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Source: The Auk, 135(2) : 171-180

Published By: American Ornithological Society

URL: https://doi.org/10.1642/AUK-17-120.1

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The Auk
Ornithological Advances
AmericanOrnithology.org

Volume 135, 2018, pp. 171–180
DOI: 10.1642/AUK-17-120.1

RESEARCH ARTICLE

Population genetics of an island invasion by Japanese Bush-Warblers in Hawaii, USA

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Submitted June 30, 2017; Accepted November 5, 2017; Published January 17, 2018

ABSTRACT

Island bird populations often provide exemplary cases of evolution based on historical colonization and diversification events. However, capturing contemporary cases of evolution is rare. Introductions of nonnative birds into the Hawaiian Islands, USA, provide numerous potential opportunities to assess evolutionary changes over a relatively short time frame. One introduced species, the Japanese Bush-Warbler (*Cettia diphone*), has a well-established history in the Hawaiian Islands, with a documented introduction in 1929 to the island of Oahu and natural colonization of the other main islands by 1997. We sampled 143 Japanese Bush-Warblers from 5 of the main Hawaiian Islands and amplified 12 microsatellite loci (9 were variable) and sequenced portions of the cytochrome *b* and cytochrome *c* oxidase subunit I (COI) genes to assess the genetic structure and potential original source of these populations. As predicted, genetic diversity, measured by allelic richness and private alleles, was greatest on Oahu (the original introduction site) and was significantly lower in birds on the islands farthest from Oahu. Accordingly, there was a clear isolation-by-distance effect, with highest F_{ST} values between island pairs farthest apart. The population on the westernmost island of Kauai appears to be diverging from the easternmost populations on the islands of Maui, Molokai, and Hawaii. The results provide a unique opportunity to document the microevolutionary process of genetic drift in action, and we speculate on the potential role of behavior in diversification.

Keywords: biological invasion, bush-warbler, *Cettia diphone*, introduced species, population genetics

Genética poblacional de una invasión de islas por *Cettia diphone* en Hawái

RESUMEN

Las poblaciones de aves de las islas a menudo brindan casos ejemplares de evolución basada en colonización histórica y eventos de diversificación. Sin embargo, es raro encontrar casos contemporáneos de evolución. Las introducciones de aves no nativas en las islas de Hawái brindan numerosos oportunidades potenciales para evaluar cambios evolutivos sobre períodos de tiempo relativamente cortos. Una especie introducida, *Cettia diphone*, tiene una historia bien conocida de establecimiento en las islas de Hawái, con una introducción documentada en 1929 a la isla de Oahu y la colonización natural de las otras islas principales hasta 1997. Muestreamos 143 individuos de *C. diphone* provenientes de las cinco islas principales de Hawái y amplificamos 12 loci micro-satelitales (9 fueron variables) y secuenciamos porciones de los genes del citocromo b y de la subunidad I del citocromo c oxidasa (COI) para evaluar la estructura genética y la fuente potencial de origen de estas poblaciones. Como predijimos, la diversidad genética, medida mediante la riqueza alélica y los alelos privados, fue mayor en Oahu (el sitio de introducción original) y fue significativamente más baja en las aves de las islas más lejanas de Oahu. En consecuencia, hubo un claro efecto de aislamiento-por-distancia, con valores más altos de F_{ST} entres los pares de islas más apartados. La población de aves en las islas más al oeste de Kauai parece estar divergiendo de las poblaciones más al este en las islas de Maui, Molokai y Hawái. Estos resultados brindan una oportunidad única para documentar el proceso de micro-evolución de deriva génica en acción y especulamos sobre el rol potencial del comportamiento en la diversificación.

Palabras clave: Cettia diphone, especie introducida, genética poblacional, invasión biológica

INTRODUCTION

Studies of birds on islands form the foundation of several important concepts in modern evolutionary biology, including some of the best-known illustrations of speciation, biogeography, and natural variation. As discrete areas of land surrounded by water, islands force landbirds to venture across inhospitable stretches in search of more favorable environments. From Charles Darwin's work on mockingbirds in the Galápagos (Darwin 1845) and Alfred Russel Wallace's work on birds of Indonesia and Malaysia (Wallace 1869) to studies by Ernst Mayr in the southwest

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Pacific (Mayr 1940) and by David Lack and Peter and Rosemary Grant on Darwin's finches in the Galápagos (e.g., Lack 1947, Grant 1986), island bird studies have provided fundamental insights into evolution.

Natural examples of unaided colonizations by birds on a contemporary timescale are exceedingly rare (but see Clegg et al. 2002a, 2002b). However, introductions of birds to islands by humans are more common and better documented, and they allow for a direct evaluation of microevolutionary processes involved with recent arrival (Blackburn et al. 2009). Although different selective pressures and other varying conditions make natural vs. human-mediated colonizations not entirely equivalent, these introductions mirror, in some ways, historical range expansions by native birds. Moreover, these introductions provide insights into how organisms respond to novel environments, a needed understanding given the increases in species invasions worldwide. Rapid changes in the morphology, behavior, and genetics of introduced bird populations have been assessed in a number of systems—including Common Chaffinches (*Fringilla coelebs*; e.g., Baker and Jenkins 1987), Common Mynas (*Acridotheres tristis*; Baker and Moeed 1987), and island (Baker 1980) and continental (Lima et al. 2012) populations of House Sparrows (*Passer domesticus*)—and indicate that there are a variety of phenotypic and genotypic changes in birds that have been introduced into new environments.

