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Genomic evidence for convergent evolution of a key trait underlying divergence in island birds

Elizabeth A. Cooper | J. Albert C. Uy

Department of Biology, University of Miami, Coral Gables, FL, USA

Correspondence
Elizabeth A. Cooper, Department of Genetics and Biochemistry, Clemson University, Clemson, SC, USA.
Email: ecoope4@clemson.edu

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Abstract

Reproductive isolation can be initiated by changes in one or a few key traits that prevent random mating among individuals in a population. During the early stages of speciation, when isolation is often incomplete, there will be a heterogeneous pattern of differentiation across regions of the genome between diverging populations, with loci controlling these key traits appearing the most distinct as a result of strong diversifying selection. In this study, we used Illumina-sequenced ddRAD tags to identify genomewide patterns of differentiation in three recently diverged island populations of the *Monarcha castaneiventris* flycatcher of the Solomon Islands. Populations of this species have diverged in plumage colour, and these differences in plumage colour, in turn, are used in conspecific recognition and likely important in reproductive isolation. Previous candidate gene sequencing identified point mutations in *MC1R* and *ASIP*, both known pigmentation genes, to be associated with the difference in plumage colour between islands. Here, we show that background levels of genomic differentiation based on over 70,000 SNPs are extremely low between populations of distinct plumage colour, with no loci reaching the level of differentiation found in either candidate gene. Further, we found that a phylogenetic analysis based on these SNPs produced a taxonomy wherein the two melanic populations appear to have evolved convergently, rather than from a single common ancestor, in contrast to their original classification as a single subspecies. Finally, we found evidence that the pattern of low genomic differentiation is the result of both incomplete lineage sorting and gene flow between populations.

KEYWORDS
convergent evolution, ddRAD sequencing, divergence, *Monarcha*, speciation with gene flow

1 | INTRODUCTION

Understanding how new species arise is a key question in evolutionary biology, and is essential in determining the origins of biodiversity (Coyne & Orr, 2004; Darwin, 1869; Nosil, 2012; Price, 2007). In sexually reproducing organisms, any trait or genetic change that restricts random mating can initiate speciation, and in the earliest stages, newly-formed species can show patterns of heterogenous differentiation, with the most differentiated regions most likely containing the genes that underlie these key traits (Butlin, Galindo, & Graham, 2008; Hey, 2006). Additionally, species with deeper divergence times can still exhibit the same patterns in the presence of ongoing gene flow. Genomewide analyses of several species have shown that levels of introgression between divergent populations or species can vary across the genome, with divergent selection actively preventing gene flow at the loci underlying adaptive traits, while having weaker or no effect on other neutral regions of the genome (Cruickshank & Hahn, 2014; Ellegren et al., 2012; Feder, Egan, & Nosil, 2012; Poelstra et al., 2014). Furthermore, population genetic and phylogenetic analyses using genomic data have revealed that similar traits critical

to reproductive isolation across populations can evolve independently and could thus lead to parallel speciation (Hohenlohe et al., 2010; Soria-Carrasco et al., 2014). Low levels of background genomic differentiation due to either gene flow or incomplete lineage sorting can be advantageous for isolating genetic changes contributing to species differences, but this heterogeneity can also result in ambiguities of taxonomic classification of recently diverged taxa because specific regions will have unique evolutionary histories.

A classic example of incipient speciation is populations of the *Monarcha castaneiventris* flycatcher of the Solomon Islands, which has several subspecies that vary in body size, plumage colour and song, and may be at different stages of divergence (Mayr, 1942; Mayr & Diamond, 2001; Filardi & Smith, 2005; Uy, Moyle, & Filardi, 2009; Uy, Moyle, Filardi, & Cheviron, 2009; Uy & Safran, 2013; Figure 1). This species complex featured prominently in Ernst Mayr's seminal book on speciation, in which he argued that geographic isolation is essential in initiating reproductive isolation (Mayr, 1942). A phylogenetic analysis of this species complex based on three markers (two mtDNA, one nuclear gene) indicated that most subspecies form well-defined clades in accordance with their geography and the region's geological history, with the exception of two subspecies found in the southeastern region of the archipelago (Uy, Moyle, & Filardi, 2009). These two subspecies are found on islands that are 8–10 km apart (Figure 1), and are essentially fixed for divergent plumage colour, yet fail to form reciprocally monophyletic groups. On the large island of Makira, birds have chestnut bellies and iridescent blue-black upper parts (*Monarcha castaneiventris megarhynchus*). In contrast, birds in nearby satellite islands to the north (Ugi and Three Sisters) and southeast (Santa Ana and Santa Catalina) of Makira are entirely blue-black or melanic (*Monarcha castaneiventris ugiensis*) (Mayr, 1942; Mayr & Diamond, 2001; Figure 1).

Only one other taxon in the entire clade exhibits a completely melanic phenotype: the polymorphic population of *Monarcha castaneiventris obscurior* found on the Russell Islands more than 300 km northwest of Makira (Figure 1). Within this population, melanic birds are relatively rare (<10% of the population) (Uy & Castro-Vargas, 2015), while a chestnut-bellied form similar to *M. c. megarhynchus* predominates. All other members of the super species complex that includes *Monarcha castaneiventris*, *M. melanopsis* and *M. frater*, as well as their outgroup *M. cinerascens*, are characterized by chestnut bellies, suggesting this phenotype is the ancestral form (Filardi & Smith, 2005; Mayr & Diamond, 2001; Uy, Moyle, & Filardi, 2009).

While it is known that the melanic and chestnut forms of *M. castaneiventris* must have diverged within the last 1–2 million years based on the geological history of Makira and the satellite islands (Mayr & Diamond, 2001), the exact timing of the origin of the derived melanic form remains unresolved. Furthermore, such an estimation is likely to be complicated by gene flow between the two subspecies, as the islands are separated by short distances (i.e. ca. 8 km). Evidence for gene flow can be deduced not only from previous estimates from limited genetic data, but also from the presence of putative hybrids or birds of intermediate plumage colour on both Makira and the satellite island of Santa Ana (Uy, Moyle, Filardi et al., 2009; Uy et al., 2016).

Previous work using a handful of markers suggested substantial gene flow among the islands of the Makira clade. However, despite gene flow between the two colour forms, it has been shown that both plumage colour and song are important in species recognition, with plumage colour playing a much larger role (Uy & Safran, 2013; Uy, Moyle, Filardi et al., 2009). In mount presentation and song playback experiments conducted in the field, territorial males responded more strongly to homotypic rather than heterotypic mounts and songs, an assay that has been used in other systems as an index of

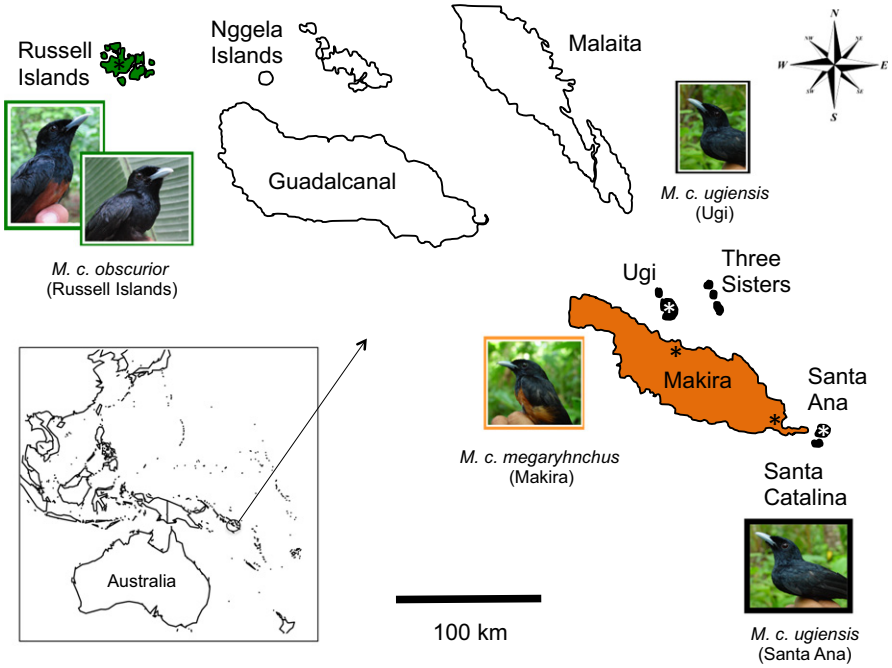

FIGURE 1 Sampling localities and the distribution of Subspecies in the Makira Clade. The southeastern region of the Solomon Islands archipelago with asterisks showing the locations sampled. Orange-coloured islands represent the populations of chestnut-bellied *M. c. megarhynchus*, while black islands indicate the range of the melanic *M. c. ugiensis* birds. Green islands indicate the range of the colour polymorphic *M. c. obscurior* subspecies, which was used as an outgroup in this study

conspecific recognition and thus reproductive isolation (Baker, 1991; Irwin, 2000; Ratcliffe & Grant, 1983, 1985). Therefore, divergent colour and song likely play a key role in premating reproductive isolation in the *Monarcha* flycatchers and in keeping plumage colour nearly fixed between islands (i.e. assortative pairing by plumage colour and song are agents of disruptive selection; Uy, Moyle, Filardi et al., 2009; Uy & Safran, 2013).

