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RESEARCH ARTICLE

Genetic structure and population history in two critically endangered Kaua'i honeycreepers

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Abstract

Population sizes of endemic songbirds on Kaua'i have decreased by an order of magnitude over the past 10–15 years to dangerously low numbers. The primary cause appears to be the ascent of invasive mosquitoes and *Plasmodium relictum*, the agent of avian malaria, into elevations formerly free of introduced malarial parasites and their vectors. Given that these declines in native bird populations appear to be continuing, last resort measures to save these species from extinction, such as conservation breeding, are being implemented. Using 200–1439 SNPs from across the genome, we assessed kinship among individuals, levels of genetic variation, and extent of population decline in wild birds of the two most critically endangered Kaua'i endemic species, the 'akikiki (*Oreomystis bairdi*) and 'akeke'e (*Loxops caeruleirostris*). We found relatively high genomic diversity within individuals and little evidence of spatial population genetic structure. Populations displayed genomic signatures of declining population size, but individual inbreeding coefficients were universally negative, likely indicating inbreeding avoidance. Diversity within the founding conservation breeding population largely mirrored that in the wild, indicating that genetic variation in the conservation breeding population is representative of the wild population and suggesting that the current breeding program captures existing variation. Thus, although existing genetic diversity is likely lower than in historical populations, contemporary variation has been retained through high gene flow and inbreeding avoidance. Nonetheless, current effective population size for both species was estimated at fewer than 20 individuals, highlighting the urgency of management actions to protect these species.

Keywords Bottleneck · Drepanids · Population structure · Islands · Hawaii · Conservation breeding

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Introduction

The contemporary rate of biodiversity loss, with extinction rates 1000 times higher than background rates (Pimm et al. 2014), has been unparalleled since the mass extinction of non-avian dinosaurs (Jablonski 1986). Species declines and extinctions may occur due to external forces such as environmental catastrophes or emerging infectious diseases (Lande 1993; Spiller et al. 1998; Courchamp et al. 2006; Prowse et al. 2013), demographic stochasticity (Shaffer 1983; Lande 1988; Wootton and Pfister 2013; Mashayekhi et al. 2014), genetic processes such as adaptive diversity loss or fixation of deleterious mutations (Lande 1994, 1998; Palkopoulou et al. 2015; Rogers and Slatkin 2017), or the interaction of these forces (Robert 2011). External forces are often the most straightforward to document, but it is also important to understand how these processes influence demographics and genetics of declining species.

With changes in climate and other anthropogenic effects, emerging infectious diseases are a primary driver of global biodiversity loss (La Marca et al. 2005; Lips et al. 2006; Smith et al. 2006). Because climatic variables can influence pathogen vector abundance and in turn pathogen distribution (Padilla et al. 2017), climate is likely to influence population dynamics of host species susceptible to infectious diseases (Samuel et al. 2015). The interaction between infectious diseases and climate is complex (Paull et al. 2012; Mordecai et al. 2017), and their combined influence on genetic variation of declining species is less well understood. Especially vulnerable are island species, which are threatened by introduced predators and pathogens, and often require specialized habitats or specific climatic regimes (Fortini et al. 2015; Glad and Crampton 2015; Harter et al. 2015; Liao et al. 2015).

Hawaiian honeycreepers (Passeriformes: Fringillidae: Carduelinae), which are endemic to the Hawaiian Islands, have experienced population declines and extinctions since humans arrived on the islands in approximately 1000 C.E. (Kirch 2011). These declines accelerated in the late nineteenth century, likely due to introduced avian pox virus and introduced predators, and in the twentieth century honeycreeper populations began to crash (Foster et al. 2004; Camp et al. 2009; Gorresen et al. 2009). *Plasmodium relictum*, the causative agent of avian malaria that was introduced by the 1940s (Fisher and Baldwin 1947) and the previously introduced mosquito vectors (*Culex quinquefasciatus*) were historically restricted by temperature to low elevations. As a result of climate warming (Diaz et al. 2011), mosquitoes have expanded their elevational range so that bird populations are within the range of malaria-infected mosquitoes for most of the year (Atkinson et al. 2014). This expansion has contributed to the extinction of several avian species and pushed most remaining honeycreeper species to the brink of extinction (Paxton et al. 2016, 2018). On Kaua‘i in particular, avian species no longer have high-elevation refuge from malaria (Atkinson et al. 2014). The past decade has witnessed the near complete collapse of the native bird community on Kaua‘i, and several endemic species have likely gone extinct (Paxton et al. 2016). Particularly alarming are the population declines and range contractions of two Kaua‘i endemic species: the ‘akeke‘e (*Loxops caeruleirostris*, 98% decline from 2000–2012) and the ‘akikiki (*Oreomystis bairdi*, 71% decline from 1981 to 2012; Paxton et al. 2016). These population crashes directly correspond to the ascent of introduced malaria and its invasive mosquito vector *Cx. quinquefasciatus* to even the highest elevations on the island (Atkinson et al. 2014), in part due to the influence of warming temperatures on disease dynamics (Samuel et al. 2011), and potentially to the replacement of a warm-adapted mosquito lineage by a cold-adapted lineage (Fonseca et al. 2006).