The Hawaiian Islands, USA, contain an exceptionally rich assemblage of introduced birds, with documented releases of >170 species, ≥54 of which have established breeding populations (Foster 2009). These releases were in response to the extirpation of native birds from lowland forests due to introduced rats and avian disease (Lever 2011). One species, the Japanese Bush-Warbler (*Cettia diphone*; hereafter "bush-warbler"), has had a particularly dynamic history. The bush-warbler was first introduced to Oahu in 1929 by the Hui Manu, a bird-naturalization club whose sole purpose was to release songbirds in Hawaii (Foster 2009). Several subsequent releases, totaling ~138 individuals, occurred on this island until 1941, and bush-warblers expanded to both mountain ranges by the 1950s (see Pyle and Pyle 2017). Oahu was the only island where bush-warblers were documented to have been released; absence of reports of their loud distinctive call elsewhere, observable even by laypeople, makes it unlikely they were successfully introduced to other Hawaiian islands. The bush-warbler has since become naturally established on all main islands in Hawaii, successfully colonizing Molokai by 1979 (Pyle 1979) and 2 other islands immediately east of Oahu, Lanai and Maui by 1980 (Carothers and Hansen 1982). Kauai, the island immediately west of Oahu, was colonized by 1983 (Pyle and Pyle 2017); and the island of Hawaii, farthest east in the archipelago, was colonized by 1997 (Nelson and Vitts 1998; Figure 1). Because the Hawaiian Islands are an isolated cluster of islands and the bush-warbler's introduction was recent, the documented history of colonizations provides a unique opportunity to characterize post-invasion evolution over known, and recent, timelines. Moreover, bush-warblers can breed as second-year birds and have correspondingly short generation times, increasing the potential for rapid change over this period.

We assessed genetic diversity and population genetic structure of 143 bush-warblers collected from the islands of Kauai, Oahu, Molokai, Maui, and Hawaii. We predicted that founder-mediated genetic drift would be an evident evolutionary change detectable over this short period and tested whether there has been an effect of time and/or geography on genetic diversity after successive island colonizations within the archipelago. This prediction stemmed from a comprehensive review of microevolutionary changes in island bird populations after colonization, in which Clegg (2009) detailed evidence for the effects of drift and natural selection on island birds. Major conclusions from Clegg's work predict that sequential founder steps over multiple islands, rather than a single event, are necessary for detectable declines in genetic diversity and increased population divergence, suggesting where one should look for likely genetic changes in our system.

Given the known colonization history, we expected (1) greatest allelic diversity on Oahu, the island on which birds were introduced, with declining diversity on each successive island that bush-warblers colonized, with the island of Hawaii having the least diversity; (2) a strong founder effect on Kauai, resulting in obvious genetic structuring between the birds of Kauai and those of the other islands because Kauai has the longest stretch of inter-island ocean; and (3) distinct isolation-by-distance and isolation-by-colonization effects.

METHODS

Sampling

We sampled 147 bush-warblers from March 2003 to June 2005; after 4 samples from putative family groups were excluded because of high genetic relatedness, final analyses included 143 individuals (12 from Hawaii, 40 from Kauai, 22 from Maui, 40 from Molokai, and 29 from Oahu). We sampled bush-warblers in shrubby or forested habitat where we knew they occurred or where we believed habitat conditions to be suitable. We limited our sampling to state-owned or private land accessible by trail or road. Tissue from bush-warblers came either from pectoral muscle of collected specimens or from whole blood taken using venipuncture during mist netting (Supplemental Material Table S1). Birds were attracted using playback of male song and alarm calls (Hawaii Audubon Society CD; Pratt 1996).