Candidate gene sequencing revealed that a single, nonsynonymous point mutation in the *melanocortin 1 receptor* (*MC1R*) is perfectly associated with the difference in plumage colour between the chestnut-bellied population of Makira and the melanic population of Santa Ana, southeast of Makira (Uy, Moyle, Filardi et al., 2009; Figure 1). This point mutation was also found to be identical to variants in *MC1R* shown to produce melanism in both sheep and pigs (Kijas, Moller, Plastow, & Andersson, 2001; Vage, Klungland, Lu, & Cone, 1999), making it very likely to be the causal mutation in *Monarcha* as well. Surprisingly, however, the same *MC1R* variant is *not* fixed in the northern melanic population of Ugi Island, with melanic birds either being heterozygous or homozygous for the ancestral or derived *MC1R* alleles (Uy, Moyle, Filardi et al., 2009; Uy et al., 2016). Instead, a single nonsynonymous mutation in the *agouti signalling protein* (*ASIP*) is perfectly associated with melanism (Uy et al., 2016). No birds from Santa Ana were homozygous for the mutant variant of *ASIP*, which suggests two genetic mechanisms for the melanic phenotype in *M. c. ugiensis*. Given the high frequency of the melanic *MC1R* allele in the northern Ugi population but very low frequency in Makira (i.e. only in putative hybrids), and that the melanic *ASIP* allele is only found in rare hybrids in Makira and Santa Ana (Uy et al., 2016), it seems plausible that the derived *MC1R* variant evolved first in the common ancestor of all melanic subspecies, with the *ASIP* variant evolving secondarily in only the northern populations of Ugi and Three Sisters. Under this hypothesis, the two populations of *M. c. ugiensis* should be sister taxa (Figure 2). On the other hand, the current distribution of candidate colour mutations could be the result of convergent evolution of melanism across island populations, followed by ongoing gene flow between island populations, and/or differential selection on the two different alleles in different populations (Figure 2).

If the *MC1R* polymorphism was truly ancestral, then it would be reasonable to expect that this variant would also underlie melanism in the polymorphic Russell Islands population, but this is not the case. In fact, melanic and partially melanic Russell Islands birds lack both the *MC1R* and the *ASIP* variant, indicating the presence of yet a third mechanism for the plumage colour differences observed in this system (unpublished data). This result also raises an alternate possibility that *MC1R* and *ASIP* are actually the spurious results of population structure, and there is a single, unidentified mutation controlling melanism in all of the populations. In the zebra finch *Taeniopygia guttata*, an association between *MC1R* variants and plumage colour was recently found to be such a spurious result of population structure (Hoffman, Krause, Lehmann, & Kruger, 2014), so it is essential to consider candidate mutations found in *Monarcha* in a genomic context. In order to differentiate between alternate speciation scenarios and confirm that the observed nonsynonymous mutations truly represent two independent mechanisms for a key evolutionary trait, it is necessary to rule out the possibility that *MC1R* and *ASIP* appear highly differentiated between colour morphs because of neutral processes instead of strong selection.

The advent of next-generation sequencing technologies has made genomewide studies of genetic variation feasible in nonmodel organisms, making this a powerful tool in the study of speciation in nature [see Seehausen et al., 2014 for a review]. In particular, restriction-site-associated DNA sequencing [RAD-Seq; Baird et al., 2008;] has been used successfully to identify gene flow and

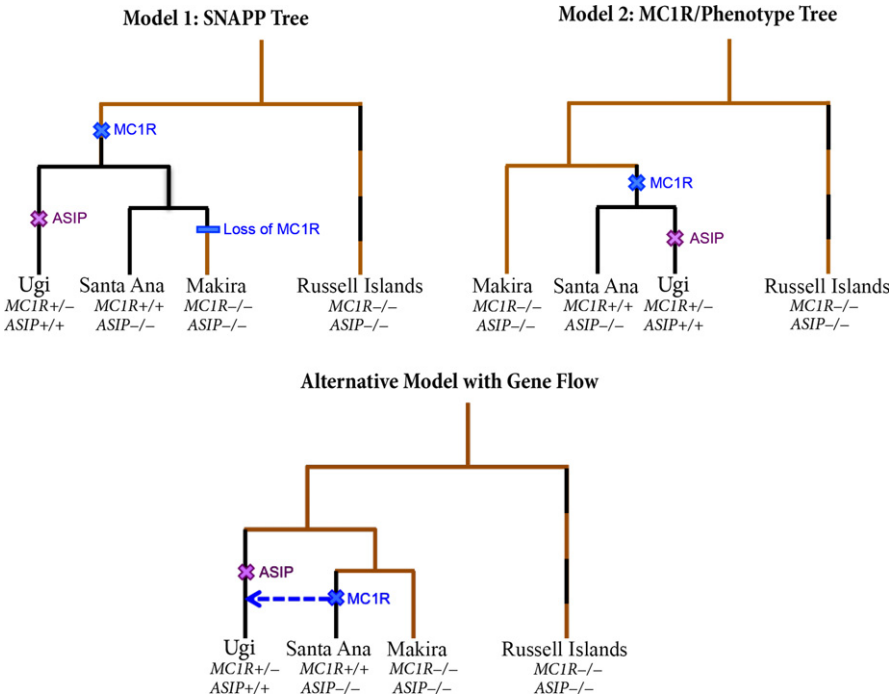

FIGURE 2 Alternative tree topologies for the Makira clade. *MC1R+/+* indicates that a population is nearly fixed for the point mutation in *MC1R* associated with melanism; *MC1R+/–* indicates a population that is polymorphic for this mutation and *MC1R–/–* indicates that a population is nearly fixed for the ancestral, nonmutant *MC1R* allele. The same notation is used to indicate presence/absence of the *ASIP* allele that is also associated with melanism. The colour of the branch indicates the phenotype of the population: brown branches correspond to chestnut-bellied populations while black branches correspond to melanic populations and mixed branch colours indicate that the population is polymorphic. 'X' symbols denote when the mutations in either *MC1R* or *ASIP* may have originated, depending on the model

population structure in a variety of closely related species (Andrew, Kane, Baute, Grassa, & Rieseberg, 2013; Bruneaux et al., 2013; Hohenlohe, Amish, Catchen, Allendorf, & Luikart, 2011; Keller et al., 2013), including several species of birds (McCormack et al., 2012; Parchman et al., 2013; Rheindt, Fujita, Wilton, & Edwards, 2013).

In the absence of a reference genome for *M. castaneiventris*, we generated a data set of de novo assembled double-digested RAD (ddRAD) marker sequences to discover and analyse a large set of widely distributed genomewide single nucleotide polymorphisms (SNPs) in several populations of the *Monarcha* flycatcher, focusing on the Makira clade. We then used these SNPs to address multiple questions important to the understanding of speciation and taxonomy of these island birds: How much variation is shared between subspecies, and how much of this shared variation is due to gene flow rather than recent divergence from a common ancestor? How recently did the melanic subspecies diverge from the chestnut-bellied ancestor? Did melanism evolve convergently between the Ugi and Santa Ana populations? What are the background levels of genomic differentiation, and how do the candidate genes for melanism compare to the genomewide SNP data? Given substantial gene flow and little genomic differentiation, what are the implications for the taxonomic classification of these taxa?

In this study, we show that the vast majority of genomewide variation is shared between subspecies that differ distinctly in plumage colour, with some population pairs showing extremely low levels of differentiation. The phylogeny predicted by our genomewide data conflicts with the previous classification of both melanic populations as a single subspecies and instead shows evidence for independent origins of melanism in each population. We also find clear evidence of gene flow between adjacent populations, with the greater migration occurring from the chestnut-bellied, main island population into the melanic satellite islands. The presence of gene flow may provide an explanation for intermediate frequencies of the *MC1R* allele in the northern satellite islands, but may also be confounding the species tree. Regardless, our results show that both candidate plumage colour genes are more highly differentiated (i.e. nearly fixed; Uy et al., 2016) than any of the polymorphisms identified in the ddRAD-Seq markers, further implicating them as causative mutations rather than by-products of genetic drift. This system therefore appears to have at least two separate mechanisms for plumage colour evolution, with selection maintaining the differences in each pigmentation gene while background loci introgress between islands. These results have intriguing implications for complex origins of subspecies differences and parallel speciation, but they also present considerable challenges to the taxonomic analysis of this clade.

