of phylogenetic relationships at the subspecific level, we selected two mitochondrial protein-coding genes, the fast-evolving NADH dehydrogenase subunit 2 (ND2), and cytochrome *b* (Cyt *b*) (Lerner et al., 2011). We also selected two nuclear loci that consistently yielded a single amplification product and exhibited genetic variation, ornithine decarboxylase (ODC) introns 6 through 7, and fibrinogen beta chain intron 5 (FGB5).

2.2.3 Gene amplification

Genes were amplified via the Polymerase Chain Reaction (PCR). Primers used and their sources are listed in Table S2. New species-specific primers were designed and assessed using Primer3 (Rozen and Skaletsky, 1998), Primer3 v. 3.4.0 (Rozen and Skaletsky, 2000) and OligoCalc (Kibbe, 2007). We used recombinant *Taq* DNA Polymerase for extracts from preserved-tissue samples, and AmpliTaq Gold DNA Polymerase for extracts from toe pad samples (Life Technologies, Grand Island, NY). PCR conditions are listed in Table S3. Amplification products were assessed for quality and length by gel electrophoresis.

2.2.4 Cycle sequencing

Amplification products were cleaned of primers and unincorporated dNTPs using ExoSAP-IT (Affymetrix, Santa Clara, CA). Cycle sequencing was carried out using the BigDye Terminator kit v3.1 (Life Technologies) following the STeP protocol (Platt et al., 2007). Sequence products were ethanol-precipitated, suspended in formamide, and read on an ABI 3130 genetic analyzer (Life Technologies).

2.2.5 Cloning

Alleles for the nuclear loci of some heterozygous samples could not be phased by visual inspection and were cloned using the TOPO TA Cloning kit (Life Technologies). We used vector pCR2.1 and chemically competent Mach1-T1^R *E. coli* cells. As primers preferentially ligated to the vector, PCR products were first cut from electrophoresis gels, and the gel fragments were filtered through filtered pipette tips (Mettler Toledo, Columbus, Ohio) under centrifugation at 5000 rpm (2348 X g) for 10 min. The filtrate was incubated for 15

min at 72°C with Taq polymerase, dATP and PCR buffer for addition of A-overhangs before proceeding to ligation and transformation. Transformed cultures were spread on agar plates containing ampicillin and incubated overnight at 37°C. In order to ensure representation of both alleles and accommodate any non-target inserts or PCR recombinants, we picked 16 transformant colonies per cloned PCR product, unless fewer than 16 grew, in which case we picked all. Inserts were amplified using the M13 primers (see Table S2) under the conditions listed in Table S3. All post-PCR steps were carried out as described above.

2.3 Phylogenetic analysis

2.3.1 Initial editing

Sequences were checked for quality, edited, and aligned in Sequencher v. 4.7/4.8 (Gene Codes Corporation, Ann Arbor, MI). Nuclear alleles of polymorphic individuals were phased by visual comparison with sequences of non-polymorphic individuals or with clones of other polymorphic individuals. Redundant sequences were collapsed in MacClade v. 4.08 (Maddison and Maddison, 2005).

2.3.2 Model selection for mitochondrial data

Best-fit substitution models were selected from 203 possible partitions of the general time-reversible (GTR) rate matrix using the Bayesian Information Criterion (BIC) as implemented in jModelTest 2.1.1 (Darriba et al., 2012; Guindon and Gascuel, 2003). Models

were compared with and without Γ -distributed rate variation (using 10 rate categories) and with and without unequal base frequencies. The proportion of invariable sites was not evaluated, due to concerns that it is correlated with the shape parameter of the Gamma distribution, and that it is sensitive to the number of taxa used to estimate it (Stamatakis, 2008 and references therein: Gu et al., 1995; Sullivan et al., 1999; Yang, 2006). HKY+ Γ (Hasegawa et al., 1985) was consistently the best-fit model for our mitochondrial data, regardless of whether we tested each locus separately, the two loci combined, included or excluded outgroup taxa, or included only fully sequenced taxa or fully represented characters.

2.3.3 Phylogenetic analysis of mitochondrial data

We reconstructed phylogenetic relationships via Maximum Likelihood (ML) heuristic searches, Bayesian Inference (BI), and bootstrap analyses. ML and BI analyses were carried out both with and without enforcing a strict clock. We carried out heuristic searches and bootstrap analyses in PAUP* v. 4.0b10 (Swofford, 2003) and RAxML 7.2.6 (Stamatakis, 2006; Stamatakis et al., 2008). We carried out BI analyses using MrBayes-MPI 3.1.2 (Altekar et al., 2004; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003).

For ML heuristic searches in PAUP*, we obtained starting trees via stepwise addition using 100 random-addition-sequence replicates. We used the tree bisection-reconnection branch-swapping algorithm and employed the HKY+ Γ model of substitution with the gamma distribution approximated with 10 rate categories. Model parameters and tree topology were estimated by successive approximation (Swofford et al., 1996). For searches

with clock enforced, we restricted the number of rearrangements per iteration to 500 and reduced the number of rate categories used to approximate the gamma distribution to 4 in order to ensure that the search finished in a reasonable period of time.

We carried out the following four sets of bootstrap analysis in PAUP*: one with 10,000 Maximum Parsimony (MP) heuristic search replicates, one with 10,000 Neighbor-Joining (NJ) replicates, one with 1,000 ML heuristic search replicates, and one with 500 ML heuristic search replicates with clock enforced. Starting trees for the MP and ML bootstrap analyses were obtained via stepwise addition using a random addition sequence. For the ML bootstrap analysis with clock enforced, we restricted the number of rearrangements per replicate to 1500 to ensure that the analysis finished in a reasonable period of time.

For ML analysis in RAxML, we additionally partitioned the data by gene and codon position. We used the GTR+ Γ (Tavaré, 1986) model of substitution, since RAxML does not employ the HKY model or other special cases of the GTR model. We did not enforce a clock, as this option is not available in RAxML. We also ran 25,000 rapid bootstrap replicates.

For BI analysis in MrBayes, we ran each analysis for 30 million generations, using two runs with four chains each (one cold and three heated). We sampled parameters and tree topology every 2000 generations, and discarded the first 25% of trees as burn-in. We used the HKY+ Γ model, default priors, and default Markov-chain Monte Carlo (MCMC) parameters. The output was inspected in Tracer 1.5 (Rambaut and Drummond, 2009) for MCMC convergence and mixing. The MCMC seemed to reach stationarity well within the

25% burn-in in each run. Effective sample sizes were greater than 7,000 for all parameters. The potential scale reduction factor (PSRF) reached 1.000 ± 0.004 for all parameters by the end of the analysis, and the average standard deviation of the split frequencies dropped below 0.003.

We estimated the marginal likelihoods for clock and non-clock models using the stepping-stone method (Xie et al., 2011) in MrBayes 3.2.6-svn(r1040) (Ronquist et al., 2012) through the CIPRES Science Gateway V 3.3 (Miller et al., 2010). The stepping-stone method is one of several methods for estimation of marginal likelihoods recently shown to outperform the more traditional harmonic mean estimation (Baele et al., 2012). We ran the algorithm multiple times for each model, increasing the number of generations each time in order to minimize the average standard deviation of the split frequencies. Our longest run for each model was 510,000,000 generations long divided into 50 steps plus an additional burn-in step and diagnosed every 300,000 generations. The average standard deviation of the split frequencies dropped below 0.003 in the beginning of each of the longest runs. All runs for a given model gave similar marginal likelihood estimates regardless of the number of generations. We used the marginal likelihoods to evaluate relative support for the non-clock and strict clock models following the rough scale provided by Kass and Raftery (1995). Our comparison is summarized in Table S4.

2.3.4 Analysis of nuclear data

Due to a high degree of heterozygosity in our nuclear data, we analyzed our nuclear loci separately from our mitochondrial data and separately from each other. Due to low

phylogenetic signal within our nuclear loci (not shown), we chose to analyze each nuclear locus as an allele network. We used TCS 1.21 (Clement et al., 2000) to calculate an allele network for *Pyrocephalus* for each nuclear locus (ODC and FGB5), using sequences from preserved-tissue samples. The connection limit was set to 95% probability of parsimony. Gaps in DNA alignments were treated as a fifth character state and each gap was only counted once (as in Bi et al., 2008). To root each network, we included outgroup alleles in the analysis and lowered the connection limit to 90% probability of parsimony. Because we obtained a partial sequence of ODC for three of our samples from toe pads, we also calculated a network for that portion of ODC using all available sequences.

2.3.5 Pairwise mitochondrial genetic distances

To obtain a measure of mitochondrial divergence, pairwise genetic distances were calculated in PAUP* for ND2 using the evolutionary model selected using BIC as implemented in jModelTest 2.1.1 [HKY+ Γ (Hasegawa et al., 1985), with shape parameter, α , set to 0.4850]. We used ND2 because we obtained sequences of this gene for the largest number of taxa. Rates of sequence evolution of ND2 have been correlated with those of other mitochondrial genes that are commonly used to calculate pairwise genetic distances (Lerner et al., 2011; Smith and Klicka, 2010) as well as with morphological change (Winger and Bates, 2015). ND2 sequences were incomplete for *P. r. dubius*, several samples of *P. r. nanus* and one sample of *P. r. mexicanus* (see Fig. S1). Missing data was ignored, and distances were calculated based on available data for each pairwise comparison. Within- and between-clade pairwise distances were averaged for seven clades of *Pyrocephalus* recovered by phylogenetic analysis of mitochondrial data.

2.3.6 Divergence-time analysis

We used our mitochondrial data to estimate divergence times for key nodes within the *Pyrocephalus* phylogeny using BEAST 2.4.0 (Bouckaert et al., 2014). We used a Yule tree model with a uniform birthrate prior. We partitioned our data by gene (Cyt *b*, ND2, ND5, tRNAs + spacers), using a separate substitution model for each partition as estimated in jModelTest 2.1.10 (Darriba et al., 2012; Guindon and Gascuel, 2003) [HKY+ Γ for Cyt *b* and ND2, HKY for ND5, and K80 for tRNA + spacers (Kimura, 1980)]. We used a separate strict clock for each partition. We fixed the clock rate for Cyt *b* according to the rate estimated by Weir and Schluter (2008) for passerines (2.07% divergence per million years = 1.035% per-lineage change per million years), and estimated clock rates for the other partitions using priors with log-normal distributions and initial values based on relative substitution rates as reported by Lerner et al. (2011) (about twice the rate for ND2, about one and a half times the rate for ND5, and about one half to one sixth times the rate for tRNA relative to Cyt *b*). We estimated time to most recent common ancestor for 12 internal nodes in our phylogenetic tree. To account for phylogenetic uncertainty, we did not restrict any of the corresponding clades to be monophyletic, and we used uniform priors ranging from 0 to 100 million years. We ran the analysis for 10 million generations, logging every 1,000 generations. We discarded the first 25% of trees as burn-in. We inspected our output using Tracer 1.6.0 (Rambaut et al., 2014). Effective sample sizes were greater than 500 for all parameters and were greater than 1000 for time to most recent common ancestor for all 12 nodes of interest. The MCMC reached stationarity well within the burn-in period for all parameters.