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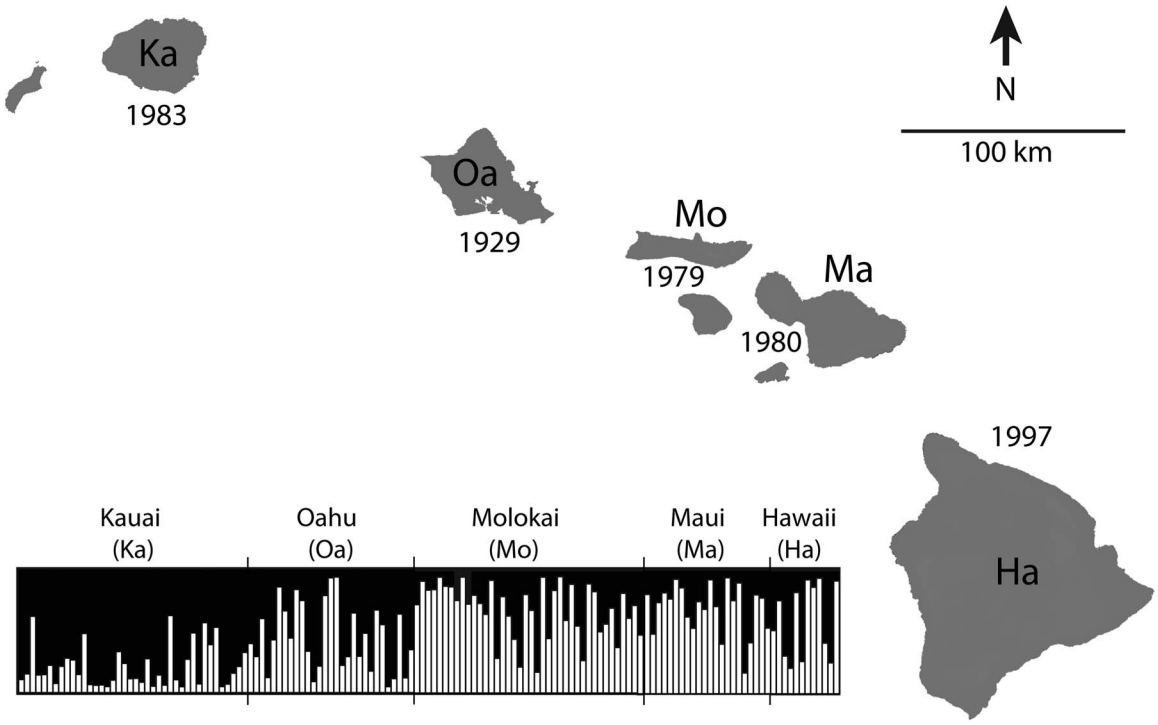


FIGURE 1. Map of the Hawaiian Islands, USA. The Japanese Bush-Warbler was introduced to Oahu from Japan in 1929 and naturally spread to the other islands by the years indicated. The bar plot shows STRUCTURE results based on 2 populations, with the genotype for each individual bird represented as a vertical bar, with black and white indicating the relative proportion of assignment to each genetic cluster.

Specimens were deposited at the American Museum of Natural History, Bernice Pauahi Bishop Museum, and Smithsonian Institution National Museum of Natural History.

Genotyping and Locus Behavior

Pectoral tissue was stored in 95% ethanol, and blood was stored in lysis buffer (0.1 M Tris-HCl, 0.1 M EDTA, 0.01 M NaCl, 0.5% w/v sodium dodecyl sulfate). DNA extractions were performed with a DNeasy Blood and Tissue Kit (Qiagen, Valencia, California, USA). We amplified 12 microsatellite markers with polymerase chain reaction according to Otsuka et al. (2003), using an MJ Research PTC-200 thermocycler; conducted fragment analysis on an Applied Biosystems 3130 Genetic Analyzer; and visualized results with GeneMapper 4.0 (Applied Biosystems, Foster City, California). All equivocal results and private alleles were reamplified, and inherently problematic samples (likely due to poor preservation) were subjected to the preamp protocol of Arandjelovic et al. (2009). We performed global tests for departure from Hardy-Weinberg equilibrium (HWE) for each population ($\alpha = 0.0056$, Bonferroni-adjusted for 9 comparisons), each locus ($\alpha = 0.01$, adjusted for 5 comparisons), and each locus-population ($\alpha = 0.001$, adjusted for 45 comparisons) in GENEPOP 4 (Raymond and Rousset 1995). GENEPOP was also used to screen for linkage disequilibrium between each locus pair in all populations ($\alpha = 0.001$, Bonferroni-adjusted for 45 multiple comparisons).

Genetic Diversity and Structure

Genetic diversity was assessed by observed and expected heterozygosity and the average number of alleles per locus. Because allelic diversity is sensitive to sample size, we performed rarefaction using the program HP-Rare (Kalinowski 2005). For both measures we then employed 2-sample *t*-tests between Oahu, the site of introduction, and each island. Population structure was investigated with analysis of molecular variance (AMOVA) via Arlequin 3.5 (Excoffier and Lischer 2010), which uses nonparametric permutation methods (20,000 iterations) to test for significance of the variance components of the within- and among-island levels of genetic structure. Genetic differentiation was also assessed using FSTAT 2.9.3 (Goudet 2001) to calculate Nei's genetic distance between population pairs and to examine significance via 1,000 permutations of the data ($P = 0.005$, adjusted for multiple comparisons). Additionally, we employed the Bayesian clustering program STRUCTURE 2.3.3 (Pritchard et al. 2000) in combination with Structure Harvester (Earl and vonHoldt 2012). We identified the most likely number of genetic clusters (K), via the methods of Evanno et al.

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(2005) and the Ln $P(D)$ as determined in CLUMPAK (Kopelman et al. 2015). We used 5 runs per K ($K = 1–6$), a burn-in of 50,000, and 200,000 Markov chain Monte Carlo (MCMC) iterations (until convergence was achieved), an admixture model with correlated allele frequencies, and withheld island sampling location information. Results from the runs for each value of K were averaged and graphed using CLUMPAK. We explored fine-scale genetic structure in Primer 6 (Clarke and Gorley 2006) by performing nonparametric multidimensional scaling (NMDS) on mean pairwise relatedness values, as estimated in GenAlEx 6.41 (Peakall and Smouse 2006), and tested results via analysis of similarity (ANOSIM; Clarke 1993). NMDS is a type of ordination that uses a similarity matrix to assign individuals a location in space, with similar individuals proximal to one another. This visualization tool is allied with ANOSIM, which is a permutation-based analogue to univariate analysis of variance.