2 | MATERIALS AND METHODS

2.1 | Sample collection, library preparation, and sequencing

From 2006 through 2011, a total of 91 individuals from four different island populations were sampled and included in this study. Melanic, *M. c. ugiensis* birds were sampled from two satellite islands: 21 birds from Santa Ana (southeast) and 24 birds from Ugi (north; Figure 1). Chestnut-bellied *M. c. megarhynchus* birds were sampled from two locations in Makira Island: 10 birds from Kirakira on the northern coast of the island across from Ugi and 14 birds from Star Harbour on the southeastern coast across from Santa Ana (Figure 1). Finally, 22 birds of the more distantly related subspecies, *M. c. obscurior*, were sampled from the polymorphic population on the Russell Islands. This cluster of islands is >300 km northwest of Makira and separated by the large island of Guadalcanal (Figure 1). There are no records, either from historical or recent museum collecting trips, that have reported the presence of *M. c. obscurior* birds on Guadalcanal or *M. c. castaneiventris* birds in the Russell Islands, so migration between the Russell Islands and Makira via Guadalcanal as a stepping stone seems unlikely (Mayr, 1942, C. Filardi, personal communications, J.A.C. Uy, personal observations). Therefore, the Russell Island population was treated as an outgroup for this study.

For each bird, blood serum was collected in the field and stored in lysis buffer (Longmire et al., 1991) until genomic DNA was extracted using Qiagen's DNeasy Blood and Tissue Kit. ddRAD-Seq libraries were constructed following a slightly modified version of the protocol described by Parchman et al. (2013). Briefly, the genomic DNA was double-digested with EcoRI and MseI, and then ligated to barcoded Illumina sequencing adapters containing sequences that were complementary to the restriction sites. Only forward adapters complementary to the EcoRI cut sites were barcoded; MseI complementary adapters were universal across samples. Adapter-ligated DNA was then purified using Beckman-Coulter's AMPure purification kit (cat #A63880) to remove any remaining adapter dimers prior to PCR amplification. This final purification step was not included in the original protocol but was found to noticeably improve library quality and greatly reduce the presence of adapter sequences compared to samples that were only purified via size selection. The adapter-ligated fragments were then amplified via PCR and size-selected for fragments between 300 and 500 bp using gel electrophoresis, which removed any unligated adapters that may have escaped AMPure clean-up. Samples were organized into libraries according to population, resulting in four libraries with a maximum of 24 barcoded individuals each. Each library was sequenced at the Hussman Institute of Human Genetics, University of Miami sequencing core facilities in one half of a lane on an Illumina HiSeq 2000, for a total of two lanes of 100-bp single-end reads. The library for one of the populations (Makira) was divided into two separate one-quarter lane runs, which allowed us to test for lane effects. Detailed laboratory protocols are available in the Supplementary Information.

2.2 | Alignment and SNP calling

Raw Illumina reads were sorted by barcode and filtered for quality (i.e. any read with a Phred quality <20 was removed) using PROCESS_RADTAGS from the Stacks package (Catchen, Amores, Hohenlohe, Cresko, & Postlethwait, 2011). After removal of adapter sequences and barcodes, the remaining reads were 94 bp in length. These reads

were then clustered together within individuals at a high similarity (99%) using USTACKS (also from the STACKS package), and any putative tag with an excessive read depth (>50) was removed from further analyses. To avoid potentially paralogous loci, the tags within each individual were re-clustered using a more relaxed identity threshold in CAP3 (Huang & Madan, 1999), and any reads clustering at 90% or higher were removed. Next, the consensus ddRAD tags across all individuals in all populations of the Makira clade were identified using USTACKS and CSTACKS with much more relaxed mismatch parameters (up to 10 mismatches allowed). These consensus tags were subsequently treated as a 'reference' genome (similar to the provisional reference genome described in Hird, Brumfield, & Carstens, 2011).

To ensure that the ddRAD tags were distributed evenly across the genome, we aligned the consensus tags against the publicly available Zebra finch *Taeniopygia guttata* reference genome (Warren et al., 2010) using Stampy with the recommended parameters for mapping to a divergent reference (i.e. set substitution rate to 0.05) (Lunter & Goodson, 2011), and checked for a strong correlation between chromosome size in the zebra finch and the number of mapped *Monarcha* consensus tags (as in Parchman et al., 2013).

For the sample that was divided into two separate quarter lane runs, raw data from each run were aligned to our consensus 'reference' genome, and the read depth for each tag sequence was used as a metric for comparing runs. To control for differences in the total number of sequences in each run, read depths were normalized by calculating the proportion of reads from each run that aligned to a particular tag sequence, and then multiplying these proportions by the total number of sequences from the combined runs. A Spearman's rank correlation test in *R* revealed that read depths at each tag were highly correlated across the two runs ($r^2 = 0.979$, $p < 2.2e\text{-}16$), and a one-way ANOVA did not find any evidence for significant lane effect ($p = .222$). Finally, only 3.7% of the total tags in the data were unique to one lane, and only 0.2% of tags that passed read depth filters (described below) were not found in both runs, indicating minimal lane effects in our data.

Individual, quality-filtered reads (output from PROCESS_RADTAGS) from both the Makira clade populations and the Russell Island population were aligned to the consensus tag 'reference' genome using BWA (Li & Durbin, 2009), allowing up to 10 mismatches (note that over 99% of reads actually had fewer than five mismatches). SNPs were called using VCFUTILS in the SAMTOOLS package (Li et al., 2009) with command line options to calculate genotype likelihood scores, require a minimum quality threshold of 20, a minimum individual read depth of 5 and a maximum individual read depth threshold of 50. Using custom Perl scripts, we further filtered the SAMTOOLS output by removing any sites with more than two alleles and verifying that heterozygous variants consisted of at least 20% of reads within an individual containing the minor allele. We also considered the overall frequency of minor alleles in the entire population, and removed polymorphisms with <5% frequency to eliminate potential sequencing errors. More details about our pipeline and the scripts we used can be found online at: (https://github.com/eacooper400/GenDivergence). Raw sequencing data are available from the NCBI SRA database (SRP082402).

Our process is similar to the methods and cut-offs used by Rheindt et al. (2013), although we used lower minimum and maximum read depth thresholds because we had lower overall coverage in our data. We determined the thresholds of 5X and 50X by considering the empirical distribution of coverage depths in our data, where it appeared that sites above or below these thresholds represented outliers (Fig. S1). However, as 5X is a lower minimum read depth than many standard pipelines, we also generated an alternate SNP data set using a 10X coverage requirement, and reran the F_{ST} analyses (Fig. S2). These analyses produced results that were very similar to the 5X data set, so we decided to continue with the 5X data in order to maximize the number of loci in our analyses and to avoid potential bias from restricting to overly conserved sites (Buerkle & Gompert, 2013).

2.3 | Population differentiation

Prior to any population genetics analyses, we first determined how much missing data could be present at a particular locus without biasing our results. Because the proportion of SNPs classified as shared across populations should be the most sensitive to missing individuals, we plotted this statistic as a function of the number of individuals present in each population. We found that a minimum cut-off of at least 15 individuals per population would allow us to effectively assess population-level sharing and differentiation (Fig. S3). This was determined based on the fact that the estimated proportion of shared variants stopped increasing once this many individuals were present (or in other words, this statistic became independent of coverage), indicating that we had reached sufficient coverage to calculate the 'true' proportion of shared polymorphisms.

After filtering for coverage in each population, the SNPs called in the Makira clade populations of Makira, Santa Ana, and Ugi were polarized using the Russell Islands (*M. c. obscurior*) data as a reference (i.e. the ancestral state for each locus was inferred based on an outgroup). Many of the sites were also polymorphic even in the more genetically distinct *M. c. obscurior*, suggesting relatively high levels of incomplete lineage sorting. Alleles that were fixed in the Russell Islands were considered to be ancestral (as this is the most parsimonious explanation) [See Rong et al., 2014 as an example]. These polarized data were used to generate the derived allele frequency spectrum both as its own analysis and for use as input for the $\partial a \partial i$ models (see further details in the Migration Analysis section).

Estimates of F_{ST} were calculated on a site-by-site basis and then averaged across loci. F_{ST} was estimated using the following equation:

$$F_{ST} = \frac{H_T - H_S}{H_T}$$

where H_T and H_S were based on transformations of the minor allele frequency (Wright, 1969). More explicitly, if $\bar{p}$ is the frequency of the minor allele in the total sample, p_i is the frequency of the allele in the *i*th population and c_i is the relative size of the *i*th population, then:

$$F_{ST} = \frac{\bar{p}(1-\bar{p}) - \sum c_i p_i (1-p_i)}{\bar{p}(1-\bar{p})}$$

Within the larger island of Makira, F_{ST} was estimated between the Kirakira and Star Harbour populations (Figure 1). To test for nonzero estimates of F_{ST} that might result from the small sample sizes within Makira, within-island levels of F_{ST} for Ugi and Santa Ana were also estimated by randomly assigning individuals into one of two groups of equal size (groups of 10 for Santa Ana, and groups of 12 for Ugi). Significance levels for the mean F_{ST} values were determined using permutation tests: individual genotypes (across all ddRAD sites) were randomly permuted into 'populations' of the same size 10,000 times, and then, mean F_{ST} was re-calculated each time. The resulting distributions of the randomly permuted F_{ST} values were then used to calculate the probability that the observed mean F_{ST} values differed significantly from zero. F_{ST} outliers were defined as values >0.7, a cut-off that was selected based on the shapes of the empirical F_{ST} distributions and which corresponded to the 99.9th percentile.