2.4 Reconstruction of ancestral migratory status and shifts in migratory behavior

We used the MultiState option in BayesTraits V2.0 (Pagel et al., 2004) to estimate the migratory status of Vermilion Flycatchers at key ancestral nodes and to estimate relative rates of gain and loss of migration in the clade that includes Vermilion Flycatchers and their close relatives (section 2.1). Terminal taxa were coded as migratory, non-migratory, or unknown (three birds from Mexico, specimens CAS ORN 61433, CAS ORN 62487, and CAS ORN 63923, that occur in a region where Vermilion Flycatchers occur year-round. We could not verify that these three individuals were not migrants). We used the set of 22,500 post-burn-in trees from our Bayesian Inference analysis in MrBayes with clock enforced, thus allowing the program to take phylogenetic uncertainty into account. We ran the analysis using both Maximum Likelihood and Markov Chain Monte Carlo (MCMC) approaches using default parameters.

We conducted this analysis with and without an *a priori* constraint that the most recent common ancestor of Galápagos and continental populations was migratory, using the 'Fossil' command in BayesTraits. The justification for this constraint is the assumption that the Galápagos clade was founded by migrant individuals that wandered off course. We expound on this assumption in the Discussion (section 4.2).

3. Results

3.1 Molecular data

We sequenced two mitochondrial and two nuclear regions (Genbank numbers KT319227-KT319596). Table S5 lists the number of unique sequences and variable and parsimony-informative characters for each region. Lengths of sequences obtained for each sample are presented schematically in Fig. S1. Genbank numbers are listed in Table S1. Annotations of the protein-coding, tRNA, intronic, and other non-coding sequence components were determined by comparison with sequences of other Tyrannids available on Genbank.

The first mitochondrial region we sequenced consisted of the entire ND2 gene (1039 bases), plus the upstream 3'-end of tRNA-Met (25 bp), and the downstream 5'-end of tRNA-Trp (26 bases). We obtained complete sequences of this region from all preserved-tissue samples, and complete or partial sequences for samples from toe pads (Fig. S1A).

The second mitochondrial region we sequenced consisted of the entire Cyt *b* gene (1143 bases), regions upstream of Cyt *b* including the 3'-end of NADH dehydrogenase subunit 5 (ND5; 105-116 bp) and a spacer region (0-11 bp), and regions downstream of Cyt *b* including another spacer region (2 bp) and the 5'-end of tRNA-Thr (24-25 bp). We obtained complete or partial sequences of this region from all preserved-tissue samples (Fig. S1B). The Cyt *b* gene was complete in all cases. We did not obtain sequences from toe pad

samples for this region. This region included a parsimony-informative mutation in the stop codon of ND5 that has been discussed previously (Carmi, 2013; Fig. S1B).

The first nuclear region we sequenced consisted of the following portions of the ODC gene: the 3'-end of exon 6 (81 bp), intron 6 (153-155 bp), exon 7 (82 bp), intron 7 (336-339 bp), and the 5'-end of exon 8 (33 bp). We obtained complete sequences of this region from all preserved-tissue samples. We also obtained sequences of a portion of this region for three samples from toe pads (*Pyrocephalus rubinus mexicanus* specimen CAS ORN 62487 from Morelos, Mexico, and *Pyrocephalus rubinus nanus* specimens CAS ORN 86209 and CAS ORN 86210 from Santa Cruz Island, Galápagos; Fig. S1C).

The second nuclear region we sequenced consisted of the following portions of the FGB gene: the 3'-end of exon 5 (17 bp), intron 5 (519-533 bp), and the 5'-end of exon 6 (12 bp). We obtained complete sequences of this region from all preserved-tissue samples. We did not obtain any sequences of this region from toe pad samples. This region was characterized by three distinct length-polymorphisms (Fig. S1D; section 3.3.2).

3.2 Phylogenetic reconstruction of mitochondrial haplotypes

3.2.1 Topological congruence among different methods of analysis, and application of a strict clock

Vermilion Flycatchers were monophyletic with respect to outgroup taxa with 100% support regardless of method of analysis. In addition, the same seven distinct mitochondrial clades were recovered within Vermilion Flycatchers in all our analyses, including three clades from the Galápagos Islands (Fig. 2, nodes E, F and D), and four continental clades (Fig. 2, nodes I, J, K and H). Relationships among these seven clades were consistent among all methods of analysis, not taking rooting into account. However, different methods of analysis resulted in different placement of the outgroup root, leading to different basal topologies.

Our topology consisted of deep nodes connected by relatively short internodal distances. The branch separating our outgroup taxa from our ingroup (*Pyrocephalus*) was particularly long relative to the basal branches within *Pyrocephalus*. It has been demonstrated that such phylogenetic topology—with long “external” branches and short “internal” branches—can lead many methods of phylogenetic reconstruction to be inconsistent (e.g. the “long edge attraction” problem described by Hendy and Penny, 1989), potentially explaining the differences in placement of the outgroup root among our different analyses. The topology of the trees resulting from our ML and BI analyses without application of a clock rendered Vermilion Flycatchers from the Galápagos paraphyletic with respect to continental Vermilion Flycatchers, which suggests an oceanic island origin for continental Vermilion

Flycatchers. Non-continental origins of continental passerine taxa are thought to occur rarely (Filardi and Moyle, 2005), but may be particularly unlikely because of the deterministic tendency for island birds to evolve reduced flight ability (Wright et al., 2016). Furthermore, the genera that are most closely related to the Vermilion Flycatcher are virtually restricted to South America (Gómez Bahamón, 2014; Tello et al., 2009). Applying a strict clock has been shown to help circumvent “long edge attraction” problems when evolutionary rates are equal (Holland et al., 2003). Therefore, we reconstructed phylogenetic relationships again under the assumption of a strict clock, using both ML and BI. The resulting topology rendered Galápagos and continental Vermilion Flycatchers sister taxa. We assessed relative support for the two models (strict clock and no clock) by comparing their marginal likelihoods estimated using the stepping-stone method (Xie et al., 2011). We found positive support for the strict clock model over the non-clock model. [$2 \times \log(\text{Bayes factor})$ was greater than 5; Table S4; Kass and Raftery (1995)]. Therefore, we favor the topology we obtained using a strict clock (Fig. 2 and section 3.2.2 below). All topologies we recovered, including those obtained without the assumption of a clock, are presented in Fig. S2 and are also available in Nexus format in Supplementary Data S1.

Our ML and BI reconstruction of phylogenetic relationships under the assumption of a strict clock yielded trees essentially identical to each other, not taking into account minor differences in branch lengths and minor rearrangements of short terminal branches. This topology was supported by ML bootstrapping carried out under the application of a strict clock. Our clock-constrained Maximum Likelihood search in PAUP* using successive approximations (Swofford et al., 1996) resulted in alternation between two sets of most-

likely trees with near identical log likelihood scores. Differences between the trees were limited to small terminal rearrangements and minor differences in branch lengths, none of which were central to our conclusions. We present the first tree from the set of 21 similar trees with the greater likelihood score in the body of the text (Fig. 2). The remainder of these trees is available in the supplementary material (Fig. S2 and Data S1).

3.2.2 Detailed topology of strict-clock tree

Nodes discussed in the text are labeled with circled letters in Fig. 2. In the following discussion, we consider Bayesian posterior probabilities greater than 0.95 to constitute strong support, those equal to or below 0.95 to constitute weak support, bootstrap values above 90% to constitute strong support, and those between 70% and 90% to constitute moderate support (Hillis and Bull, 1993).

Pyrocephalus was monophyletic with respect to the outgroup genera, with strong support (Bayesian posterior probability of 1.00 and bootstrap value of 100% in all cases).

Pyrocephalus was separated from the base of the outgroup clade by a relatively long branch (nearly 10 times the length of the *Pyrocephalus* crown group). Within *Pyrocephalus*, two monophyletic sister clades were recovered: a clade of all specimens from the Galápagos Islands (the clade corresponding to node A in Fig. 2), and a clade of all continental specimens (the clade corresponding to node B in Fig. 2). Support for monophyly of these clades was mixed. The Galápagos clade was recovered with low posterior probability (0.87) and moderate ML bootstrap and MP bootstrap support (72% and 80%, respectively), but strong NJ bootstrap support (99%). The continental clade was recovered with high

posterior probability and strong ML bootstrap support (1.00 and 99%, respectively), but weak MP and NJ bootstrap support (<50%). Differences in support for monophyly of these clades resulted from differences in outgroup rooting among different methods of analysis (see section 3.2.1).

Within the Galápagos clade, three well-supported clades were recovered. A clade corresponding to subspecies *dubius* from San Cristóbal Island (node D) was sister to two clades comprising the subspecies *nanus*. One *nanus* clade, labeled “*P. r. nanus* Clade 1” in Fig. 2, included populations on the more northerly and central islands (Pinta, Marchena, Santiago, Rábida, Pinzón, and Santa Cruz). The other *nanus* clade, “*P. r. nanus* Clade 2” in Fig. 2, comprised populations on more southerly and westerly islands (Fernandina, Isabela, and Floreana).

Continental birds were divided into two monophyletic sister groups with high posterior probability and strong ML bootstrap support: a South American clade (node G) and a North American clade (node H). South American birds, in turn, were divided into three clades with high posterior probability and strong ML and MP bootstrap support: a clade corresponding to subspecies *saturatus* from northern South America (node I), a clade corresponding to the austral migrant South American subspecies *rubinus* (node J), and a clade encompassing the four contiguous resident western South American subspecies *piurae*, *ardens*, *obscurus* and *cocachacrae* (node K).

Additional internal structure within each of these four continental clades varied. Within the *saturatus* clade, our single sequence from the western portion of the range (specimen UMMZ 227704 from Zulia, Venezuela) was sister to sequences from the eastern portion of the range with strong support (node L). An additional strongly supported split within the eastern portion (node M) did not correspond to any obvious geographic pattern. Similarly, several divergent haplotypes within the *rubinus* clade did not correspond to any obvious geographic pattern. The four subspecies from western South America formed a strongly supported monophyletic clade with substantial haplotype diversity, but with extensive haplotype sharing among localities and weak geographic structure from the Peru-Chile border to northwest Peru and the Marañon Valley. Likewise, the two North American subspecies that we sequenced (*P. r. flammeus* and *P. r. mexicanus*) comprised a single clade with no evident geographic structure.