We examined the relationship between genetic structure (F_{ST}, estimated by theta) and both geographic distance and the putative number of colonization steps from Oahu by performing Mantel tests involving 999 permutations of the data, in GenAlEx. Geographic distance was measured from the closest edges of island pairs; we used both the simple (single) number of colonization steps as well as a weighted measure for Kauai (2 extra colonization steps) because the flight distance from the closest neighboring island (Oahu) was ~3 times greater than the distance between other island pairs. We assumed a stepwise colonization pattern and, consistent with the known establishment history, that skipping the closest island during colonization did not occur. With GPS coordinates of the sampling location of each bird, we also used Mantel tests to investigate the relationship between each bird pair's genetic and geographic distance.

Finally, to further investigate our finding of potentially 2 separate populations on each island, denoted by "black" or "white" genotypes in the STRUCTURE plot, we sequenced a 538–base pair (bp) region of mitochondrial cytochrome *c* oxidase subunit I (COI) according to the protocol of Emura et al. (2013). From birds sampled across the islands, we used a subsample of individuals ($n = 7$ black, $n = 5$ white) with high Q values, indicating high probability of assignment to one of these groups. This gene region exhibits variation within and among bush-warbler subspecies from island and mainland Japan (Emura et al. 2013).

Inferences about Dispersal

To test for inter-island movement patterns, we tested recent migration with BAYESASS 3.0 (Wilson and Rannala 2003), a Bayesian method that estimates relative migration rates. The tests evaluated migration among islands for all birds. Mixing parameters were optimized and were run 3–4 times with different starting seeds for 50 million iterations, with intervals between samples set to 1,000. To ensure chain stationarity, 15 million iterations were discarded as burn-in and trace files were assessed in Tracer 1.6 (http://beast.community/tracer). All runs converged and had concordant log probability distributions, indicating consistent results. Given the stochastic nature of MCMC analysis, it is expected to have slight variation between posterior mean probabilities across runs, so we averaged the migration rates and their standard deviations across related runs and calculated 95% confidence intervals for those values (Wilson and Rannala 2003).

Bayesian assignment tests in GeneClass2 (Piry et al. 2004) were used to determine whether there were age or sex biases in dispersal, or differences between islands. These tests employ population allele frequencies to evaluate the likelihood of each individual's genotype arising in the sampled population. Our protocol used the approach of Rannala and Mountain (1997), the leave-one-out procedure, and 1,000 simulated individuals. In instances where sex was not apparent from morphology, we employed a sex-linked marker to identify sex. This involved amplifying 2 fragments of the CHD gene, following the protocol of Lee et al. (2010). Because samples from females were limited, we treat the female dispersal data with caution (detailed below).

Region of Origin in Japan

We sequenced the *C. diphone cantans* mitochondrial cytochrome *b* gene following Hamao et al. (2008) for all birds, in order to determine the native region of origin of Hawaiian *C. diphone*. As we did with the COI gene, sequences were generated on an Applied Biosystems 3130 Genetic Analyzer using BigDye 3.1 Terminator Chemistry. We aligned forward and reverse sequences for all samples, created a consensus sequence in Sequencher 5.0, and using this same program then compared these sequences to 13 *C. diphone* sequences from Japan ($n = 5$: *C. d. cantans*), Korea ($n = 2$ of each *C. d. cantans* and *C. d. borealis*), Russia ($n = 2$: *C. d. borealis*), and China ($n = 2$: *C. d. canturians*). Comparisons were to GenBank accessions AB159194–AB159199, D38315, DQ008510, HQ121522, HQ608838, and JN808923–JN808925 (from Alström et al. 2006, 2011, Chikuni et al. 1996, Nishiumi and Kim 2004, Lei et al. 2010).

RESULTS

Microsatellite Locus Characteristics

In bush-warblers of the Hawaiian Islands, 9 of the 12 loci (Cdi1, 2, 8, 10, 25, 31, 32, 38, 39) were polymorphic (mean $H_E = 0.49$) and thus were used in analyses. All populations were in HWE except for Molokai. This departure was driven by loci Cdi8 and Cdi25, which had significant ($P = 0.001$ and 0.0002, respectively) heterozygote deficit for

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TABLE 1. Population genetic parameters for *Cettia diphone* in the Hawaiian Islands, USA, including mean number of individuals genotyped at each locus, observed heterozygosity (H_O), expected heterozygosity (H_E), and rarified allelic richness.

Island	Mean number of individuals per locus	H_O	H_E	Rarified allelic richness	Number of private alleles
Oahu	28.4	0.52	0.53	3.32	3
Kauai	39.9	0.49	0.51	3.11	1
Molokai	39.4	0.42	0.52	2.89 [a]	0
Maui	21.0	0.47	0.49	2.80 [a]	0
Hawaii	11.9	0.46	0.51	2.73 [a]	0

[a] Significantly different from Oahu.