It should be noted that F_{ST} is a relative measure of differentiation between populations, which has been shown to be inflated in genomic regions of low diversity (Cruickshank & Hahn, 2014). With the sparse genome coverage of our ddRADtag data (on average 9–10 SNPs per 100-kb window, but often lower), it is not possible to use an estimate of absolute divergence such as D_{XY} in this data set as a means of controlling for this; instead, our F_{ST} results must be interpreted with caution and in the context of other analyses.

Population structure was inferred directly from the genotype data using the Bayesian MCMC program STRUCTURE 2.3 (Pritchard, Stephens, & Donnelly, 2000). A subset of SNPs with minor allele frequencies ≥10% were selected from the larger data set, and only one SNP per ddRAD tag locus was included to ensure that each locus could be treated as independent, which left 37,265 SNPs to be included in the analysis. We performed 10 runs each for values of K between 1 and 6, with a burn-in length of 20,000 iterations followed by 100,000 estimation steps. Plots of the STRUCTURE output were generated by *distruct* (Rosenberg, 2004). The optimal value of K was evaluated both by considering the log-likelihood of each K value, as well as the average 'clusteredness', which is described in (Rosenberg et al., 2005) and is given by the following equation:

$$G = \frac{1}{I}\sum_{i=1}^{I}\sqrt{\frac{K}{K-1}\sum_{k=1}^{K}\left(q_{ik} - \frac{1}{K}\right)^2}$$

G denotes 'clusteredness', I represents the total number of individuals, K is the number of clusters and q_{ik} is the estimated membership coefficient of the ith individual in the Kth cluster (Fig. S4). For this measure, a G value of one would indicate that individuals were each perfectly assigned to a single cluster. A $G < 1$, on the other hand, would indicate that some or all individuals were assigned to more than one cluster. The lower the G value, the less perfect the assignment would be (i.e. a lack of population structure).

To corroborate the STRUCTURE analysis, a principal components analysis (PCA) was also performed using the same set of SNPs. The PCA was implemented in the software package PLINK version 1.9 (Purcell et al., 2007). A second PCA was performed with the subset of SNPs mapped to the Z chromosome as well. Results are shown in Fig. S5.

2.4 | Species trees

For the phylogenetic analysis, we randomly selected three individuals from each of the four populations, for a total of 12 birds, and included only SNPs with no missing data in any population (6,648 SNPs; as in Rheindt et al., 2013). We performed the subsampling of individuals 10 separate times and reran the analysis with each data set to ensure consistency regardless of which individuals were used. We used the software SNP AND AFLP PACKAGE FOR PHYLOGENETIC ANALYSIS (SNAPP; Bryant, Bouckaert, Felsenstein, Rosenberg, & RoyChoudhury, 2012) to infer species tree topologies, along with posterior probabilities for each node. Using mostly default settings (e.g. *Yule prior* for species tree and branch length estimations; gamma distributions for ancestral theta), we performed one million burn-in iterations, followed by nine million sampling runs, which resulted in 9,000 saved trees that were used to generate the consensus species tree. To insure convergence, we confirmed that our runs reached effective sample size values >500 after burn-in using the program TRACER (Rambaut, Suchard, Xie, & Drummond, 2014). Maximum clade credibility trees were generated using TREEANNOTATOR, and posterior distributions of species trees were visualized using DENSITREE (Bouckaert, 2010).

In a previous phylogenetic study of the *M. castaneiventris* species complex (Uy, Moyle, & Filardi, 2009), a ND2-specific molecular clock of 0.02–0.022 substitutions per site per lineage million years was used to estimate the divergence times of key nodes within the entire complex. This ND2-specific molecular clock was calibrated for island Passerines with evolutionary processes similar to those experienced by *M. castaneiventris*, such as small effective population sizes and potential founder effects (Arbogast et al., 2006). Unfortunately, genetic samples for the *M. c. obscurior* subspecies were unavailable at the time of the Uy, Moyle, & Filardi (2009) study. Therefore, as we are using *M. c. obscurior* as the outgroup in this study, we sequenced 764 bp of the ND2 gene for 13 *M. c. obscurior* birds to supplement the Uy, Moyle, & Filardi (2009) published data set. We calculated the genetic distance and thus the timing of the split between the Makira and Russell Islands clades using the 12 birds from the Makira clade included in the Uy, Moyle, & Filardi (2009) study (i.e. 3 birds from Makira, three birds from Ugi and six birds from Santa Ana), and the 13 birds from the Russell Islands sequenced in this study. As the divergences between the Makira, Ugi and Santa Ana populations within the Makira clade are less resolved using the ND2 gene (Uy, Moyle, & Filardi, 2009), the nodes for the more recent splits within the Makira clade were dated by calibrating the branch lengths that lead to each node within the species tree generated by SNAPP (note, the mutation rates in SNAPP are rescaled so that the average number of mutations per unit time is

one). Genetic distance was calculated as D_a using the Jukes–Cantor model of evolution and implemented in DNASP.

2.5 | Migration analysis

To simultaneously investigate the divergence times and levels of gene flow between all pairs of populations in the Makira clade, we used the software *∂a∂i*, which implements a diffusion approach based on the joint allele frequency spectrum (Gutenkunst, Hernandez, Williamson, & Bustamante, 2009). To select the SNPs to use in calculating the allele frequency spectrum, we started with the set of all quality-filtered SNPs that were present in the data prior to filtering for population-level coverage. We then removed all sites where the alleles were *not* fixed in the Russell Islands population in order to use the Russell Islands as an outgroup to polarize the Makira clade SNPs. This resulted in a set of 90,051 SNPs that was used to create a *∂a∂i* spectrum object as described in the *∂a∂i* manual. Because we had missing data, we projected down to a sample size of 24 (i.e. 12 individuals per population) in order to increase the number of SNPs used in the analysis.

We used the resulting frequency spectrum to test a total of three different models of demographic history: (1) a model with no migration between any populations, (2) a model with migration only between adjacent populations (Makira and Santa Ana, Makira and Ugi; see Figure 1), and (3) a model with migration between all population pairs. Because of the uncertainty with regard to the true divergence order in the Makira clade (See Section 3.3), we chose to run each of the three models with two alternate tree topologies: the maximum-likelihood tree inferred by SNAPP, and the tree that is most parsimonious with respect to the candidate plumage colour genes (Figure 2). For each model, maximum-likelihood parameter values were estimated with the BFGS optimization method, with a total of 100 random perturbations and iterations per run to ensure convergence on a global optimum. To check for convergence, we also ran 10 independent replicates of each analysis for each demographic model, and looked for similarity among the final optimized likelihood scores. Scripts for running *∂a∂i* were adapted from Rittmeyer and Austin (2015) (Scripts can be found in the Supplemental Information).

To remove runs that were possible outliers, we used an extreme studentized deviate (ESD) test (Rosner, 1983) to determine which runs had optimized likelihood values that differed significantly from the mean likelihood across all ten runs. We removed runs with either an unusually high or low ESD statistic based on the assumption that these likely represented local rather than global optima. Most models showed convergence of likelihood scores across all 10 runs (Table S1); however, both models testing for migration between all population pairs contained up to three outliers. To ensure consistency of our results, we removed the three runs with the highest ESD score for every model, such that all further analyses were based on parameter means from seven independent replicates of every model. To determine the best model, we used the second-order Akaike information criterion (AIC_c; Burnham & Anderson, 2002) in order to control for both the number of parameters in each model and the sample size. Models with different population migration histories were compared for the same tree topologies, but models were not compared across tree topologies.

To translate divergence time estimates into years, we used the equivalency given in the *∂a∂i* manual that $T_{\partial a \partial i} = 2N_e$ generations, and assumed that generation time in *Monarcha* is roughly 2 years. To calculate N_e, we used *∂a∂i*'s estimate of θ for the ancestral population (which was similar for both topologies), and determined θ per site based on how many SNPs passed filtering when estimating the allele frequency spectrum ($\approx 7,000$). A mutation rate of $2(10)^{-8}$ sites per year was used based on data from ND2, and N_e was calculated as $N_e = \theta_A/4\mu$.