3.3 Nuclear allele networks

3.3.1 ODC

All alleles of ODC sequenced from *Pyrocephalus* were joined in a single network using a connection limit of 95% probability of parsimony. Outgroup species formed separate subnetworks. Lowering the connection limit to 90% probability of parsimony resulted in a single network for all alleles, allowing us to include a putative root for the *Pyrocephalus* network (Fig. 3A).

Within *Pyrocephalus*, one allele of ODC (allele d in Fig. 3A) was the most common, and occurred in nearly all continental populations. Most other alleles clustered within one to two substitutions or indel events of allele d. Of the five alleles that differed from allele d by more than two substitutions, three were unique to the austral migrant South American subspecies *rubinus* (alleles a, b and n in Fig. 3A). The possible root of the network was closer to two of these *P. r. rubinus* alleles (a and b) than to any other allele.

Some ODC structure was apparent among populations. Most alleles were unique to subspecies or geographic regions: alleles a, b, m, n, p, q and r were unique to austral migrant South American subspecies *rubinus*; allele c was unique to subspecies *nanus* from the Galápagos; allele e was unique to North American subspecies *flammeus*; allele g was unique to subspecies *saturatus* from northern South America; and alleles h, i, j, k, l, and o were unique to resident western South American subspecies as a group.

However, there was no ODC structure apparent among the four resident western South American subspecies. Of the seven alleles found in resident western South American populations, four occurred in more than one subspecies (alleles d, i, k, and o). By contrast, our allele network suggests structure within northern South American subspecies *saturatus*. Our one specimen from the western portion of the range of this subspecies (UMMZ 227704 from Zulia, Venezuela; allele g in Fig. 3A) was homozygous for a 3-bp deletion relative to sequences from the eastern portion of the range (alleles d and f). Our results suggest little differentiation between the two North American subspecies we sampled. A partial ODC sequence from a toe pad sample of subspecies *mexicanus* could not

be included in the network estimate, but it matched the corresponding portions of alleles d through n and allele r, which includes both alleles recovered from subspecies *flammeus* (alleles d and e in Fig. 3A).

The one allele we recovered from Galápagos birds (allele c) was separated from all other alleles by at least four substitutions. Sequences for this allele came from toe pad samples of two individuals of subspecies *nanus* from Santa Cruz Island, Galápagos, and were 36% complete. Placement of allele c is therefore approximate (Fig. 3A). A complete sequence would be expected to reveal additional divergence.

Allele diversity varied among populations. The highest diversity was recovered in the austral migrant South American subspecies *rubinus*, with 14 individuals characterized by 9 alleles spanning 21 base-pair substitutions. By contrast, the 24 individuals from resident western South American subspecies were characterized by only 7 alleles spanning 10 base-pair substitutions, the 9 individuals from the northern South American subspecies *saturatus* were characterized by 3 alleles spanning one base-pair substitution and one 3-bp indel, and the 13 individuals of North American subspecies *flammeus* were characterized by only 2 alleles separated by 2 base-pair substitutions.

3.3.2 FGB5

All alleles of FGB5 sequenced from *Pyrocephalus* were joined in a single network using a connection limit of 95% probability of parsimony. Outgroup species formed separate subnetworks. Lowering the connection limit to 90% probability of parsimony failed to

connect ingroup and outgroup alleles in a single network. We therefore show an unrooted network (Fig. 3B).

Within *Pyrocephalus*, FGB5 length-polymorphisms resulted in three distinct allele lengths. Most alleles of the same length clustered close to each other in the network.

Eight alleles matched the outgroup genera in length (alleles i through p in Fig. 3B; 562 bp). Allele i was the most common of these, and the others clustered within one to two substitutions of it. This group of alleles was found mainly in resident western South American populations, but allele i also occurred in two birds from Guyana.

Three alleles were characterized by a 14-bp deletion relative to the outgroup genera and to alleles i through p (alleles q, r and s in Fig. 3B; 548 bp). The most common of these, allele q, was identical to allele i except for the 14-bp deletion. Alleles r and s differed from allele q by only one base-pair substitution each and occurred only once each. This group of alleles was found mainly in North American subspecies *flammeus* and northern South American subspecies *saturatus*, but allele s occurred in an individual from Lambayeque, Peru (specimen MSB:Bird:33800).

The remaining FGB5 alleles were characterized by a 3-bp deletion relative to the outgroup taxa and to alleles i through p (alleles a through h in Fig. 3B; 559 bp). The two most common of these (alleles a and b) were one base-pair substitution apart from each other, and several alleles (c through g) were clustered within one or two base-pair substitutions

of them. One allele (allele h) was separated by six base-pair substitutions from allele a. This group of alleles was found mainly in the austral migrant South American subspecies *rubinus*, but birds from the northern South American subspecies *saturatus* were also represented in this group.

Structure among populations was clearly evident. Alleles from the austral migrant South American subspecies *rubinus* were all characterized by the 3-bp deletion. Alleles from North American samples were all characterized by the 14-bp deletion. Alleles from resident western South American subspecies were almost all of the same length as outgroup alleles, the only exception being alleles of specimen MSB:Bird:33800 from northern Peru. The northern South American subspecies *saturatus* exhibited all three length-polymorphisms, and had one private allele (allele d).

As with the ODC network, structure among the subspecies resident in western South America was not apparent. Nor was there geographic structure apparent within any subspecies of *Pyrocephalus*.

As with ODC, the diversity of FGB5 alleles varied among populations. The 14 individuals of the austral migrant South American subspecies *rubinus* again showed relatively high genetic variation with 7 alleles spanning 12 base-pair substitutions. The 24 individuals of the resident western South American subspecies were characterized by 9 alleles spanning 9 substitutions or indel events. The 13 individuals of North American subspecies *flammeus*

again showed the lowest genetic variation with only 2 alleles separated by 1 base-pair substitution.

3.4 Pairwise mitochondrial genetic distances

In Table S6, we present average within-clade and between-clade pairwise mitochondrial genetic distances (corrected using HKY+ Γ) for seven clades of *Pyrocephalus* that received strong support in our phylogenetic analysis of mitochondrial data. Average within-clade pairwise distances ranged from 0.14% to 0.37% and were generally lower for clades from the Galápagos Islands (0.14% to 0.25%) than for continental clades (0.21% to 0.37%). The average pairwise distance was highest within the austral migrant South American subspecies *rubinus* (0.37%) and within the group of four subspecies resident in western South America (0.31%).

Average between-clade pairwise distances were greatest for pairs consisting of one clade from the Galápagos and one continental clade (3.0-4.3%). Average between-clade pairwise distances for pairs of clades from the Galápagos ranged from 2.0% to 2.7%. Those for pairs consisting of the *flammeus* + *mexicanus* clade and another continental clade hovered around 2% (1.7-2.4%). Average pairwise distances were 0.7-1.2% among the three continental South American clades—subspecies *saturatus* from northern South America, austral migrant subspecies *rubinus*, and the clade of four resident western South American subspecies.

3.5 Divergence dating

We used our mitochondrial data to estimate the ages of most recent common ancestors for 12 clades within *Pyrocephalus* in BEAST 2.4.0 (Bouckaert et al., 2014) (Fig. 4). We used Weir and Schluter's (2008) estimated divergence rate for Cyt *b* in passerines (2.07% per million years), with rates for other mitochondrial genes in our dataset estimated by the program based on the relative rates found by Lerner et al. (Lerner et al., 2011). Given the many concerns that have been raised about potential pitfalls of divergence dating (e.g. Arbogast et al., 2002; Bromham and Penny, 2003; Ho et al., 2005), particularly in birds (e.g. Lavinia et al., 2016; Lovette, 2004; Nguyen and Ho, 2016), we present node ages as conditional hypotheses, subject to reevaluation if different evolutionary rates, clock models, or biasing population or evolutionary processes are demonstrated for Vermilion Flycatchers for our loci.

In our analysis, the entire *Pyrocephalus* clade coalesces on average around 1.15 mya [0.89 to 1.44 mya, 95% highest posterior density interval (HPD)]. The Galápagos clade coalesces on average about 0.82 mya (0.56 to 1.11 mya, 95% HPD), while the continental clade coalesces on average about 0.76 mya (0.60 to 0.92 mya, 95% HPD). Within the Galápagos clade, our sequences of subspecies *nanus* (encompassing all Galápagos Vermilion Flycatchers outside of San Cristóbal Island) coalesce around 0.610 mya (0.39 to 0.86 mya, 95% HPD), while our two clades within *nanus* coalesce on average about 0.15 and 0.17 mya. Our two sequences of subspecies *dubius* from San Cristóbal Island coalesce on average about 80 kya. Within our continental clade, South American Vermilion Flycatchers coalesce

on average about 0.56 mya (0.44 to 0.68 mya, 95% HPD). Our three South American clades (subspecies *saturatus* from northern South America, the clade of four resident western South American subspecies, and the austral migrant subspecies *rubinus*) and our North American clade each coalesce on average roughly a quarter of a million years ago, with very similar 95% HPD intervals.

3.6 Ancestral migratory status and shifts in migratory behavior

Loss of migratory behavior was more likely than gain regardless of method of analysis (ML or MCMC) and regardless of whether the most recent common ancestor of Galápagos and continental forms (=most recent common ancestor of all Vermilion Flycatchers) was constrained to be migratory (Table 3). When this ancestor was constrained to be migratory, loss of migratory behavior was more than 2½ times as likely as gain. The probabilities of ancestral migratory behavior at key nodes varied little between ML and MCMC approaches, and were similar for most nodes regardless of whether the ancestor was constrained to be migratory (Table 3). However, without the constraint to be migratory, the most recent common ancestor of all Vermilion Flycatchers was estimated to have a low probability of migratory behavior (Table 3). The MCMC estimates of migratory status at each node under the constraint that the most recent common ancestor of Vermilion Flycatchers was migratory imply that there were at least three or four losses of migratory status during the evolution of Vermilion Flycatchers: one in the Galápagos clade, one in the North American clade, and one or two in the South American clade (Fig. 2).

4. Discussion

4.1 Distinctness of currently recognized subspecies

Our genetic evidence is consistent with the distinctness of several of the currently recognized taxa within the Vermilion Flycatcher: the austral migrant subspecies *P. r. rubinus*, the northern South American subspecies *P. r. saturatus*, and subspecies *P. r. dubius* from San Cristóbal Island, Galápagos. However, our genetic evidence cannot distinguish several subspecies from each other: we found that the resident western South American subspecies *piurae*, *ardens*, *obscurus* and *cocachacrae* are genetically indistinguishable from each other, and that the North American subspecies *flammeus* and *mexicanus* are genetically indistinguishable from each other. In addition, our analyses identify a formerly unrecognized genetic division within subspecies *P. r. nanus* from the Galápagos. We discuss each subspecies or group of subspecies in detail below, and assess species and subspecies hypotheses in light of our genetic findings in combination with other available evidence.