Both the ‘akeke‘e and the ‘akikiki are listed as Critically Endangered by the International Union for the Conservation of Nature (IUCN; BirdLife International, 2018a, b), Endangered by the U.S. Fish and Wildlife Service (USFWS 2010), and of Greatest Conservation Need by the State of Hawai‘i (Hawai‘i Division of Forestry and Wildlife 2015). ‘Akikiki are estimated to number ~ 450 individuals and occupy ~ 25 km^2 of habitat; ‘akeke‘e are estimated to number ~ 1160 and occupy ~ 40 km^2 (Paxton et al. 2020). Both species are currently restricted to the remote interior high elevation ‘ōhi’a (*Metrosideros polymorpha*, Myrtaceae) forests in the Nā Pali Forest Reserve and Alaka‘i Wilderness Preserve (Fig. 1), which has long been a refuge from mosquito-borne avian diseases because temperatures were historically too low for mosquito and parasite development. However, mean temperatures on Kaua‘i have risen in the last several decades (Fortini et al. 2015; Atkinson et al. 2014), allowing the incursion of mosquitoes and malaria into this former refuge (Atkinson et al. 2014) and threatening the survival of these avian species in the absence of intervention.

Given the catastrophic declines documented in ‘akeke‘e and ‘akikiki populations and the lack of means to control mosquitoes across the landscape, two key conservation strategies are gaining knowledge about the distribution and degree of genetic variation in the species and establishing conservation breeding populations for each species. As a critical element of the ‘akeke‘e and ‘akikiki conservation management programs, egg collections were initiated in 2015 by the state and San Diego Zoo Wildlife Alliance to establish a conservation breeding (also known as captive propagation, captive breeding, ex situ management, or managed care) population for each species. The ultimate