3 | RESULTS

3.1 | Sequencing and SNP calling

After filtering reads with low-quality sequences or ambiguous barcodes, we retained an average of nearly three million reads per individual, which corresponded to 409,030 consensus 94-bp ddRAD-Seq loci after clustering with Stacks and CAP3 (3.4% of the *Monarcha* genome, assuming a 1.2 Gb genome size). Approximately 78% of these tag sequences aligned to the zebra finch reference genome, where a strong correlation between chromosome size and number of aligned tags indicated a roughly even distribution of the markers (Fig. S6).

Of the 409,030 total tags or loci, there were 213,205 loci with at least 15 individuals present from each island population, and 23.5% of these were variable, resulting in a total of 70,808 SNP sites called with a minor allele frequency ≥5%. There were no fixed differences between any of the Makira clade populations, and 68% of the variation was shared between all three island populations (Fig. S7). The northern melanic population on Ugi Island contained the highest number of unique SNPs, and both melanic populations shared more variants with the chestnut-bellied population of Makira than with each other. Many of the SNPs shared across all three Makira clade populations were also shared with the Russell Islands (57%), reflecting the very recent ancestry of this species complex (Fig. S7).

A total of 24,550 sites that were polymorphic in the Makira clade were fixed in the Russell Islands. Based on the assumption that alleles fixed in the Russells represent the ancestral alleles, the derived allele frequency spectrum for Makira followed the neutral expectation of new mutations being rare, while Ugi and Santa Ana show slightly increased levels of intermediate frequency alleles (Figs S8 and S10). At the derived sites, transitions were more than twice as likely to occur as transversions (16,699 transitions vs. 7,851 transversions).

3.2 | Analyses of population differentiation

In accordance with the amount of shared variation that was observed, the mean F_{ST} values were very low between island

populations, with the lowest occurring between the main island of Makira and the southeastern melanic population of Santa Ana (Figure 3). Despite being low, all mean F_{ST} values between islands were significantly different from zero, except for the mean F_{ST} between Makira and Santa Ana. By contrast, no within-population F_{ST} values were significantly different from the permuted means (Fig. S9), with a distribution similar to the F_{ST} values between Makira and Santa Ana. F_{ST} was highest between Santa Ana and the Russell Islands, which are the most geographically distant populations (Figure 1). For all comparisons within the Makira clade, the distribution of F_{ST} values never extended to 1 (F_{ST} maximum is 0.96), consistent with the fact that there are no fixed differences.

A small number of high F_{ST} (≥ 0.7) variants were detected in the Makira–Ugi comparison (15 SNPs) and the Ugi–Santa Ana comparison (20 SNPs); 11 of these were the same markers for both comparisons, indicating the presence of high-frequency SNPs unique to the Ugi population. Of the 24 total tags that contained a high F_{ST} variant, 22 aligned to the Zebra finch genome, and half (11) of the aligned tags mapped to the Z chromosome. There were 12 high F_{ST} variants that fell within an annotated protein-coding region, although none fell within known melanic genes (Table S2). F_{ST} between Makira and Santa Ana for the genomewide SNPs never reached a value as high as the observed value for the *MC1R* allele, and only one SNP in the comparison between Makira and Ugi had an F_{ST} equivalent to the observed value for *ASIP*, with none being higher (Figure 3).

Despite the overall low levels of differentiation based on the low F_{ST} values, the pattern of clustering found by STRUCTURE and the PCA corresponded well with geography (Figure 4 and Fig. S5). Under the assumption of two groups ($K = 2$), all birds from the Makira clade clustered together, while birds from the more distant Russell Islands populations formed a distinct group. As the number of assumed groups (K) increased, the more geographically isolated populations started to form distinct clusters. At $K = 3$, the melanic Ugi population formed a distinct group from the chestnut-bellied Makira and melanic Santa Ana populations. At $K = 4$, Santa Ana started to differentiate from Makira, although not as markedly as the other populations. The most likely value for K was 2 (Fig. S4), with all of the populations from the Makira clade grouping together and the Russell Islands forming a second group, which is not unexpected based on the low F_{ST} values for the SNPs we uncovered for the three Makira clade populations.

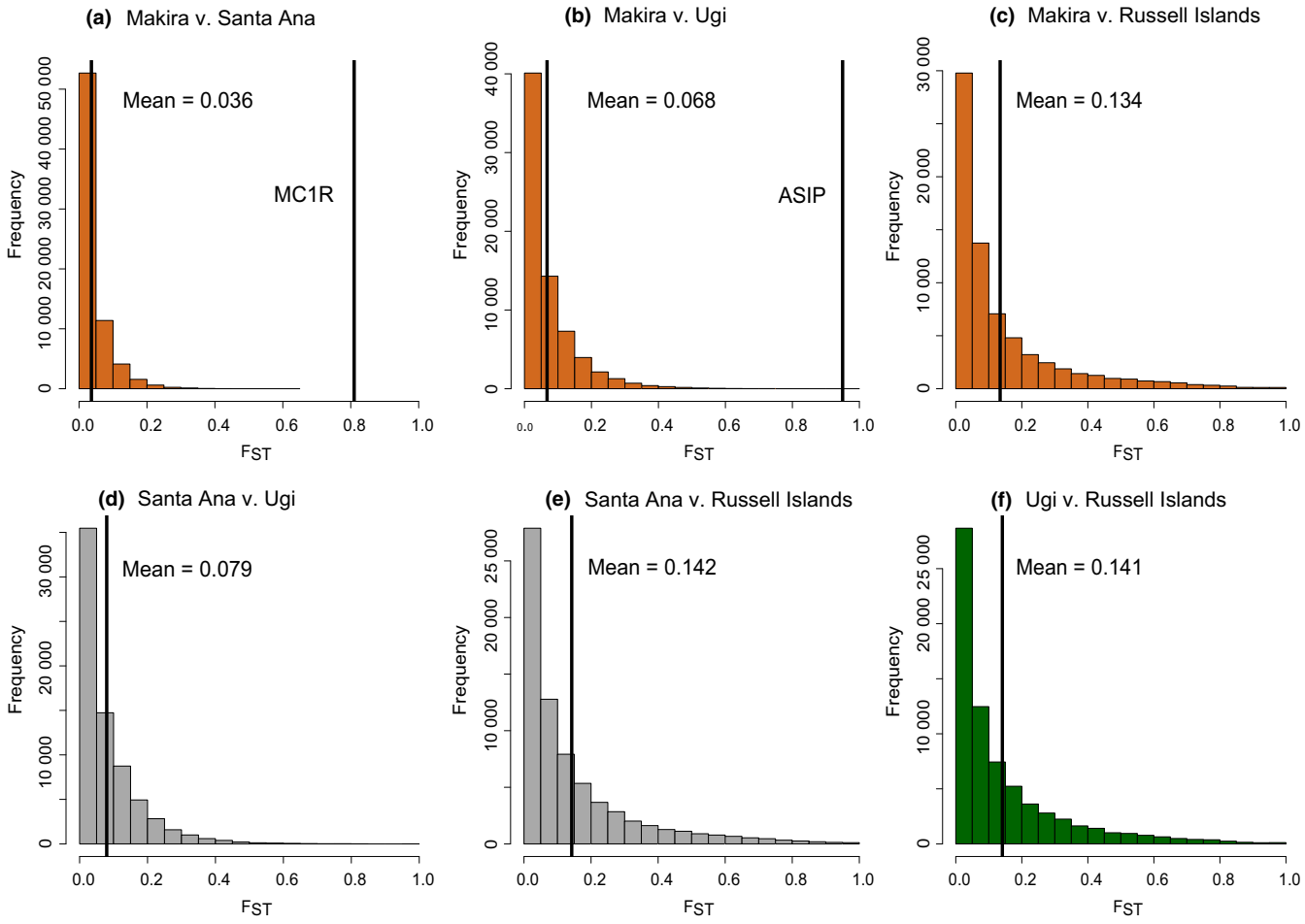

FIGURE 3 F_{ST} distributions for all ddRAD tag loci in each pair of populations. Vertical lines indicate the mean F_{ST} value for each comparison