In considering potential species status for a given clade, we followed the approach described in the AOU Checklist, 7th edition (Committee on Classification and Nomenclature of the American Ornithologists' Union, 1998), which involves assessing essential reproductive isolation via multiple lines of evidence. We assessed the degree of mitochondrial divergence, corroborating evidence from nuclear loci, differences in size and plumage, and differences in song, migratory habit, and other behavior. We considered evidence bearing directly on reproductive isolation where such evidence was available. In

assessing subspecies status, we followed the views recently articulated by the American Ornithologists' Union's Committee on Classification and Nomenclature (<http://www.aou.org/committees/nacc/subspecies.php>), that subspecies must be diagnosably distinct, though not necessarily genetically distinct, and that they should not merely represent points along a cline.

4.1.1 *Pyrocephalus rubinus rubinus*

Mitochondrial DNA of the austral migrant South American subspecies *Pyrocephalus rubinus rubinus* was monophyletic with strong support (Fig. 2, node J), suggesting genetic isolation. The two nuclear loci we sequenced, ODC and FGB5, provide further evidence for the isolation of *P. r. rubinus*. Seven of the nine alleles of ODC that occurred in *P. r. rubinus* were unique to the subspecies (Fig. 3A). Six of the seven alleles of FGB5 that we recovered from *P. r. rubinus* were unique to the subspecies (Fig. 3B). Furthermore, FGB5 alleles from *P. r. rubinus* clustered together separately from all other subspecies except *P. r. saturatus* (Fig. 3B). Thus, our molecular results suggest that recognition of *P. r. rubinus* as a distinct taxon reflects its distinct evolutionary history.

Our molecular evidence coincides with morphological, geographic, and vocal evidence that *P. r. rubinus* is distinct from other populations of *P. rubinus*. Even before its migratory status was clarified definitively by Zimmer (1941), Hellmayr easily distinguished *P. r. rubinus* from other South American populations based on size, dorsal coloration and tail markings (Cory and Hellmayr, 1927). In addition, Zimmer found that *P. r. rubinus* had pointier wings, on average, in comparison with non-migratory South American forms

(Zimmer, 1941), a common adaptation of migratory birds (Lockwood et al., 1998). The breeding range of *P. r. rubinus* is discontinuous with the ranges of other subspecies (Fig. 1), and it breeds at different times of year than most other South American populations (Farnsworth and Lebbin, 2004). Finally, the song of *P. r. rubinus* is distinct from the song of other continental Vermilion Flycatchers. The two song types are easily distinguished by the human ear, and comparisons of the two show consistent differences in structure, including in several frequency characters and in duration (Carmi and Jaramillo, unpublished data).

The distinct difference in song between *rubinus* and other continental populations is particularly worthy of note, since song differentiation plays an important role in speciation in many bird systems (reviewed in Slabbekoorn and Smith, 2002), and is especially important in suboscines, where song is typically innate (Kroodsma, 1984; reviewed in Seddon and Tobias, 2007). Song playback experiments between recently diverged populations of another suboscine species suggested that even subtle differences in song could contribute to reproductive isolation (Seddon and Tobias, 2007). It's likely, therefore, that the strong difference in song between *rubinus* and other continental populations is an accurate indicator of reproductive isolation. Preliminary song playback experiments carried out by one of the authors (Jaramillo, unpublished) support this notion. Based on the combination of molecular, morphological, and behavioral evidence that we have described above, we propose elevating *P. r. rubinus* to full species status.

4.1.2 Resident western South American subspecies

Our samples of the resident western South American subspecies *piurae*, *ardens*, *obscurus* and *cocachacrae* all came from Peru. Our mitochondrial data strongly supports a monophyletic grouping of these four subspecies (Fig. 2, node K). Nuclear evidence provides further support for the shared evolutionary history of the resident Peruvian populations. Six of the seven alleles of ODC in resident Peruvian birds were unique to this group (Fig. 3A). Eight of the nine alleles of FGB5 in resident Peruvian birds were unique to the group (Fig. 3B). In addition, eight of the nine FGB5 alleles in resident Peruvian birds clustered close together separately from all other subspecies except *P. r. saturatus* (alleles i through p in Fig. 3B). Our molecular evidence, therefore, supports a distinct evolutionary history for resident western South American Vermilion Flycatchers.

However, we do not feel that we have sufficient evidence to recommend that the four resident western South American subspecies as a group be recognized as a separate species. Most notably in this regard, the song of resident western South American Vermilion Flycatchers closely resembles that of northern South American and North American birds (De Benedictis, 1966; Jaramillo, unpublished). More work is necessary to determine whether western South American birds may be reproductively isolated from northern South American and North American forms. Moreover, the northern portion of the range of *P. r. piurae* in western South America approaches the western portion of the range of *P. r. saturatus* in northern South America (see Fig. 1). The possibility of gene flow between the two populations deserves examination.

Our molecular markers did not resolve the four resident western South American subspecies as lineages distinct from each other. Neither mitochondrial haplotypes (Fig. 2) nor nuclear alleles (Fig. 3) of any of the four subspecies clustered separately from the others. Initially, Hellmayr treated the resident western South American subspecies that we studied as a single subspecies, *Pyrocephalus rubinus obscurus* (Cory and Hellmayr, 1927). Zimmer separated Hellmayr's *obscurus* into subspecies *piurae*, *ardens*, *obscurus*, and *cocachacrae* (Zimmer, 1941) based on differences in size and coloration. Zimmer's detailed work described subspecies that are highly, though not perfectly, diagnosable on the basis of morphology, with only narrow zones of intergradation or taxonomic ambiguity between them. The absence of genetic distinctness is not sufficient to invalidate these subspecies, and we therefore continue to recognize them. Central and southern coastal Peru is very dry, thus potentially restricting Vermilion Flycatcher populations to isolated points along the coast, and it's possible that the subspecies described by Zimmer are recently isolated lineages. However, a systematic review of the distributions and phenotypic characteristics of Vermilion Flycatcher populations along the Peruvian coast is needed to test Zimmer's hypotheses. Future studies should also include sampling of the ecologically distinctive northern part of the range of *P. r. piurae* (Ecuador and Colombia) in which Zimmer (1941) noted geographic variation.

It's important to mention the dark, or melanic, morph of the Vermilion Flycatcher, which co-occurs with the non-melanic morph in coastal Peru within the range of subspecies *obscurus* and within part of the range of subspecies *cocachacrae* (Cory and Hellmayr, 1927; Zimmer, 1941). Some early authors believed that this morph represented a separate

taxonomic form (e.g. Berlepsch and Stolzmann, 1892; Gould, 1839; Taczanowski, 1884), but it is now clear that it has a simple genetic basis, and that melanic and non-melanic alleles occur in the same populations (Schmitt et al., 2016; van Grouw and Nolasco, 2012). Our study included samples from two specimens of *P. r. obscurus* that had been previously shown to be homozygous for the melanic allele (MSB:Bird:31417 and MSB:Bird:32963; Schmitt et al., 2016). We found no evidence of genetic differentiation between these and other central Peruvian samples.

4.1.3 *Pyrocephalus rubinus saturatus*

Mitochondrial sequences of birds within the range of the northern South American subspecies *Pyrocephalus rubinus saturatus* formed a monophyletic group with strong support (Fig. 2, node I). Our two nuclear loci, however, presented a more complex picture. Of the three ODC alleles recovered from *P. r. saturatus*, only one was unique to the subspecies (allele g in Fig. 3A). Similarly, of the four FGB5 alleles recovered from *P. r. saturatus*, only one was unique to the subspecies (allele d in Fig. 3B). It is particularly striking that all three length-polymorphisms of FGB5 were recovered from *P. r. saturatus*. In the case of both FGB5 and ODC, *P. r. saturatus* shared alleles with all other continental populations. This small degree of sorting of nuclear alleles may simply reflect the recent divergence of continental clades (roughly 760,000 years for continental birds, and about 560,000 years for South American birds; Fig. 4). However, in combination with our mitochondrial data, it constitutes imperfect evidence for genetic isolation. Previous work suggests that *saturatus* is diagnosably distinct from other populations of Vermilion Flycatcher—*saturatus* individuals average smaller and darker than other continental birds,

with females more extensively red on the ventral side than most other continental populations (Berlepsch and Hartert, 1902; Cory and Hellmayr, 1927; Zimmer, 1941). However, the song of northern South American, western South American, and North American birds is similar (De Benedictis, 1966; Jaramillo, unpublished). Further work is needed to determine whether individuals of *P. r. saturatus* recognize vocalizations from these other populations as conspecific, but in the meantime, the similarity in song is suggestive of a lack of reproductive isolation. As noted above, the possibility of gene flow between *P. r. saturatus* and *P. r. piurae* of western South America should also be investigated. In sum, *saturatus* is genetically and morphologically diagnosable and should continue to be recognized as a subspecies. However, we do not feel that there is enough evidence yet to determine whether it constitutes a full species.

Our data strongly suggests geographic structure within *P. r. saturatus*. We found a strongly supported sister relationship between our single mitochondrial sequence from the western portion of the range of *P. r. saturatus* (from Zulia, Venezuela) and our remaining mitochondrial sequences of *P. r. saturatus* from the eastern portion of the range (from Guyana; nodes I and L in Fig. 2). The individual we sequenced from Zulia, Venezuela was also homozygous for a 3-bp deletion in ODC relative to all other *Pyrocephalus* individuals and our outgroups. The possibility of a genetic split between western and eastern populations of *P. r. saturatus* deserves further investigation.

4.1.4 North American subspecies

Our mitochondrial evidence strongly suggests that *P. r. flammeus* and *P. r. mexicanus* as a group are genetically distinct from other forms of the Vermilion Flycatcher sampled in our study. Mitochondrial haplotypes from these two subspecies formed a monophyletic clade with strong support (Fig. 2, node H). Evidence from nuclear loci is less definitive. Only two alleles of ODC were recovered from *P. r. flammeus* (Fig. 3A). One was unique to *P. r. flammeus* (allele e), but the other was shared with all other continental subspecies (the most abundant and widespread allele, allele d). Similarly, only two alleles of FGB5 were recovered from *P. r. flammeus* (alleles q and r in Fig. 3B), only one of which was unique to the subspecies. For reasons similar to those we outlined for *P. r. saturatus* above, we do not recommend separate species status for North American Vermilion Flycatchers. Previous work suggest that they are morphologically diagnosable—they tend to be lighter on the back and males tend to be more orange on the ventral side (Ridgway, 1907; Sclater, 1888). However, the song of North American, northern South American, and western South American Vermilion Flycatchers is very similar (De Benedictis, 1966; Jaramillo, unpublished), and our mitochondrial evidence for genetic isolation is not mirrored by nuclear evidence. Therefore, we cannot say with certainty that North American Vermilion Flycatchers are different from other continental Vermilion Flycatchers (except *P. r. rubinus*) to an extent that is consistent with essential reproductive isolation. We note, however, that North American Vermilion Flycatchers are geographically isolated from South American forms by a substantial distance (Fig. 1), and could therefore be evolving on their own trajectory. To further test the species limits in this group, future work should assess whether there is continuing gene flow between North and South American Vermilion

Flycatchers, and the extent to which those two groups may have diverged in display behavior, song, or song recognition.