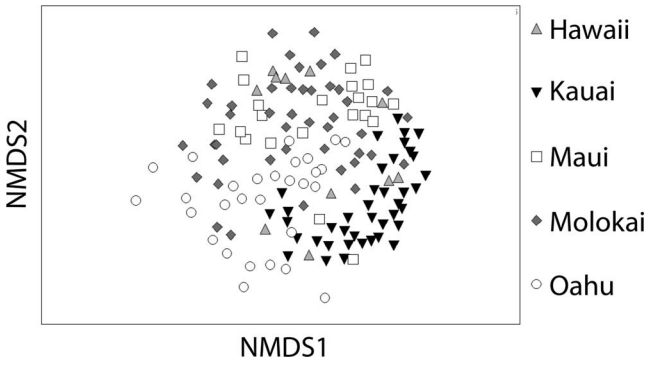


FIGURE 2. Relatedness structure of 143 individual *Cettia diphone* in the Hawaiian Islands, USA. Proximal individuals are more closely related (NMDS plot; global ANOSIM, $P = 0.001$; pairwise ANOSIM: birds of Oahu and Kauai each differ significantly from those of all other islands).

samples from this island but not the other islands. No other loci were out of HWE, and the fact that these 2 loci were in HWE on the other islands likely reflects a sampling effect on Molokai rather than null alleles, and thus both loci were retained in analyses. Although LD was not reported initially in these markers in a bush-warbler population in Japan (Otsuka et al. 2003), significant linkage disequilibrium was found between Cdi2 and Cdi39 ($P < 0.0001$) across populations and also in each of 4 populations. Thus, Cdi39 was removed from further analyses.

Genetic Diversity and Structure

Bush-warblers from Oahu had significantly greater allelic richness (rarified) than birds from all islands except Kauai (Hawaii, $P = 0.03$; Molokai, $P = 0.04$; Maui, $P = 0.02$; Kauai, $P = 0.26$), and there were no inter-island differences in expected heterozygosity (H_E; $P > 0.32$ for all pairs) (Table 1). Further, Oahu birds had 3 private alleles and Kauai birds had 1 private allele, whereas no private alleles were found on the other islands. Genetic structure among the islands was low but discernible. In the AMOVA, a significant proportion of the variance in allele frequencies was among islands (2.7%, $P < 0.003$). Post hoc analyses on the STRUCTURE results indicated that the most likely number of populations was 2 (Figure 1). Kauai birds were significantly differentiated from birds of all other islands, as evidenced by the significance of pairwise comparisons of Nei's genetic diversity (Table 2). Oahu was also genetically different from all other islands, although Molokai was the only comparison that remained significant after Bonferroni correction. These results are concordant with those of mean pairwise relatedness (NMDS and ANOSIM), which indicated that birds of Oahu and Kauai each differed significantly from birds of all other islands (Figure 2).

TABLE 2. Nei's genetic distance (below diagonal) and associated *P* values (above diagonal) between island pairs of *Cettia diphone* in the Hawaiian Islands, USA. Bold indicates significance, after adjustment for multiple tests ($P = 0.005$).

	Hawaii	Kauai	Maui	Molokai	Oahu
Hawaii	–	**0.001**	0.228	0.318	0.007
Kauai	0.116	–	**0.001**	**0.001**	**0.001**
Maui	0.039	0.109	–	0.900	0.006
Molokai	0.026	0.097	0.011	–	**0.001**
Oahu	0.072	0.060	0.052	0.049	–

Both geographic distance and the number of colonization steps from Oahu were significantly correlated with F_{ST} (geographic distance: $R^2 = 0.84$, $P = 0.007$; colonization steps: $R^2 = 0.60$, $P = 0.01$; weighted colonization steps: $R^2 = 0.87$, $P = 0.01$; Figure 3). Additionally, even a coarse examination of genetic and geographic distance of bird pairs across the islands yielded a significant positive correlation (Mantel $R^2 = 0.01$, $P = 0.001$). There were no inter-island, sex, or age differences in assignment scores ($P > 0.1$).

Pairwise genic differentiation was significant across islands for each locus, and it did not appear that particular islands, loci, or alleles were driving the pattern. Additional analyses showed little support for the potential of one or a few loci creating patterns of differentiation into 2 populations (data not shown).

Insight from mtDNA

We found identical cytochrome *b* sequences in all Hawaiian birds. The cytochrome *b* sequence of our samples was an exact match to *C. d. cantans* from Chiba, Japan (near Tokyo; GenBank accession AB159194), as well as to *C. d. cantans* from southern South Korea (AB159196). The other Japanese *C. d. cantans* sequences differed by 1 bp (Saitama, Honshu Island; Miyake Island; and a sample with unknown location) or 2 bp (Ibaraki, Honshu Island). Hawaiian samples clearly differed from *C. d. canturians* (now *C. canturians*) from China by 18 bp and *C. d. borealis* from Russia and Korea by 18–19 bp.