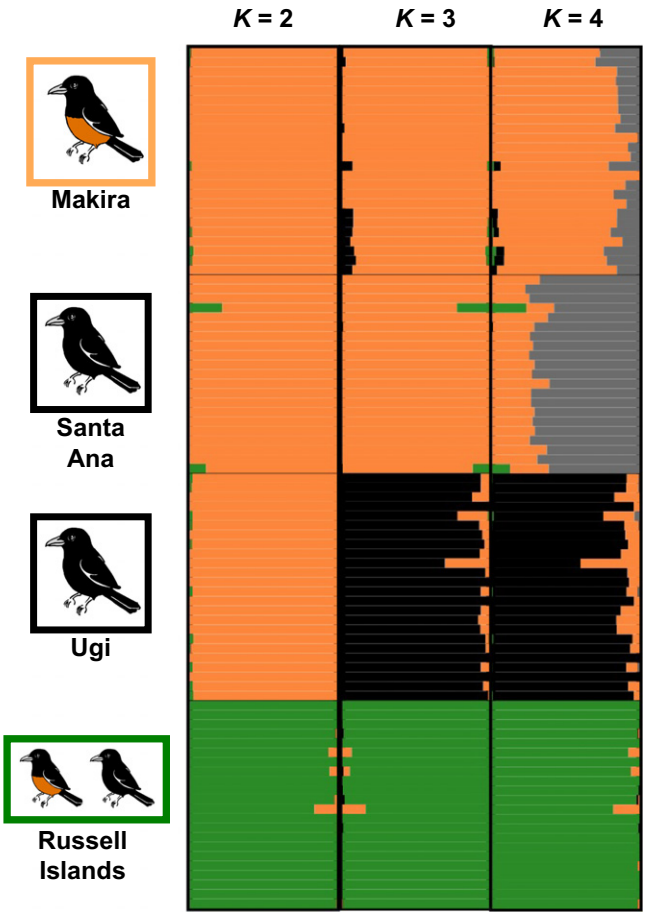

FIGURE 4 Structure analysis. Structure plots for $K = 2$, $K = 3$ and $K = 4$. Mean likelihoods for each K value are −2,859,500.2, −2,686,716.2 and −2,644,972.1, and the mean 'clusteredness' values (G) for each K are 0.980, 0.955 and 0.835, respectively. Horizontal bars represent individuals, and colours indicate cluster assignments

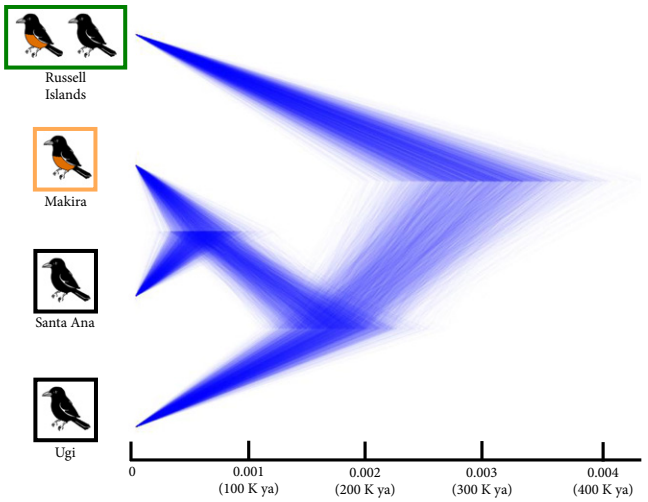

FIGURE 5 Species tree. Bayesian species tree estimated based on 3 individuals from each population, and approximately 7,000 loci in SNAPP. Branch lengths, a relative measure of substitutions per site and divergence time estimates in millions of years are provided in the scale below each tree

A PCA of the 1,428 filtered SNPs that mapped to the Z chromosome showed a clustering pattern almost identical to that of the genomewide data set (Fig. S5). One key difference was that the Z chromosome SNPs did not distinguish the Makira and Santa Ana populations as well as the full SNP data set, likely reflecting the fact that many of the highly differentiated SNPs on this chromosome are actually specific to the Ugi population.

3.3 | Species tree estimation

SNP AND AFLP PACKAGE FOR PHYLOGENETIC ANALYSIS converged on a single tree topology with perfect support at each node, which presents the Russell Islands as an outgroup, and the chestnut-bellied (Makira) population as sister to the southeastern melanic population of Santa Ana (Figure 5). The results were consistent across all individual subsamples. Based on 764 bp of the ND2 gene, the genetic distance between the Russell Islands *M. c. obscurior* and Makira *M. c. ugiensis* and *M. c. meharhynchus* clades was 0.0175 (unpublished data). Using the ND2-specific substitution rate of 0.02–0.022 substitutions per site per lineage per million years (Arbogast et al., 2006), the node that splits the Russell Islands and Makira clade is dated at 407,000–438,000 years ago. As such, the node that separates Ugi from the Makira and Santa Ana clade is estimated at 165,000–178,000 years ago, and the node that splits Makira from Santa Ana is estimated at 76,000–82,000 years ago (Figure 5).

This arrangement mirrors the relationships present in the STRUCTURE analyses and further suggests that the current classification of the melanic Ugi and Santa Ana populations as a single subspecies may be inaccurate. Under the divergence scenario predicted by our data, the mutations in *MC1R* and *ASIP* appear to be convergent mechanisms for the evolution of melanic plumage colour in each satellite island. Because the phylogenetic analysis cannot account for the presence of gene flow, both tree topologies (the one based on phenotype and the one predicted by our data) were examined under different isolation-with-migration conditions to get a more complete picture of the possible selective and demographic forces acting in this system.

3.4 | Estimates of gene flow

The *∂a∂i* analyses of different migration scenarios indicated that a demographic model with migration only between adjacent islands (Makira and Santa Ana, or Makira and Ugi, but NOT Ugi and Santa Ana) was the most likely model regardless of tree topology; the models with no migration or migration between all island pairs were statistically less significant after correcting for the number of parameters (Table 1 and Table S1). Furthermore, the parameter estimates for population sizes (θ) and migration rates (m) were remarkably similar for the two topologies across all models, but most especially in the adjacent migration model (Table 1). The two

TABLE 1 Parameter estimates and likelihoods for each migration scenario tested in dadi

				Population size estimates				Div. times		Migration rates					
		Ln (LL)	AICc	Mak	SA	Ugi	Anc	T1	T2	M-S	S-M	M-U	U-M	S-U	U-S
SNAPP	Adj.	−19,866.6	39,757.1	5.86	2.08	2.33	6.52	1.31	7.35	3.04	1.21	0.99	0.29	NA	NA
	Mig.	(675.85)		(1.24)	(0.52)	(0.30)	(1.54)	(0.31)	(1.11)	(0.67)	(0.50)	(0.11)	(0.09)		
	No	−58,431.5	49,907.9	0.76	1.66	0.72	2.34	0.02	0.15	NA	NA	NA	NA	NA	NA
	Mig.	(23,915.9)		(0.46)	(1.38)	(0.52)	(1.20)	(0.01)	(0.13)						
	Full	−29,631.2	59,291.9	1.01	0.85	0.42	4.38	1.09	0.91	1.85	8.76	0.90	1.00	0.57	1.04
	Mig.	(3,288.61)		(0.30)	(0.14)	(0.28)	(1.43)	(0.27)	(0.32)	(0.34)	(0.39)	(0.13)	(0.64)	(0.12)	(0.14)
MC1R	Adj.	−18,250.5	36,524.7	4.74	1.99	2.12	7.01	4.19	4.06	3.15	1.36	1.38	0.35	NA	NA
	Mig.	(206.38)		(0.93)	(0.59)	(0.42)	(1.43)	(1.25)	(1.59)	(0.84)	(0.42)	(0.29)	(0.09)		
	No	−24,891.3	49,796.1	0.05	0.016	0.01	7.78	0.0016	0.0015	NA	NA	NA	NA	NA	NA
	Mig.	(3.54)		(2e-3)	(2e-3)	(2e-3)	(0.74)	(2e-4)	(2e-4)						
	Full	−26,337.4	52,704.4	3.11	0.65	0.21	5.82	2.64	0.71	5.32	4.21	1.05	0.26	1.05	1.77
	Mig.	(3,729.4)		(0.71)	(0.14)	(0.12)	(1.45)	(0.66)	(0.43)	(1.47)	(1.02)	(0.09)	(0.08)	(0.70)	(0.44)

The top three rows of the table show estimates for the tree topology estimated by SNAPP, with Makira and Santa Ana as sister taxa. The bottom three rows indicate estimates for models based on a tree topology with the two melanic subspecies, Ugi and Santa Ana, as sister taxa. The log-likelihood values are the mean values from seven independent runs of the model, after removal of potential outlier runs. Standard errors are given in parentheses below each value. The AIC was calculated as described in the Methods.

satellite island populations are estimated to have very similar θ values, and the estimated θ for the main island population is roughly twice as large as either satellite island and only slightly smaller than the estimate for the ancestral population, which supports the hypothesis that the common ancestor originated on the main island of Makira. Migration rates are noticeably asymmetrical; gene flow is consistently estimated to be roughly three times higher from the main island to either satellite island than it is in the reverse direction (Table 1 and Figure 6). Migration rates are highest between the least geographically separated islands of Makira and Santa Ana: the highest estimated migration parameter occurs from the main island of Makira to the southeastern island of Santa Ana.