Our genetic data does not provide evidence that the North American subspecies *P. r. flammeus* and *P. r. mexicanus* are distinct from each other. The three mitochondrial sequences of *P. r. mexicanus* did not cluster separately from sequences of *P. r. flammeus*. Our nuclear data also failed to separate the two subspecies. The partial sequence of ODC we obtained from *P. r. mexicanus* was identical to the corresponding portion of both alleles recovered from *P. r. flammeus*, although it matched the corresponding portion of multiple alleles from other populations as well.

Van Rossem, who described subspecies *flammeus*, suggested an extreme eastern type locality for *P. r. mexicanus* because of an implied broad clinal gradation between the two forms (van Rossem, 1934). He claimed that other ornithologists had long noticed a racial distinction between eastern and western Vermilion Flycatchers in North America, but he failed to cite any. Ridgway, who was otherwise a voracious splitter of *Pyrocephalus*, failed to note any east-to-west variation in *Pyrocephalus* in North America, despite suggesting the existence of two or three geographic forms in North America along a north-to-south axis (Ridgway, 1907). Taken together with our molecular results, the apparent subtlety of morphological differentiation between subspecies *flammeus* and *mexicanus* suggests that the two forms should not be considered distinct. However, a morphological reassessment should be carried out to confirm this conclusion.

Our North American samples showed a low diversity of nuclear alleles as compared with other continental populations (section 3.3 and Fig. 3). In addition, nuclear alleles recovered from our North American samples were either shared with South American populations, or differentiated from them by only one or two substitutions (Fig. 3). This contrasts sharply with a strong mitochondrial differentiation that places our North American birds in a sister relationship with all our South American samples (Fig. 2). Low genetic diversity at both nuclear loci raises the possibility of a recent bottleneck, or perhaps a relatively recent origin of the population as a whole.

Two North American subspecies were not included in our study, *blatteus* and *pinicola*. Future genetic studies should include samples from these populations. The range of *P. r. blatteus* is contiguous with that of *flammeus* and *mexicanus*. It was described on the basis of differences in size and slight differences in coloration (Bangs, 1911; Ridgway, 1907). Future work should test whether these differences are geographically discrete or clinal. Preliminary song playback experiments suggest that males of subspecies *blatteus* recognize song of subspecies *flammeus* as conspecific (Jaramillo, unpublished). Subspecies *pinicola* is geographically isolated, significantly smaller than other North American forms, and occurs in a distinct ecosystem (Howell, 1965). Future work should evaluate the degree to which *pinicola* is reproductively isolated from other populations of Vermilion Flycatchers.

4.1.5 Galápagos taxa

We recovered a deep sister relationship between Galápagos and continental mitochondrial clades of the Vermilion Flycatcher (Fig. 2, nodes A and B), with the divergence dating to

roughly a million years ago (Fig. 4). In addition, the single partial allele of a nuclear gene (ODC) that we recovered from a Galápagos population (allele c in Fig. 3A, recovered from two individuals from Santa Cruz Island) was unique and differentiated from continental alleles by at least four substitutions. It is also noteworthy that the largest average pairwise genetic distances we calculated were between Galápagos clades and continental clades (Table S6).

Our finding of strong genetic differentiation between Galápagos and continental Vermilion Flycatchers coincides with substantial morphological and behavioral evidence distinguishing the two clades. The difference in size between continental and Galápagos birds is well established and unmistakable (Farnsworth and Lebbin, 2004; Gould, 1839; Ridgway, 1907; Sclater, 1888). The coloration of Galápagos birds differs from that of continental ones. Typical continental adult females have varying extents of rosy or reddish coloration on the underparts, depending on the subspecies, whereas adult Galápagos females are commonly buffy or yellow over most of the underparts (Farnsworth and Lebbin, 2004; Ridgway, 1907; Zimmer, 1941). In addition, adult Galápagos males show a rounder crown and reduced red on the crown compared to mainland males (Jaramillo, unpublished data). Steadman considered osteological differences between continental and Galápagos Vermilion Flycatchers to be substantial enough to warrant recognition of separate species on the Galápagos (Steadman, 1986). Also, the song and display flights of males observed in the Galápagos are distinct from those observed in continental birds (De Benedictis, 1966; Smith, 1967). We therefore strongly believe that Galápagos Vermilion Flycatchers represent at least one species separate from continental Vermilion Flycatchers.

Evidence also exists that Galápagos subspecies *dubius*, from San Cristóbal Island, and *nanus*, from the remainder of the archipelago, represent two separate species. The mitochondrial divergence between *dubius* and *nanus* is deep, >2% (Fig. 2, Table S6). In addition, individuals of *dubius* are distinctly smaller than birds from the remainder of the archipelago, and subtly, but discernably and consistently, different in coloration (Farnsworth and Lebbin, 2004; Gould, 1839; Ridgway, 1907). While we do not have nuclear or behavioral data available to compare the two clades, given the deep mitochondrial divergence and the distinct difference in size, we propose recognition of two separate species in the Galápagos, *P. nanus* and *P. dubius*. *P. dubius* is restricted to San Cristóbal Island, where the Vermilion Flycatcher is now generally considered to be extinct. Its posthumous recognition as a distinct species would render its extinction the first documented extinction of a Galápagos-endemic bird species (Jiménez-Uzcátegui et al., 2007).

In addition to the mitochondrial divergence between *P. dubius* and *P. nanus*, we also found a deep mitochondrial divergence within *nanus*, dividing *P. nanus* into two lineages (nodes E and F in Fig. 2). These two mitochondrial lineages separate geographically in a previously unrecognized pattern, with one lineage encompassing birds from Pinta, Marchena, Santiago, Rábida, Pinzón and Santa Cruz Islands in the north and center of the archipelago, and the other encompassing birds from Floreana, Isabela and Fernandina Islands in the south and west of the archipelago (Fig. 5E). The deep mitochondrial divergence within *nanus* raises the possibility that two taxa should be recognized within *P. nanus*. However, there isn't

sufficient evidence to fully assess this possibility. Previous treatments that recognized multiple forms within the range of *P. nanus* were all based on subtle differences in coloration. These previous treatments all conflict with one another as well as with the geographical structure we found (Ridgway, 1907, 1897, 1894; Rothschild and Hartert, 1899; Snodgrass and Heller, 1904; Swarth, 1931; Fig. 5). Behavioral comparisons among different Vermilion Flycatcher populations within the Galápagos archipelago have been limited (De Benedictis, 1966; Gifford, 1919). The apparent difficulty of identifying consistent and substantial morphological distinctions within *nanus* suggests to us that subspecies status is not warranted for the two lineages we recovered within this clade. However, future work should compare nuclear DNA sequences, song recordings and other traits from population samples on multiple islands, as well as test for reciprocal song recognition, in order to assess the possibility that the two mitochondrial lineages within *P. nanus* correspond to two reproductively distinct species, despite the apparent lack of morphological differentiation.

Additional sampling of Galápagos Vermilion Flycatchers will be needed to confirm our finding of geographic isolation for the two mitochondrial lineages that we recovered from subspecies *nanus*, particularly because we were only able to obtain sequences from one or a few specimens from each island. Vermilion Flycatchers have on rare occasions been recorded on Wolf and Española, islands at extreme ends of the archipelago where the Vermilion Flycatcher is not known to breed (Harris, 1982). This suggests a dispersal capability of Vermilion Flycatchers within the Galápagos, despite our finding of a clean geographic signal in the data. The single record of the Vermilion Flycatcher from Wolf was

an individual collected in 1906, housed at the California Academy of Sciences. Harris (1973) suggested that Wolf could have previously been humid enough to support a breeding population of *Pyrocephalus*. The specimen from Wolf, therefore, may have come from a relictual breeding population on the island, as opposed to having dispersed there from another island.

It's worth noting that if *P. r. nanus* is deemed to consist of more than one species or subspecies, it will be a taxonomic challenge to sort out the appropriate available names from the literature. Darwin's original type specimens lack specific localities (Gould, 1839; Sulloway, 1982), and subsequent taxonomic treatments failed to properly address this problem (Carmi, 2013).

Regardless of the number of forms officially recognized within *nanus*, any conservation plans for the Vermilion Flycatcher on the Galápagos Islands should take into consideration the strong mitochondrial differentiation between the three Galápagos clades recovered in our analysis. As the Vermilion Flycatcher is now generally considered to be extinct on San Cristóbal and Floreana and is in serious decline on Santa Cruz and possibly elsewhere (Merlen, 2013; Vargas, 1996; Wiedenfeld, 2006; B. Fessl, personal communication), efforts to save populations from extinction are urgently needed and should ideally include populations from both surviving mitochondrial lineages of *nanus*. Introduction of birds from one lineage of *P. r. nanus* to the geographic area of the other lineage should be considered with caution, as it could lead to decimation of a distinct genetic form.

No specific continental subpopulation is implicated as the source of colonization for the Galápagos Vermilion Flycatchers by our mitochondrial tree. Recent studies of other passerines that breed on the Galápagos have found close phylogenetic relationships with North and Central American taxa (Galápagos Flycatcher, *Myiarchus magnirostris*; Sari and Parker, 2012), Caribbean taxa (Mockingbirds, *Mimus spp.*; Lovette et al., 2012), Central and South American taxa (Yellow Warbler, *Setophaga petechia aureola*; Chaves et al., 2012), and Caribbean and South American taxa (Darwin's Finches; Burns et al., 2002). Our nuclear data suggests that the greatest genetic diversity within *Pyrocephalus* lies in South America (Fig. 3 and section 3.3). By contrast, in North America we found the smallest number of nuclear alleles among continental populations (Fig. 3, section 3.3.). In addition, the closest known relatives of *Pyrocephalus*—genera *Fluvicola*, *Arundinicola*, *Gubernetes*, *Alectrurus* and *Heteroxolmis*—are virtually restricted to South America (Gómez Bahamón, 2014; Tello et al., 2009). Thus, the common ancestor of extant *Pyrocephalus* taxa most likely lived in South America, and we hypothesize that the Galápagos Vermilion Flycatchers arose after island colonization by a migratory South American population.