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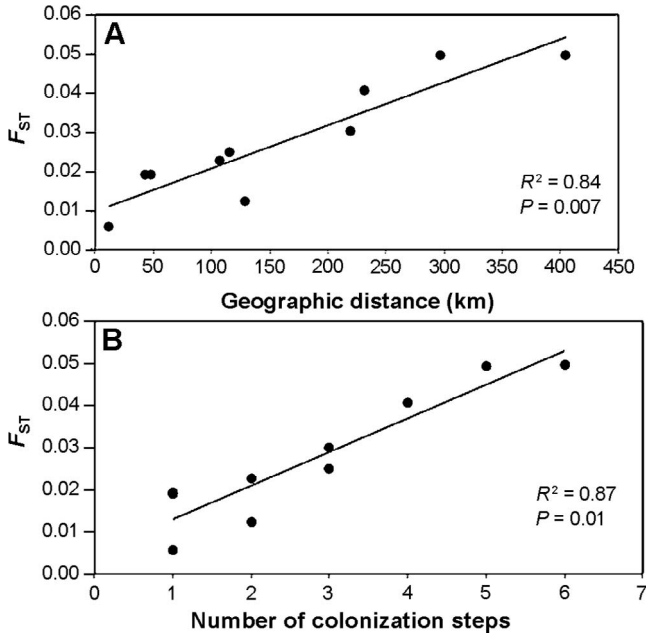


FIGURE 3. Genetic structure based on F_{ST} of *Cettia diphone* in the Hawaiian Islands, USA, is correlated with (**A**) geographic distance between islands and (**B**) simple number of colonization steps from Oahu, the island of introduction. Each point represents an island pair.

Thus, mtDNA suggested possible Japanese origins of bush-warblers in Hawaii but was not informative to colonization history within the Hawaiian Islands because only a single haplotype was found in our Hawaiian samples.

Similarly, "black" and "white" populations shared a single COI haplotype (GenBank accessions MF688616–MF688627), which was also found in *C. diphone* populations sampled in southern Japan (Honshu), as well as on surrounding islands other than the Ogasawara Islands (Emura et al. 2013).

Signatures of Dispersal

Consistent with expectations, we did not detect recent immigration into Kauai, whereas the other 4 islands had higher migration rates and non-overlapping confidence intervals (Table 3). Migration was symmetrical between the close neighbor islands of Molokai and Maui, with a higher rate toward Maui. The sex of most birds ($n = 111$) was morphologically determined, and there was no evidence of sex-biased dispersal. The 32 birds of unknown sex were juveniles when collected. Of these, sex was successfully determined for 16 birds via the sex-linked marker. In sum, only 15 females were identified in the entire dataset ($n = 143$), a markedly smaller number than males, which is due to males responding much more strongly to audio playback song or call. A consequence of this heavily male-biased sampling was that the ability to pick up signal for sex-biased dispersal was limited. Indeed, assignment tests revealed that females were no more likely to have been born locally than males, although the trend was toward female-biased philopatry ($P = 0.09$, Mann-Whitney U-test), a likely consequence of more than half of females being sampled during their hatch year as predispersal individuals. Because more females were from Kauai ($n = 7$) than from other islands, we also investigated sex-biased dispersal on this island only, with nonsignificant results ($P = 0.14$). There were also no inter-island or age differences in assignment scores ($P > 0.1$).

DISCUSSION

A prediction of population genetic theory is that genetic drift in small populations can lead to losses of allelic diversity as well as lowered heterozygosity (Allendorf and Luikart 2007). Allelic diversity patterns in bush-warblers matched these expectations, with highest genetic diversity on Oahu, the island of original introduction(s), and lower diversity on the other islands. Genetic richness was clearly reduced on the 3 easternmost Hawaiian Islands, as might be expected from successive colonization events by a few individuals per island. Despite the relatively long distance from Oahu, Kauai birds had nearly equal allelic richness to their presumed source, which may indicate that multiple individuals colonized Kauai and adequately represented the allelic diversity of Oahu. The private allele on Kauai also suggests that the full extent of genetic diversity was not sampled on Oahu, the likely origin. By contrast, analysis of H_E indicated no inter-island differences, which suggests that not all aspects of population genetic changes

TABLE 3. Mean migration rates between islands (with 95% confidence intervals) of *Cettia diphone* in the Hawaiian Islands, USA, for analysis using all birds in BAYESASS. Values in bold had significant rates of migration, and italics signify asymmetric migration.

Recipient population	Donor population				
	Kauai	Oahu	Molokai	Maui	Hawaii
Kauai	0.75 (0.66 to 0.84)	0.04 (–0.05 to 0.13)	0.04 (–0.06 to 0.14)	**0.16 (0.01 to 0.31)**	0.01 (–0.03 to 0.05)
Oahu	0.02 (–0.02 to 0.07)	0.69 (0.64 to 0.73)	0.05 (–0.07 to 0.18)	***0.20 (0.06 to 0.35)***	0.03 (–0.03 to 0.09)
Molokai	0.01 (–0.01 to 0.04)	0.02 (–0.02 to 0.05)	0.82 (0.71 to 0.93)	**0.13 (0.01 to 0.25)**	0.02 (–0.02 to 0.05)
Maui	0.01 (–0.01 to 0.04)	0.02 (–0.02 to 0.05)	**0.25 (0.17 to 0.33)**	0.70 (0.64 to 0.76)	0.02 (–0.02 to 0.07)
Hawaii	0.02 (–0.02 to 0.07)	0.02 (–0.02 to 0.07)	***0.16 (0.04 to 0.27)***	0.11 (–0.01 to 0.22)	0.69 (0.65 to 0.73)