The most striking difference between the estimates for the two different possible tree topologies was the estimates of divergence time (Figure 6 and Table 1). When Makira and Santa Ana are treated as sister taxa, their split is predicted to be almost twice as old as the estimated time of divergence between Ugi and Santa Ana in the alternative tree (385,000 years ago vs. 213,000). The Makira–Santa Ana split is also predicted to have occurred very soon after the earlier divergence of the common ancestor from Ugi, which is another notable difference from the predictions with the phenotype/candidate gene-based model (Figure 6). In both scenarios, the first split from the common ancestor is estimated to have occurred around the same time at roughly 450,000 years ago. All of these estimates are older than previous estimates as well as the ones initially calculated for the SNAPP tree, but still within a feasible range given the geophysical history of the islands and the fact that gene flow will lead to longer divergence times. The models including possible migration between all islands, including between the two satellite islands, had the lowest likelihood scores in *∂a∂i*.

4 | DISCUSSION

4.1 | Incomplete lineage sorting and long-term gene flow result in reduced genomic differentiation

Despite distinct differences in plumage colour (Figure 1), the genomewide patterns of shared polymorphisms based on over 70,000 SNPs indicate that the chestnut-bellied *M. c. megarhynchus* and melanic *M. c. ugiensis* subspecies are very similar at most background loci. These results are similar to what has been reported in other recently diverged and hybridizing bird species (Ellegren et al., 2012; Mason & Taylor, 2015; Poelstra et al., 2014), indicating that striking phenotypic variation may often be maintained by selection at only a handful of loci. Based on the results of the migration estimates and phylogenetic analysis, the low level of genomic differentiation in *Monarcha* appears to be the result of both incomplete lineage sorting due to very recent shared ancestry and gene flow among adjacent island populations, which is consistent with earlier findings based on a few markers (Uy, Moyle, Filardi et al., 2009). Finally, according to the F_{ST} estimates, STRUCTURE results and the comparison of shared versus private polymorphisms, chestnut-bellied Makira individuals are more closely related to melanic individuals from the southeastern melanic population of Santa Ana than they are to individuals from the northern melanic population on Ugi Island. Surprisingly, both melanic populations, which are currently considered as a single subspecies (Mayr, 1942), are likewise more closely related to the Makira Island chestnut-bellied population than they are to each other. This relationship appears to be at least in part driven by direct gene flow between adjacent populations, as Santa Ana and Ugi are 8–10 km away from Makira, but are over 150 km from each other (Figure 1). Further, this pattern may also reflect independent origins of melanism in each population.

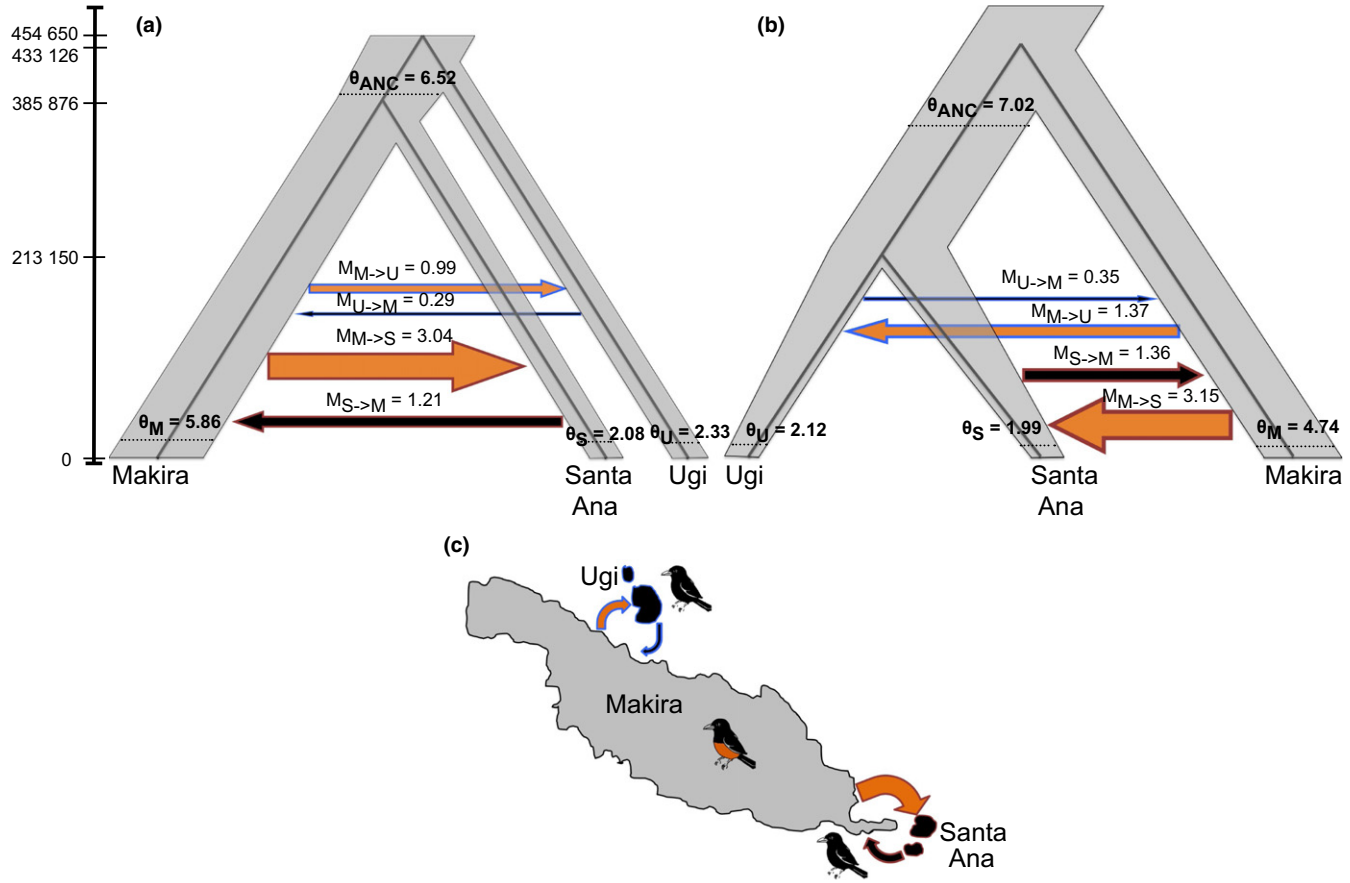

FIGURE 6 Tree topologies scaled to reflect parameter estimates in a model with migration between adjacent islands. Topology a indicates a scenario predicted by SNAPP, where Makira and Santa Ana are sister taxa. Topology b indicates an alternate scenario, where Ugi and Santa Ana are sister. Branch width and length are proportional to $\partial a \partial i$ estimates of population size and divergence times, respectively. Arrow direction and size are reflective of migration rate estimates. Panel c indicates the geographic relationship of the populations, with arrows proportional to mean estimates of gene flow from the two models

Considering the three populations from the Makira clade and the more distant Russell Islands clade, all of the results from STRUCTURE, regardless of the starting K value or predicted likelihood, corroborated the pattern of geographic distance influencing the genetic differentiation, with the most geographically isolated populations forming clusters first. The STRUCTURE model with the highest likelihood was at $K = 2$, with one cluster containing all the birds from the Makira clade and the second cluster containing birds from the Russell Islands. At $K = 3$, the model with the second highest likelihood score, the Russell Islands clade, once again formed its own group, but the two melanic populations of *M. c. uginesis* do not form their own cluster. Rather, the melanic birds of Santa Ana strongly clustered with chestnut-bellied birds of Makira. At $K = 4$, Santa Ana and Makira birds do form their own respective groups, but are less clearly differentiated than the other populations (Figure 4 and Fig. S5). Interestingly, the clustering patterns observed in the STRUCTURE data not only correspond to current geographic distances but also seem to follow a northwest to southeast trajectory. That is, the northwestern-most population of the Russells is differentiated first, followed by the Ugi population on the northern side of Makira, and finally, the partial differentiation of the southeastern-most island of Santa Ana. This may be reflective of the history of colonization as well as their current isolation, similar to what has been observed in crows (Poelstra et al., 2014).

Despite their separation, isolation-with-migration analyses performed in $\partial a \partial i$ support the occurrence of gene flow between adjacent populations. A model in which migration is occurring between the large island of Makira and the satellite islands to the north and to the southeast, but not directly between the satellite islands themselves, had the highest likelihood compared to the models with no migration or with migration between all islands. Furthermore, migration was estimated to be highest between the two closest populations, Makira and Santa Ana, with the highest migration rate for introgression from the larger population of Makira into the smaller Santa Ana population (Figure 6). Given that the area of Makira (3,089.9 km^2) is almost 75 times the size of Ugi (42.2 km^2) and over 200 times that of Santa Ana (14.7 km^2; Diamond, Gilpin, & Mayr, 1976), the asymmetry in gene flow is most likely due to the small population size of the satellite islands. Not only are there fewer potential migrants from Santa Ana and Ugi, but the smaller population size of these islands means that any birds coming from Makira

and reproducing will have a larger effect on the gene pool than birds moving in the opposite direction.