We estimated that the Galápagos and continental clades of Vermilion Flycatchers diverged roughly 1.15 mya using mitochondrial data (Fig. 4). This divergence date is somewhat older than that estimated for the Galápagos Flycatcher (*Myiarchus magnirostris*) and its sister continental clade—about 0.85 mya based on mitochondrial data (Sari and Parker, 2012). Our estimate is also much older than the divergence date estimated for Galápagos populations of the Yellow Warbler (*Setophaga petechia aureola*) and their sister continental clade—about 0.268 mya, also based on mitochondrial genes (Chaves et al.,

2012). By contrast, divergence date estimates recently obtained for Darwin's Finches—a group restricted to the Galápagos archipelago and Cocos Island—are about 0.9 mya based on genome-wide nuclear data (Lamichhaney et al., 2016) and about 1.4 mya using mitochondrial data (Lamichhaney et al., 2015). This compares with an estimated divergence date of only 0.82 mya for Galápagos lineages of *Pyrocephalus* (Fig. 4). Arbogast et al. (2006) provided a wide range as an estimate of the colonization date for Galápagos mockingbirds—between 0.6 and 5.5 mya. Keeping in mind the uncertainty inherent in divergence dating, and the limited number of Galápagos-breeding landbirds for which divergence dating analyses have been carried out, our results suggest a median colonization date for Vermilion Flycatchers relative to other landbirds in the Galápagos.

4.2 Shifts in migratory behavior in the Vermilion Flycatcher

The estimates of ancestral states for migratory behavior that we present in Fig. 2 are partly contingent on the *a priori* assumption that the most recent common ancestor of Galápagos and continental forms was migratory and, accordingly, that the Galápagos Islands were colonized by a migratory ancestral population. We favor this assumption because of evidence that dispersal ability is poor in small, non-migratory Neotropical birds, and because continental relatives of other small Galápagos landbirds include migratory representatives.

Body size in birds has been shown to be positively correlated with dispersal distance (Paradis et al., 1998) and to predict the incidence of vagrants crossing long distances of water (McLaren et al., 2006). Moreover, experiments on Neotropical birds have shown that small, non-migratory insectivorous bird species are sometimes physically incapable of crossing even short distances over water (Moore et al., 2008). By contrast, there is a strong association between over-water vagrancy and migratory distance in landbirds (McLaren et al., 2006). In this light, we find it unlikely that a small, insectivorous landbird would be able to survive an oceanic flight unless it were migratory.

Evidence in the literature for a correlation between migratory behavior and capacity for oceanic island colonization is mixed. A recent study, for instance, found an association between migratory behavior and the capacity to colonize oceanic islands in passerine and “near passerine” birds on a global scale (Lees and Gilroy, 2014), but this association did not extend to low-latitude (i.e. tropical) islands. This study, however, has limitations in its applicability to our system. Most notably, it did not distinguish between large and small-bodied species. Regardless of the dataset used, however, gauging the role of migratory behavior in oceanic island colonization is challenging, because migratory behavior is a labile trait with high rates of gain and loss (Pulido, 2007). Nonetheless, a role for migration in oceanic island colonization would be suggested by a consistent pattern of colonizers that have close migratory relatives. In the Galápagos, aside from Vermilion Flycatchers, there are seven species or groups of closely related species of small or medium sized breeding landbirds that have colonized the archipelago without the aid of humans (Wiedenfeld, 2006). Of these, two have conspecific continental relatives that are migratory [the

Galápagos subspecies of Yellow Warbler, *Setophaga petechia aureola* (Chaves et al., 2012; Curson, 2010) and the Dark-billed Cuckoo, *Coccyzus melacoryphus* (Payne, 1997)]. Four have congeneric continental relatives that are migratory [the Galápagos Flycatcher, *Myiarchus magnirostris* (Joseph, 2004; Sari and Parker, 2012), the Galapagos Dove, *Zenaida galapagoensis* (Baptista et al., 1997; Johnson and Clayton, 2000) the Galapagos Martin, *Progne modesta* (Turner, 2004), and the Galápagos mockingbirds, *Mimus parvulus*, *trifasciatus*, *macdonaldi* and *melanotis* (Arbogast et al., 2006; Cody, 2005; Lovette et al., 2012)]. The remaining group, Darwin's finches (genera *Pinaroloxias*, *Certhidea*, *Platyspiza*, *Camarhynchus*, and *Geospiza*), has no congenetics outside of the Galápagos and Cocos Island. However, its closest continental relatives include species with migratory or nomadic populations (for example, *Tiaris obscurus* and *Tiaris fuliginosus*; Jaramillo and Rising, 2011). This pattern of small-bodied Galápagos resident landbird species with migratory continental relatives is consistent with our hypothesis of migratory status for the colonizing ancestors of small Galápagos landbirds, including Vermilion Flycatchers.

While it is our favored hypothesis that the Galápagos Vermilion Flycatchers were founded by a migrant, we acknowledge that alternative interpretations are possible. Studies on movements and dispersal capacities of small Neotropical insectivores have focused on forest species (Develey and Stouffer, 2001; Laurance et al., 2004; Moore et al., 2008), whereas Vermilion Flycatchers are birds of more open habitat, and it's possible that small, non-migratory, open habitat insectivores are more capable dispersers.

If our assumption is correct that Galápagos populations of Vermilion Flycatcher were founded by a migrant form, then this form represents the most recent common ancestor of Galápagos populations and their continental sister clade. Since in our analysis, the sister continental clade of Galápagos Vermilion Flycatchers consists of all remaining Vermilion Flycatchers, we conclude that the most recent common ancestor of all extant Vermilion Flycatchers was migratory. The fact that the closest relatives of *Pyrocephalus* comprise five genera that are virtually restricted to South America (Gómez Bahamón, 2014; Tello et al., 2009) suggests that the ancestor of extant Vermilion Flycatchers occurred in South America, and may have been an austral migrant similar to the modern taxon *P. r. rubinus*. Bayesian estimates of the posterior probability of migratory habit at each node indicate that migratory behavior was lost three or four times during the diversification of this species complex (Fig. 2). It is also conceivable that additional loss of migration occurred in the unsampled subspecies *blatteus* and *pinicola*. These findings are consistent with the overall tendency for migratory behavior to be lost more often than it is gained within the Tyrannidae (Gómez Bahamón, 2014). It is possible that the transitions from migratory to sedentary behavior may have caused initial divergence events (Kondo et al., 2008) that ultimately led to the diversification of this species complex.

4.3 Proposed taxonomic revision

Our work strongly suggests several taxonomic changes to the genus *Pyrocephalus*, as detailed in section 4.1, including elevating three currently recognized subspecies to full species status: the austral migrant South American subspecies *rubinus*, and the two Galápagos subspecies *dubius* and *nanus*. As such, we propose a revised taxonomy. We invite future research to assess additional taxonomic hypotheses suggested by our study, such as (1) the possibility that North American forms *flammeus* and *mexicanus* are clinal, undiagnosable forms; (2) the possibility that North American, northern South American, and western South American Vermilion Flycatchers are best treated as biological species; and (3) the possibility that the deep mitochondrial divergence within Galápagos form *nanus* corresponds to two phenotypically distinct forms that should be recognized at the subspecies or species level.

Our proposed taxonomy follows. In addition to scientific names, we include suggested common names. We exclude the purported subspecies *major* because the evidence for its existence is sparse.

Genus *Pyrocephalus* Gould, 1838

Species *Pyrocephalus rubinus* (Boddaert, 1783), Austral Vermilion Flycatcher

Species *Pyrocephalus obscurus* Gould, 1839, Vermilion Flycatcher

Subspecies *piurae* Zimmer, 1941

Subspecies *ardens* Zimmer, 1941

Subspecies *obscurus* Gould, 1839

Subspecies *cocachacrae* Zimmer, 1941

Subspecies *saturatus* Berlepsch and Hartert, 1902

Subspecies *mexicanus* Sclater, 1859

Subspecies *blatteus* Bangs, 1911

Subspecies *flammeus* van Rossem, 1934

Subspecies *pinicola* Howell, 1965

Species *Pyrocephalus nanus* Gould, 1838, Galápagos Vermilion Flycatcher

Species *Pyrocephalus dubius* Gould, 1839, San Cristóbal Vermilion Flycatcher

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Table 1. Number and type of samples of *Pyrocephalus rubinus* used in this work, by subspecies.

Subspecies	Range	No. of samples	Source
<i>P. r. flammeus</i>	W North America	13	Tissue collections
<i>P. r. mexicanus</i>	E North America	3	Toe pads
<i>P. r. blatteus</i>	S Mexico to N Belize and N Guatemala	None	
<i>P. r. pinicola</i>	Pine savanna of NE Nicaragua and E Honduras	None	
<i>P. r. saturatus</i>	N South America	9	Tissue collections
<i>P. r. piurae</i>	W South America from Colombia to N Peru	8	Tissue collections
<i>P. r. ardens</i>	Marañón River valley in interior N Peru	3	Tissue collections
<i>P. r. obscurus</i>	Central coastal Peru	7	Tissue collections
<i>P. r. cocachacrae</i>	S coastal Peru and N Chile	3	Tissue collections
<i>P. r. unassigned</i> ^a	Western Peru	3	Tissue collections
<i>P. r. rubinus</i>	S South America; winters in Amazon basin	14	Tissue collections
<i>P. r. nanus</i>	Galápagos archipelago, excl. San Cristóbal ^b	12	Toe pads
<i>P. r. dubius</i>	San Cristóbal Island, Galápagos	2	Toe pads

^a Specimens MSB:Bird:34079 and MSB:Bird:34216 from Huarney, Ancash, Peru lie in-between the described ranges of subspecies *piurae* and *obscurus* (Zimmer, 1941). Specimen USNM 637892 from Huancavelica, Peru lies in-between the described ranges of subspecies *obscurus* and *cocachacrae* (Zimmer, 1941). We left these unassigned to subspecies.

^b We sampled toe pads of subspecies *nanus* from seven islands on which it has been known to breed (Pinta, Marchena, Santiago, Pinzón, Santa Cruz, Fernandina, and Isabela), one island on which the breeding population has recently become extinct (Floreana; Merlen, 2013; O'Connor et al., 2010; B. Fessl, personal

communication) and one island on which the breeding population went extinct during the 20th century but which has recently been recolonized (Rábida; Harris, 1982; Wiedenfled, 2006; B. Fessl, personal communication). Our sample from Rábida came from the extinct rather than the recolonizing population. We did not sample *nanus* from Santa Fé (Breeding population now extinct; Harris, 1982; Wiedenfled, 2006), Wolf (known from a single specimen; Harris, 1973), or Española (known from a single sighting; Wiedenfled, 2006).

Table 2. Specimens used in this work.