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have been affected. This is perhaps expected, given that H_E is not very sensitive to bottlenecks (Allendorf and Luikart 2007) and that significant changes in H_E are not typically seen in bird colonizations of islands (Clegg 2009). In bottleneck events where the population is reduced to a few individuals, alleles may be lost, particularly low-frequency alleles—directly affecting this measure of genetic diversity—but loss of heterozygosity is not always observed (Luikart et al. 1998). Founder effects have been well documented in Silvereye (*Zosterops lateralis*) colonizations in the southwest Pacific by Clegg et al. (2002a, 2002b), who found that allelic diversity and heterozygosity were not affected by single colonizations—rather, multiple sequential colonizations were required. One major difference between the system of Clegg et al. and ours is that their modeling suggested that Silvereyes colonize new islands in large flocks. Because flocking is not known to occur in bush-warblers, movement among islands is likely by solo individuals. The effect of this difference is that genetic drift in these smaller founding populations of bush-warblers should occur more quickly, as observed on Kauai. Why bush-warblers apparently took >50 yr to start colonizing other islands from Oahu is unknown. Furthermore, it is possible that after the initial colonization of Molokai in the late 1970s, bush-warblers colonized all remaining islands within a few years (Pyle and Pyle 2017). Time lags in population growth of introduced species are well documented, with 2 possibilities being more favorable environmental conditions or improved fitness based on adaptation to the new environment (Crooks and Soulé 1999). Our data do not inform either alternative, but we suspect that it took decades for the bush-warbler population on Oahu to be sizable enough to start colonizing the other islands. But what then allowed for their rapid expansion onto subsequent islands and apparent rapid population growth provides substantial room for speculation.

Patterns of gene flow are an important determinant of standing genetic variation. Nothing prevents bush-warblers from back-colonizing islands where they are already established and potentially blurring patterns of genetic differentiation. The relative amount of back colonization will determine, to a large extent, the potential for population divergence on different islands over time. If current patterns hold, this would suggest that birds on Kauai may be on a different evolutionary trajectory than those on the easternmost islands of Molokai, Maui, and Hawaii. Analyses of genetic structure revealed 2 clusters on each island, with Kauai populations dominated by one cluster and Molokai, Maui, and Hawaii having predominantly a second cluster. Oahu had individuals assigned to each cluster roughly equally. Results from mean pairwise genetic relatedness support these findings of 2 groups, with birds from Kauai and Oahu significantly different than birds from the other 3 islands. Because flight distance between Kauai and Oahu is ≥3 times the distance between other neighboring islands, we were not surprised that it was more genetically differentiated and that allelic frequencies are different from those found on other islands. The difficulty of dispersal between Oahu and Kauai and the ease of dispersal between Oahu and the eastern islands were also found by VanderWerf et al. (2010), who determined that ancestors of elepaio (*Chasiempis* spp.) naturally colonized Kauai from Asia and later, after a substantial amount of time, colonized Oahu, whence they quickly spread to the other islands.

The finding of 2 genetic groups must be approached with caution, however. Determining the true value of K (i.e. the number of populations) has considerable uncertainty, and use of results from STRUCTURE can be complicated by many factors, including complex population structuring (Meirmans 2015), closely related individuals within the dataset (Rodríguez-Ramilo and Wang 2012), and uneven sampling (Puechmaille 2016). Indeed, the maintenance of 2 separate genetic groups on each island is perhaps the most surprising result from our study—so surprising that we are cautious about overinterpreting this finding. The number of individual bush-warblers introduced to Oahu and the number of introduction attempts are not well known but are likely similar to other introductions at the time that involved limited numbers of birds and few release attempts (Foster 2009, Lever 2011). We do not yet know the biological reason for the presence of genetic structure on each of the islands, despite years of potential mixing of genotypes. No discernible ecological basis was found for the pattern of the location of individuals from "black" or "white" populations, including no pattern for elevation, habitat type, or sex that would potentially account for the separate populations. Although we sampled birds at multiple sites on each island, these localities did not match group assignment (e.g., not all members from high-elevation sites were part of the white group in Figure 1). In fact, individuals from black and white populations often shared apparently adjacent territories. Separate breeding populations based on assortative mating due to differences in calls (i.e. cultural evolution; see below) are possible and could be a productive area for future research. These data suggest that the 2 different groups may have existed prior to introduction, possibly because ≥2 different collecting sites in Japan or mainland Asia were sources. Unfortunately, our mitochondrial loci did not have enough variation to determine whether there are different source population(s) for the apparent black and white groupings.