Both the migration models and phylogenetic analysis implicate recent divergence and incomplete lineage sorting as a major factor contributing to genomic similarities between populations, and the large proportion of polymorphism still shared with the more distant Russell Islands population could only come from a recent common ancestor. However, the exact order and timing of divergence of the melanic subspecies remains uncertain. The two melanic populations have been regarded as a single subspecies with one origin (Mayr, 1942), and the alleles observed at the candidate plumage locus *MC1R* seemed to support this scenario, even with the discovery of a second plumage colour mutation in *ASIP* (Figure 2). Specifically, the homozygous *MC1R* variant is still present at an intermediate frequency in the northwestern (Ugi) population, even though it does not seem to be responsible for melanism in this group. This is in contrast to the southeastern (Santa Ana) population, where the homozygous *ASIP* variant is completely absent, and *MC1R* appears to be the sole mechanism for melanic plumage. However, despite the distribution of the *MC1R* mutation, all results based on the genome-wide ddRADseq data, including F_{ST}, STRUCTURE and the phylogenetic estimation, clearly indicate that the southeastern melanic population is sister to the chestnut-bellied Makira population instead of the other melanic population. F_{ST} results further suggest that both melanic populations are actually more closely related to the chestnut-bellied Makira population than they are to each other, which argues against their current assignment to a single subspecies.

4.2 | Species tree and taxonomy

The phylogenomic analysis of nearly 7,000 SNPs revealed an alternative explanation for the unexpected patterns of genetic similarity between phenotypically distinct populations. According to the best-supported species tree, the two melanic populations of Santa Ana to the southeast and Ugi to the north of Makira do not form a monophyletic group. Rather, the melanic birds of Santa Ana clearly clustered with the chestnut-bellied birds of Makira, with the melanic Ugi population sister to the two (Figure 5), mirroring the relationship recovered by the STRUCTURE and F_{ST} analyses.

Under the tree topology suggested by the phylogenomic analysis, the northern (Ugi) and southeastern (Santa Ana) melanic populations are actually the result of two separate divergence events. This is further supported by the presence of two different mutations underlying melanism in each population, neither of which appear to be spurious based on the results presented here. However, it remains difficult to account for the relatively high frequency of derived *MC1R* alleles in Ugi (despite their rarity in Makira). If the *MC1R* polymorphism had been present in the common ancestor of the Makira clade, then its current distribution could be explained by a combination of strong positive selection driving it to fixation in Santa Ana, strong purifying selection eliminating it in the Makira population and relaxed selection allowing it to remain polymorphic in Ugi. The signalling protein encoded by *ASIP* operates upstream of *MC1R* in the melanin-producing pathway, so it is feasible to suggest that changes in *MC1R* may have little consequence in individuals with mutant *ASIP*. Alternatively, the *MC1R* mutant may have arisen later in the Santa Ana population and been introduced to Ugi via gene flow, although none of the migration models suggest that direct gene flow between these two satellite islands is likely. That is, introgression of the derived *MC1R* allele would have to indirectly reach Ugi through Makira, while simultaneously being selected against in Makira. Additionally, such gene flow would have to be strongly asymmetrical (SA to Ugi) to produce the observed patterns where mutant *ASIP* alleles are completely absent in the southeastern melanic population (Uy et al., 2016).

The tree topology recovered from the nearly 7,000 SNPs showing Makira and Santa Ana as sister taxa may also be confounded by significant gene flow between Makira and Santa Ana rather than reflective of the true species tree. The predicted phylogeny is highly correlated with the current geography of the populations and thus is consistent with the pattern we would expect if it were confounded by gene flow between adjacent islands, especially between Makira and Santa Ana. There are previously reported instances where gene flow confounds species tree estimation. For instance, in the *Anopheles* mosquito complex, where historical hybridization is known to be extensive, it was recently shown that a well-supported species tree based on complete genome resequencing data was incorrect, and that the true species tree was only reflected in a subset of markers located on the X chromosome, where gene flow had been restricted (Fontaine et al., 2015). In our data, SNPs aligned to the Z chromosome did exhibit higher differentiation than SNPs aligned to the autosomal loci (Table S2 and Fig. S11), but a separate PCA of Z chromosome SNPs produced results nearly identical to genomewide patterns, and in fact showed even less differentiation between Makira and Santa Ana than the full data set (Fig. S5). The consistency among even highly differentiated loci strengthens the argument that we are in fact recovering the true species tree, but because there is evidence for geographic population structure in this system, it is difficult to definitively distinguish past divergence events from the effects of current and ongoing genetic drift. Our study highlights the difficulties in uncovering the true evolutionary histories of very closely related species experiencing gene flow.

Ultimately, although we may not be able to fully resolve the correct tree topology with the available data, there is some additional evidence to argue in favour of the SNAPP phylogeny (Figure 5) and against the two melanic populations of *M. c. ugiensis* being classified as a single monophyletic subspecies. First, the melanic Ugi birds, which are shown to diverge first under the SNAPP scenario, had a higher frequency of derived alleles when compared to Santa Ana and Makira and also contained the most unique polymorphisms of any population. While these patterns could be attributed to genetic drift having a larger impact on the smaller and more isolated Ugi island, they could also reflect an earlier divergence of the Ugi population that allowed more time for the effects of drift to accumulate. Second, and more importantly, evidence of different pigmentation genes mediating melanism between Santa Ana and Ugi (Uy et al.,

2016) suggests that the two populations have evolved melanism convergently and should be reclassified as distinct subspecies. If the subspecies are indeed independently diverged from their chestnut-bellied ancestors, then they may be currently undergoing parallel speciation.

4.3 | Patterns and potential implications of the few highly differentiated loci

Only a small handful of SNPs were well differentiated (but not fixed) between populations, and none of these appeared well differentiated between the two closest populations of Makira and Santa Ana (Table S2 and Fig. S11). Instead, many of the most divergent sites corresponded to high-frequency polymorphisms unique to the Ugi population. While none of these SNPs aligned to other well-known melanism genes like *MC1R* or *ASIP*, three of them were in or near (within 50 kb of) genes found to be associated with pigmentation in other species (*YPEL5* Nan et al., 2009; *EDEM2* Liu et al., 2015; *SKIV2L2* Yang, Hindes, Hultman, & Johnson, 2007). One additional SNP was located near a gene implicated in glaucoma (Carnes, 2014), suggesting a potential role in vision. While the functions of most of these genes have not been directly verified, and none have been studied in an avian system, their possible involvement in either plumage colour or the recognition of plumage colour is highly intriguing.

Strikingly, half of the highly differentiated loci that aligned to the Zebra finch were localized to the Z chromosome, particularly those that were strongly differentiated between the two melanic populations of Santa Ana and Ugi (Table S2). A study comparing the European pied and collared flycatchers (*Ficedula hypoleuca* and *Ficedula albicollis*) uncovered increased levels of genomic divergence evenly distributed across the Z chromosome, which was in contrast to the pattern of tightly clustered 'islands' of divergence found across the autosomes (Ellegren et al., 2012). These patterns found on the Z chromosome may be indicative of a more advanced stage of speciation (Ellegren, 2013; Ellegren et al., 2012), but could also be reflective of increased genetic drift due to the smaller effective population size of the sex chromosomes. It is not possible with the data in this study to draw any strong conclusions about the exact role the Z chromosome may be playing in maintaining species differences in this clade, but this is a promising avenue for further investigation.

5 | CONCLUSIONS

Previous field experiments indicating that divergent plumage colour is used in conspecific recognition between the melanic birds of Santa Ana and the chestnut-bellied birds of Makira (Uy & Safran, 2013; Uy, Moyle, Filardi et al., 2009) provide a potential mechanism for selection on the pigmentation allelic variants. Diversifying social selection, either through competition for mates or assortative mate choice for distinct plumage colour, favours the maintenance of colour differences across islands. The clear contrast in the observed differentiation between these pigmentation alleles and over 70,000 genomic markers used in this study provides additional evidence that strong diversifying selection is essential in driving speciation and keeping variants fixed between populations in the face of gene flow between adjacent islands. These data suggest that despite the recent emergence of the Makira clade less than 500,000 years ago, the mutations in *MC1R* and *ASIP*, known pigmentation genes, have independently given rise to melanic forms derived from chestnut-bellied ancestors, suggesting that parallel selection to maintain melanism in the southeastern and northern satellite islands could drive parallel speciation in this clade (Uy et al., 2016).

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DATA ACCESSIBILITY

1. Raw sequencing reads are available from the NCBI SRA database (SRP082402).
2. Pipeline scripts and accompanying documentation can be found at: https://github.com/eacooper400/GenDivergence
3. Nexus input files for SNAPP are also available for download at https://github.com/eacooper400/GenDivergence

AUTHOR CONTRIBUTIONS

E.A.C. who is the corresponding author involved in intellectual merit, research design, laboratory procedures, bioinformatics, analysis and writing; J.A.C.U. who is a co-author involved in intellectual merit, research design, field work, funding, analysis and writing.

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SUPPORTING INFORMATION

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