Taxon ^a	Locality	Date	Museum no. ^d	Nuclear alleles ^e	
				ODC	FGB5
<i>Prf</i>	United States: Arizona: Greenlee County: Duncan, 13 mi NNW; Gila River	15 Sep 2003	UWBM 77983	d	q
<i>Prf</i>	United States: Arizona: Greenlee County: Duncan, 10 mi NNW; Gila River	14 Sep 2003	UWBM 77927	d,e	q
<i>Prf</i>	United States: Arizona: Pima County: Sonoita, 10 mi N, 4 mi E	12 Aug 2004	UWBM 77577	d,e	q
<i>Prf</i>	United States: Arizona: Pima County: Sonoita, 12 mi NNW	19 Sep 2003	UWBM 77994	d,e	q
<i>Prf</i>	United States: Arizona: Cochise County: San Bernardino National Wildlife Refuge	8 Sep 2003	UWBM 77907	d	q
<i>Prf</i>	United States: Arizona: Cochise County: San Bernardino National Wildlife Refuge	8 Sep 2003	UWBM 77908	d	q,r
<i>Prf</i>	United States: New Mexico: Sandoval County: Bernalillo Bosque	4 May 1994	MSB:Bird:18066	d	q
<i>Prf</i>	United States: New Mexico: Socorro County: San Marcial	8 May 2009	MSB:Bird:28623	d	q
<i>Prf</i>	United States: New Mexico: Socorro County: San Marcial	8 May 2009	MSB:Bird:28624	d	q
<i>Prf</i>	United States: New Mexico: Socorro Co: 8.2 km SSW San Marcial	16 Jul 2008	MSB:Bird:26864	d	q
<i>Prf</i>	United States: New Mexico: Grant County: Red Rock	8 Dec 1994	MSB:Bird:18053	d	q
<i>Prf</i>	United States: Texas: Jeff Davis County: Fort Davis, 6.6 mi ESE	4 May 2009	USNM 642014	e	q
<i>Prf</i>	United States: Texas: Jeff Davis County: Fort Davis, 6.3 mi SE	6 May 2009	USNM 642077	d,e	q
<i>Prme</i>	México: México: Lerma, 1 mi north, in marsh-corn fields	20 Jul 1952	CAS ORN 61433		

<i>Prme</i>	México: Morelos: Acatlipa	15 Aug 1958	CAS ORN 62487	d-n,r ^f	
<i>Prme</i>	México: Guerrero: Chilpancingo	1 Dec 1949	CAS ORN 63923		
<i>Prs</i>	Venezuela: Zulia: Ancon de Iturre, ca. 1 km NE of town	10 Feb 1989	UMMZ 227704	g	a,q
<i>Prs</i>	Guyana: Upper Takutu-Upper Essequibo: Karasabai, ca 17 km SSW at Ireng River	2 Aug 2004	USNM 632711	d,f	d,i
<i>Prs</i>	Guyana: Upper Takutu-Upper Essequibo: Karasabai, ca 17 km SSW at Ireng River	3 Aug 2004	USNM 632832	f	a,q
<i>Prs</i>	Guyana: Wiwitau Mountain, East Rupununi Savannah	12 Apr 2000	USNM 626191	d,f	d,q
<i>Prs</i>	Guyana: Wiwitau Mountain, East Rupununi Savannah	12 Apr 2000	USNM 626193	d	q
<i>Prs</i>	Guyana: Kusad Mountain, NE Flank, South Rupununi Savannah	4 Nov 2000	USNM 627485	d,f	d,q
<i>Prs</i>	Guyana: Kusad Mountain, NE Flank, South Rupununi Savannah	7 Nov 2000	USNM 623051	d,f	q
<i>Prs</i>	Guyana: Karaudanawa, ca. 4 km South	23 Mar 2000	USNM 625781	f	q
<i>Prs</i>	Guyana: Karaudanawa, ca. 4 km South	23 Mar 2000	USNM 625973	f	i,q
<i>Prpiu</i>	Peru: Lambayeque: Chiclayo: Olmos: ca. 9.8 km N. of Olmos	17 Dec 2009	MSB:Bird:33770	h,i	i
<i>Prpiu</i>	Peru: Lambayeque: Chiclayo: Olmos: ca. 9.8 km N. of Olmos	18 Dec 2009	MSB:Bird:33794	i	i,j
<i>Prpiu</i>	Peru: Lambayeque: Chiclayo: Olmos: ca. 9.8 km N. of Olmos	19 Dec 2009	MSB:Bird:33800	i	i,s
<i>Prpiu</i>	Peru: Lambayeque: Chiclayo: Olmos: ca. 9.8 km N. of Olmos	19 Dec 2009	MSB:Bird:33807	d,h	i,j
<i>Prpiu</i>	Peru: Lambayeque: Chiclayo: Olmos: ca. 9.8 km N. of Olmos	22 Dec 2009	MSB:Bird:33853	i,o	i
<i>Prpiu</i>	Peru: Lambayeque: Chiclayo: Olmos: ca. 9.8 km N. of Olmos	24 Dec 2009	MSB:Bird:33867	i	i

<i>Prpiu</i>	Peru: La Libertad: Viru: 1.6 km S.E. Huacapongo	1 Jul 2010	MSB:Bird:34682	d,i	i
<i>Prpiu</i>	Peru: La Libertad: Viru: 1.6 km S.E. Huacapongo	2 Jul 2010	MSB:Bird:34714	d,i	i,o
<i>Pr^b</i>	Peru: Ancash: Huarmey	21 Jan 2010	MSB:Bird:34079	d,k	i
<i>Pr^b</i>	Peru: Ancash: Huarmey	21 Jan 2010	MSB:Bird:34216	d,i	i
<i>Prar</i>	Peru: Amazonas: Chachapoyas: Balsas: Río Marañón	6 Nov 2009	UF 47634	d,j	i
<i>Prar</i>	Peru: Amazonas: Chachapoyas: Balsas: Río Marañón	9 Nov 2009	UF 47435	j	i,j
<i>Prar</i>	Peru: Amazonas: Chachapoyas: Balsas: Río Marañón	10 Nov 2009	UF 47355	d,i	i
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa; corn field	8 Jan 2008	MSB:Bird:31417	i	i,k
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa	8 Jan 2008	MSB:Bird:31423	d	i
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa	8 Jan 2008	MSB:Bird:31443	d,i	l,p
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa	18 Oct 2008	MSB:Bird:32963	i,o	i,j
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa	19 Oct 2008	MSB:Bird:32977	d,i	j
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa	19 Oct 2008	MSB:Bird:32987	i	j,m
<i>Pro</i>	Peru: Lima: San Juan de Lurigancho: Urb. La Capitana, Huachipa	19 Oct 2008	MSB:Bird:32997	i,l	i,n
<i>Pr^c</i>	Peru: Huancavelica: Pisco-Ayacucho Road, Km 102	21 Jan 2007	USNM 637892	d,i	k,m
<i>Prco</i>	Peru: Tacna: Candarave: Curibaya: Curibaya Pampa	2 Aug 2010	MSB:Bird:35468	d,i	i,k
<i>Prco</i>	Peru: Tacna: 11.0 km NE Locumba	30 Jul 2010	MSB:Bird:35038	i,k	i,m
<i>Prco</i>	Peru: Tacna: 11.0 km NE Locumba	31 Jul 2010	MSB:Bird:35055	d,i	i,k

<i>Prr</i>	Peru: Madre de Dios: Tahuamanu: Iberia: Alerta	27 Jun 2011	MSB:Bird:37039	p	a,b
<i>Prr</i>	Peru: Madre de Dios: Tahuamanu: Iberia: Alerta	27 Jun 2011	MSB:Bird:37052	p	a,f
<i>Prr</i>	Bolivia: Departamento Santa Cruz:Provincia Cordillera: Gutiérrez, 10 km E; Laguna Caucaya	6 May 2002	UWBM 77465	b,m	a,b
<i>Prr</i>	Bolivia: Departamento Santa Cruz:Provincia Cordillera: Gutiérrez, 10 km E; Laguna Caucaya	6 May 2002	UWBM 77467	f,p	a,b
<i>Prr</i>	Argentina: Provincia de Corrientes: Corrientes, 45 sm S; Manuel Derqui	17 Oct 1995	UWBM 70764	p,r	a,b
<i>Prr</i>	Argentina: Provincia de Corrientes: Corrientes, 45 sm S; Manuel Derqui	18 Oct 1995	UWBM 70766	b,p	a,h
<i>Prr</i>	Argentina: Provincia de Corrientes: Corrientes, 45 sm S; Manuel Derqui	21 Oct 1995	UWBM 70187	q,r	a,b
<i>Prr</i>	Argentina: Buenos Aires: Estancia Vitel Lauchen, Eastern Edge Laguna Vitel	27 Feb 1992	USNM 608276	d,q	a
<i>Prr</i>	Uruguay: Rivera: Vichidero, ca 11 km E at Arroyo Ceibal	31 Oct 2005	USNM 636112	p,q	a,b
<i>Prr</i>	Uruguay: Tacuarembó: Rio Negro, ca 14 km N confluence with Rio Tacuarembó, at Estancia La Rosada	29 Aug 2002	USNM 630482	f,p	a,b
<i>Prr</i>	Uruguay: Tacuarembó: Rio Negro, ca 14 km N confluence with Rio Tacuarembó, at Estancia La Rosada	30 Aug 2002	USNM 630392	a,f	b,e
<i>Prr</i>	Uruguay: Cerro Largo: Rio Yaguaron, Paso del Sarandi	30 Nov 2003	YPM 100989	n	b,c
<i>Prr</i>	Uruguay: Cerro Largo: Rio Yaguaron, Paso del Sarandi	1 Dec 2003	YPM 100994	a,r	a,g
<i>Prr</i>	Uruguay: Maldonado: Aigua, 16 km SE, at Arroyo del Yermal	21 Oct 2005	USNM 636027	a,p	a,b

<i>Prn</i> (1)	Ecuador: Galápagos Islands: Pinta Island	25 Jun 1899	CAS ORN 76689	
<i>Prn</i> (1)	Ecuador: Galápagos Islands: Marchena Island	17 Sep 1906	CAS ORN 3199	
<i>Prn</i> (1)	Ecuador: Galápagos Islands: Santiago Island	23 Dec 1905	CAS ORN 3067	
<i>Prn</i> (1)	Ecuador: Galápagos Islands: Rábida Island	21 Dec 1905	CAS ORN 3209	
<i>Prn</i> (1)	Ecuador: Galápagos Islands: Pinzón Island	5 May 1899	CAS ORN 76681	
<i>Prn</i> (1)	Ecuador: Galápagos Islands: Santa Cruz Island: trail to Fortuna	25 Nov 1961	CAS ORN 86210	c
<i>Prn</i> (1)	Ecuador: Galápagos Islands: Santa Cruz Island: Academy Bay, approx. 1/2 mi N.	22 Nov 1961	CAS ORN 86209	c
<i>Prn</i> (2)	Ecuador: Galápagos Islands: Fernandina Island (S)	5 Apr 1899	CAS ORN 76696	
<i>Prn</i> (2)	Ecuador: Galápagos Islands: Isabela Island: Banks Bay	14 Apr 1906	CAS ORN 3254	
<i>Prn</i> (2)	Ecuador: Galápagos Islands: Isabela Island: Tagus Cove	29 Dec 1898	CAS ORN 76662	
<i>Prn</i> (2)	Ecuador: Galápagos Islands: Isabela Island: Elizabeth Bay	23 Feb 1899	CAS ORN 76668	
<i>Prn</i> (2)	Ecuador: Galápagos Islands: Floreana Island	10 May 1899	CAS ORN 76686	
<i>Prd</i>	Ecuador: Galápagos Islands: San Cristóbal Island	22 May 1899	CAS ORN 76703	
<i>Prd</i>	Ecuador: Galápagos Islands: San Cristóbal Island	17 Oct 1905	CAS ORN 3287	
<i>F(p)a</i>	Bolivia: Departamento Santa Cruz:Provincia Cordillera: Gutiérrez, 10 km E; Laguna Caucaya	30 Apr 2002	UWBM 77103	
<i>F(p)a</i>	Uruguay: Departamento Artigas: Arroyo Mandiyu	18 Nov 2003	YPM 101041	
<i>Al</i>	Suriname: Sipaliwini Distrikt: Sipaliwini Savanna, Mamia Pakoro	11 Jun 2007	YPM 137461	