Mitochondrial DNA can often be used to determine an organism's origins if the species is well sampled in its home range. Based on cytochrome *b* sequence, birds from Hawaii were indistinguishable from *C. d. cantans* specimens collected from Chiba, Japan, on the island of

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Honshu. However, some birds from South Korea, those from the subspecies *C. d. cantans*, also share identical sequence to Hawaiian birds. Bush-warbler sequences from Hawaiian birds did differ from *C. d. cantans* sequences from other sites on Honshu, ruling out those areas as sources for the Hawaiian introduction. The single COI haplotype of the birds from Hawaii does not support introductions from multiple genetically divergent locales as the reason for population genetic structure, nor does it refute it. This haplotype was found in several locations in southern mainland Japan and islands to the north and south, apart from the Ogasawara Islands (Emura et al. 2013, Saitoh et al. 2015). These findings are consistent with plumage-based assignment of subspecies of Hawaiian birds (Hamao 2015). To determine the more precise Asian source(s), additional sampling is needed in Japan and possibly Korea, and additional genetic markers should be applied.

Genetic structure of bush-warblers was strongly correlated with both geographic distance between islands and number of colonization steps from Oahu, suggesting limits on dispersal, at least over the period of less than a century. Results from the migration analyses support these findings, with migration predominantly in the expected direction from earlier- to later-colonized islands. Dispersal is a key component structuring populations, with female-biased dispersal predominating in many bird species (Greenwood 1980). In the Hawaiian system, we are uncertain whether there is a sex-biased difference in colonization based on a paucity of females in our sampling. Sex-biased dispersal in bush-warblers is likely a productive area of future research because of strong sexual dimorphism; females are half the size of males and typically skulk and make only short flights, whereas males are strong and active fliers.

Phenotypic differentiation in introduced species is possible upon introduction to novel environments or could simply reflect existing differences among the introduced individuals. Hamao (2015) morphologically compared the Oahu birds from our study to birds from Honshu sites and found significantly smaller wing, tail, and bill lengths in the Hawaiian birds but longer tarsus lengths. As for vocalizations, we noted apparent differences in male bush-warbler songs among islands (J. Foster personal observation), and distinct vocalizations in individual bush-warblers in Japan have been well documented (Hamao 1993). Given that song in Old World warblers is largely learned rather than having a pure genetic basis, we would not expect differences in song to follow the same genetic patterns, such as isolation-by-distance, seen in the present study. However, subtle differences in songs between black and white populations could easily explain why these 2 genetic groups remain distinct. Furthermore, research into bush-warbler song in Hawaii could be fruitful for studying cultural evolution in small founder populations. Recent data indicate that bush-warblers in Hawaii have a simpler population-level acoustic pattern and sing at a lower frequency compared to the likely source population on Honshu, Japan; although, interestingly, birds on Oahu have a larger song repertoire (Hamao 2015). This latter finding may reflect more diverse origins of Hawaiian bush-warblers than a single Japanese source.

Conclusions

Our study documents genetic drift in action in an introduced bird population. We also found a distinct isolation-by-distance effect suggesting that successive colonizations of islands affect population genetic structure. The differentiation of bush-warblers into potentially 2 separate populations on each island was surprising and merits future research into the mechanisms potentially generating this pattern. It remains to be seen whether this differentiation of bush-warblers in Hawaii continues over the years or if continued dispersal of birds among islands will blur these differences. Moreover, we suggest that dispersal behavior, specifically in colonization by individuals rather than flocks, could be allowing for losses in genetic diversity not seen by Clegg et al. (2002a). The bush-warbler is just one example of inter-island colonizations by introduced birds in the Hawaiian Islands, and this study system can serve as a model for contemporary evolution. Genetic studies of microevolutionary processes could be investigated in other introduced bird species in Hawaii such as White-rumped Shama (*Copsychus malabaricus*) and Japanese White-eye (*Zosterops japonicus*) (Foster 2009), allowing for a contrast in genetic differentiation in species with a range of dispersal capabilities. The wealth of avian introductions should provide abundant opportunities to assess these and related rapid evolutionary changes in both genotypes and phenotypes in these populations over decades, based on comparisons with source mainland populations—and, ultimately, to assess evolutionary changes typically seen only on the scale of millennia.

ACKNOWLEDGMENTS

Land access and/or permits were provided by The Nature Conservancy of Hawaii on Molokai and Maui, State of Hawaii Division of Forestry and Wildlife and its Natural Areas Reserve System and State Forest Reserves, Parker Ranch, and Haleakala Ranch. We thank P. Sweet of the American Museum of Natural History, C. Kishinami of the Bishop Museum, and J. Dean of the National Museum of Natural History for specimen preparation and curation, despite the many preservation challenges we gave them. R. Fleischer of the Smithsonian Institution assisted with sample preparation. **Funding statement:** Research was funded by the U.S. Environmental Protection Agency (Science To Achieve Results fellowship to J.T.F.) and the American Museum of

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Natural History (Chapman Memorial Grant to J.T.F.). F.M.W. was supported by a postdoctoral fellowship from the Office of the Vice President for Research, Northern Arizona University. Funders did not have any input into the content of the manuscript.

Ethics statement: The University of Illinois approved research protocols, including mist-net capture and specimen collecting (IACUC permit 02016). This research was conducted in compliance with the *Guidelines to the Use of Wild Birds in Research*.

Author contributions: J.T.F. devised the study and collected the samples. J.T.F., F.M.W, B.D.R., and D.E.S. collected and analyzed the data. J.T.F. and F.M.W. wrote the paper.

Data deposits: Mitochondrial sequence data are deposited in GenBank, accessions MF688616–MF688630.

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