<i>Al</i>	Suriname: Sipaliwini Distrikt: Sipaliwini Savanna, Mamia Pakoro	12 Jun 2007	YPM 137376
<i>Al</i>	Suriname: Sipaliwini Distrikt: Sipaliwini Savanna, Mamia Pakoro	12 Jun 2007	YPM 137469
<i>Al</i>	Suriname: Sipaliwini Distrikt: Sipaliwini Savanna, Mamia Pakoro	12 Jun 2007	YPM 137470
<i>Gy</i>	Argentina: Provincia de Misiones: Posadas, 25 km E; Estancia San Juan	26 Oct 1995	UWBM 70533

^a *Prf*, *Pyrocephalus rubinus flammeus*; *Prme*, *Pyrocephalus rubinus mexicanus*; *Prs* *Pyrocephalus rubinus saturatus*; *Prpiu*, *Pyrocephalus rubinus piurae*; *Prar*, *Pyrocephalus rubinus ardens*; *Pro*, *Pyrocephalus rubinus obscurus*; *Prco*, *Pyrocephalus rubinus cocachacrae*; *Prr*, *Pyrocephalus rubinus rubinus*; *Prn* (1), *Pyrocephalus rubinus nanus*, clade 1; *Prn* (2), *Pyrocephalus rubinus nanus*, clade 2; *Prd*, *Pyrocephalus rubinus dubius*; *Pr*, *Pyrocephalus rubinus*, unassigned to subspecies; *F(p)a*, *Fluvicola (pica) albiventer*; *Al*, *Arundinicola leucocephala*; *Gy*, *Gubernetes yetapa*. Subspecies of *Pyrocephalus rubinus* were assigned on the basis of geographic range when not provided by collection manager.

^b The locality of specimens MSB:Bird:34079 and MSB:Bird:34216 falls in the gap between the ranges of Peruvian subspecies *piurae* and *obscurus* as described by Zimmer (1941).

^c The locality of specimen USNM 637892 falls in the gap between the ranges of Peruvian subspecies *obscurus* and *cocachacrae* as described by Zimmer (1941).

^d CAS ORN, California Academy of Sciences Bird Collection; MSB:Bird, University of New Mexico Museum of Southwestern Biology Division of Birds; UF, Florida Museum of Natural History (University of Florida) Ornithology Collection; UMMZ, University of Michigan Museum of Zoology Birds Division; USNM, Smithsonian Institution National Museum of Natural

History Division of Birds; UWBM, University of Washington Burke Museum Ornithology Collections; YPM, Yale Peabody Museum of Natural History Division of Vertebrate Zoology, bird collection.

^e Letters correspond to allele designations in Fig. 3.

^f The 246-bp portion of ODC obtained for this specimen was identical to the corresponding region of the alleles indicated.

Table 3. Gain and loss of migratory behavior and migratory status at key ancestral nodes in *Pyrocephalus* + close relatives. ^a

Ancestor		P(1) ± S.D.							
fixed as	Analysis				<i>Pyrocephalus</i>	Galápagos	Continental	N. Amer.	S. Amer.
migratory	method	q ₀₁ ± S.D.	q ₁₀ ± S.D.	q ₁₀ /q ₀₁ ± S.D.	ancestor	ancestor	ancestor	ancestor	ancestor
None	ML	13.64 ± 1.08	25.16 ± 2.69	1.85 ± 0.16	0.19 ± 0.08	0.03 ± 0.02	0.68 ± 0.07	0.98 ± 0.01	0.19 ± 0.08
	MCMC	28.50 ± 16.80	51.40 ± 23.31	2.43 ± 2.10	0.34 ± 0.18	0.13 ± 0.09	0.64 ± 0.18	0.95 ± 0.03	0.31 ± 0.17
<i>Pyrocephalus</i>	ML	15.95 ± 2.06	43.14 ± 6.85	2.70 ± 0.23	1.00 ± 0.00	0.08 ± 0.04	0.78 ± 0.05	0.97 ± 0.01	0.34 ± 0.08
	MCMC	31.20 ± 18.17	57.71 ± 21.66	2.69 ± 2.85	1.00 ± 0.00	0.15 ± 0.09	0.66 ± 0.17	0.95 ± 0.03	0.35 ± 0.16

^a Analysis carried out using the MultiState option in BayesTraits V2.0 (Pagel et al., 2004). Abbreviations: q₀₁, transition rate from non-migratory to migratory; q₁₀, transition rate from migratory to non-migratory; P(1), probability of being migratory at given ancestral node; S.D., standard deviation

Figure 1. Geographic ranges of currently recognized subspecies of *Pyrocephalus*. Subspecies sampled in this study are coded by color. Subspecies not sampled in this study are shown in gray. Solid fill represents year-round resident ranges, diagonal lines represent breeding ranges, and dotted fill represents winter ranges. Zigzagged boundaries join the ranges of subspecies that intergrade. Specimen localities are indicated with red dots (filled for preserved-tissue samples, and unfilled for toe pad samples). Several island populations in the Galápagos archipelago have recently become extinct, as indicated by crosses. The island of Rábida has been recently recolonized, as indicated by an asterisk. Ranges were drawn based on descriptions provided in previous treatments of the genus (Farnsworth and Lebbin, 2004; Traylor, 1979; van Rossem, 1934; Zimmer, 1941), as well as on georeferenced specimens listed on ORNIS (<http://ornisnet.org>), observational data from eBird (Sullivan et al., 2009), data associated with specimens used in this work, and the authors' personal experience.

Figure 2. Phylogenetic relationships within the Vermilion Flycatcher. Shown here is one of 21 equally most likely trees obtained by Maximum Likelihood analysis of unique mitochondrial haplotypes with clock enforced. Localities are indicated at the branch tips. Some specimens had identical sequences, which resulted in multiple localities for some branch tips. "(winter)" indicates that the given specimen was collected outside of the known breeding range or breeding season, and is therefore not likely to have been on its breeding grounds. Branch support values are shown where there is space (boxed numbers). Values are, in order, posterior probabilities from Bayesian analysis (with strict clock enforced), support values from ML bootstrap analysis (with clock enforced), support values

from MP bootstrap analysis, and support values from NJ bootstrap analysis. A dash indicates that the branch was recovered in the given analysis with a frequency of less than 50%. High support values are shown in bold (posterior probabilities of greater than 0.95, and bootstrap values of greater than 70%). Branches are thickened in black if the posterior probability was high (>0.95), and additionally in gray if the ML bootstrap support value was high ($>70\%$). Some of the deeper tree branches were shortened and marked with a double diagonal line across the branch, and the original branch length is indicated to the left of the double diagonal line. Monophyletic clades corresponding to recognized subspecies or groups of subspecies are bracketed and labeled on the right. Fill patterns and colors designate currently recognized subspecies, as shown in the legend. Fill patterns match those used in Fig. 3, and colors correspond to ranges shown in Fig. 1. Nodes discussed in the text are labeled with circled letters. Pie charts at selected ancestral nodes indicate probability of migratory status (black = migratory, white = non-migratory) as reconstructed in BayesTraits using MCMC methods with the most recent common ancestor of Vermilion Flycatchers restricted to be migratory. (Probabilities from alternative analyses are presented in Table 3). The coded migratory status of terminal taxa is indicated to their right (black = migratory, white = non-migratory, dash = unknown). Locality abbreviations: ARG, Argentina; BOL, Bolivia; GUY, Guyana; MEX, Mexico; PER, Peru; URU, Uruguay; USA, United States; VEN, Venezuela.

Figure 3. Nuclear allele networks. Alleles are represented by circles, with the area of each circle proportional to the total number of copies of the allele. The number of line segments connecting alleles corresponds to the number of substitutions or indel events separating

them. The populations where alleles were found are distinguished by the fill patterns indicated in the legend, with area proportional to the number of copies. Fill patterns match those used in Fig. 2, and colors correspond to ranges shown in Fig. 1. Lowercase letters correspond to the alleles listed in Table 2. Wavy gray lines indicate indel events. Dashed arrows indicate possible placement of outgroup alleles. Placement of allele c in panel A, sequenced from toe pads of subspecies *nanus*, is approximate, due to incomplete data (see section 3.3.1 and Fig. S1), and is therefore represented by dashed lines—a complete sequence would likely render allele c more divergent. Two identical allele copies were assumed to characterize each toe pad sample, as no polymorphic base was observed. (A) ODC. (B) FGB5.

Figure 4. Estimated time to most recent common ancestor for 12 clades within *Pyrocephalus*. Median node heights are given in millions of years. Horizontal purple bars indicate 95% HPD intervals for node heights. Taxa or groups of taxa are labeled to the right of the branch tips, with colors corresponding to those used in Figs. 1-3.

Figure 5. Alternative treatments of the Galápagos forms of the Vermilion Flycatcher. (A) through (D) Conflicting hypotheses advanced by various authors based on subtle differences in coloration. (E) Mitochondrial evidence from our work. Names of hypothesized species or subspecies are labeled in bold black lettering, and their ranges are circled in thick black lines. Dashed lines indicate uncertainty on the part of a given author. Localities of type or cotype specimens are indicated with asterisks when they are known.

Figure 1

Figure 2

Figure 3

A. ODC

B. FGB5

Legend:

Haplotype frequency:

Figure 4

Figure 5

A. Ridgway, 1894, 1897

B. Rothschild and Hartert, 1899

C. Snodgrass and Heller, 1904

D. Swarth, 1931

E. Our mitochondrial data

PYROCEPHALUS

ND2 + CYTB

FGB

ODC

mexicanus

flammeus

blatteus

pinicola

saturatus

piurae

ardens

obscurus

cocachacrae

rubinus

GÁLAPAGOS

nanus

dubius †

migratory ●
non-migratory ○

OUT-GROUPS

OUT-GROUPS

548 bp

562 bp

559 bp

Graphical Abstract