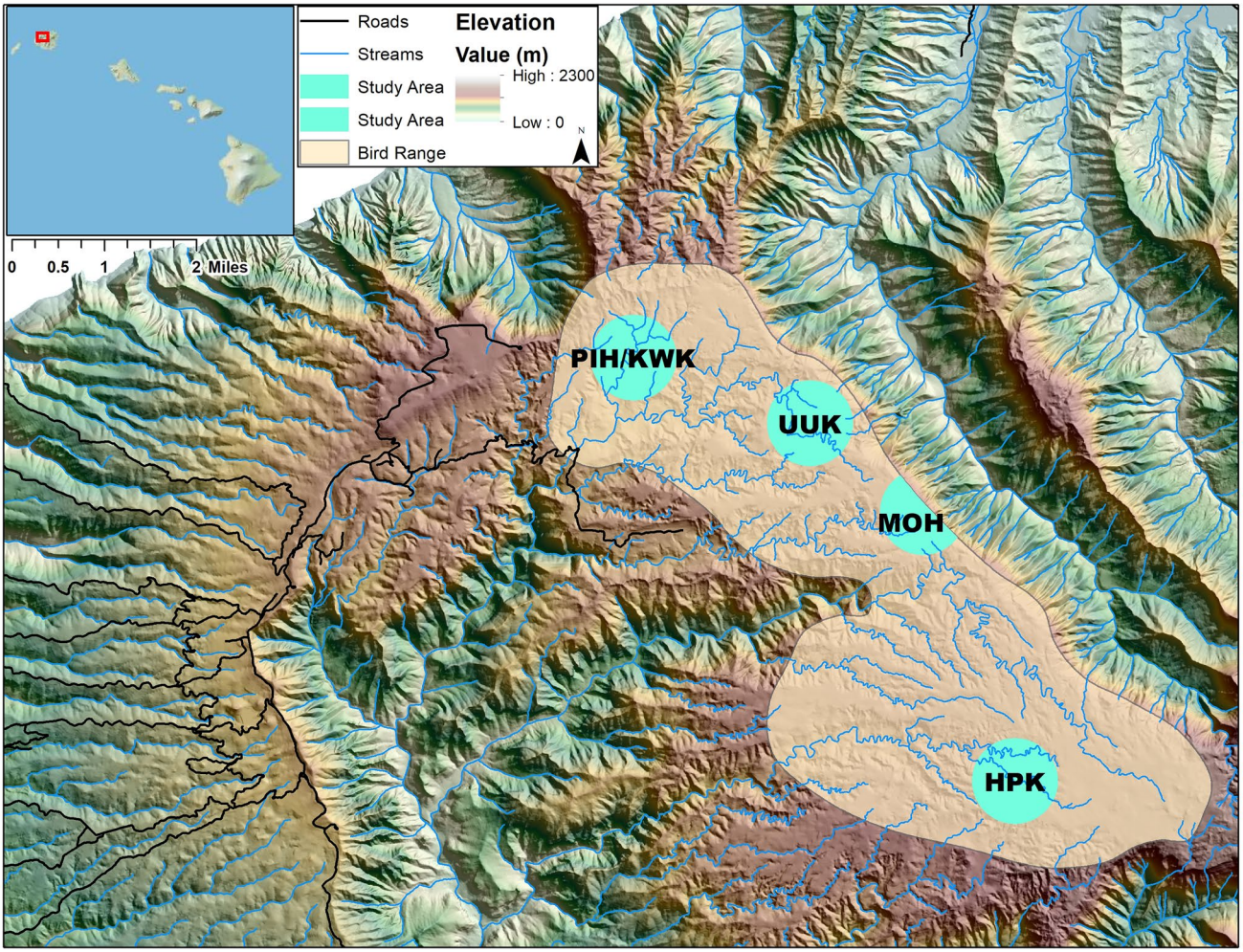

Fig. 1 Map of Kaua'i study area and field sites, with tan outline (extending across approximately 40 km^2) encompassing the geographic range of both 'akeke'e and 'akikiki, which occurs at the highest elevations on the island. PIH = Pihea, KWK = Kawaikōī, UUK = Upper Kawaikōī, MOH = Mohihi, HPK = Halepa'akai

goal of conservation breeding programs is to ensure species survival (Rodrigues 2006; Farhadinia et al. 2020), and in several well-known species these programs likely have been the primary or only factor preventing extinction (e.g., California condor (*Gymnogyps californianus*), black-footed ferrets (*Mustela nigripes*), 'alalā (*Corvus hawaiiensis*), whooping cranes (*Grus americana*), Butchard et al. 2006, Santymire et al. 2014). The viability, productivity, and success of a conservation breeding population depends largely on the genetic diversity of the founding individuals and how well it represents the neutral and adaptive genetic variation contained in wild populations. Maximizing the degree of genetic diversity and the extent of outbreeding in a conservation breeding population minimizes the risk of inbreeding depression that occurs due to the unmasking of deleterious recessive alleles and reduces the risk of mortality from the expression of lethal equivalents (Ralls et al. 1988; Roelke et al. 1993). Moreover, species' persistence in the wild is likely affected by the degree of genetic diversity contained in wild populations (Palkopoulou et al. 2015). Therefore, genomic methods provide a powerful means to assess the patterns of neutral and adaptive diversity within and among individuals (Cassin-Sackett et al. 2019b). Here, in the first study to characterize the genetics of 'akeke'e and 'akikiki, we assess the kinship and genetic diversity of wild individuals and those that were used to initiate the conservation breeding population for each species. Our goals were to (1) characterize the degree and distribution of genetic variation in wild populations of each species, (2) evaluate whether their genomes show evidence of recent declines, and (3) determine the genetic characteristics and inbreeding levels of the initial conservation breeding population of each species, including whether

these populations adequately represent the genomic variation currently present in the wild.

Materials and methods

Sampling

For wild birds, we sampled from five sites where ʻakekeʻe and ʻakikiki occur in the higher elevations of Kauaʻi (Fig. 1). Field sites were as follows: Pihea (PIH), Kawaikōī (KWK), Upper Kawaikōī (UUK), Mohihi (MOH), and Halepaʻakai (HPK). Mist nets were set intermittently from 2012 to 2018 in the canopy, both actively (i.e., with audio playback targeting each species separately) and passively (without playback). In addition, some samples from wild birds were obtained from fieldwork in the region dating back to the mid-1990s. Wild birds were banded and blood was collected as described elsewhere (Atkinson et al. 2014); wild samples included 32 ʻakekeʻe and 52 ʻakikiki. For the conservation breeding populations, eggs were collected from nests from 2015 to 2018 in UUK, MOH and HPK, the locations with the highest density of birds. Behavioral clues were used to find nests, and eggs were collected 10–15 days after the clutch was completed. To reduce the risk of inbreeding in the conservation breeding population, color band patterns and plumage differences were used to avoid collecting from the same pair more than once. All eggs (1–4 per nest) from sampled nests, which were accessed by ladder, were collected to encourage the pair to lay an additional clutch. Eggs were transported to a conservation breeding facility for subsequent artificial incubation and hand rearing aviculture. All individuals in the conservation breeding population for this study were collected from the wild (i.e., none were F1 offspring of founding individuals). As of late 2018 (when our analyses were conducted), these populations included 10 ʻakekeʻe and 46 ʻakikiki founding individuals; a subset of these individuals with blood samples with sufficiently high DNA quantity and quality were included in this manuscript.

Upon first capture (for wild birds) or after fledging (for birds in managed care; hereafter ʻmanagedʼ), birds were fitted with a unique combination of color bands and/or a Federal bird band with a unique identifier. For wild birds, approximately 50 µL of blood was collected via brachial venipuncture in heparinized capillary tubes, while for birds in managed care, approximately 20 µL of blood was collected via jugular venipuncture. Blood was then transferred to Queen's Lysis buffer to preserve the DNA and shipped on dry ice for storage in the Smithsonian Cryo-Collection at the National Zoo in Washington, D.C.

DNA preparation and sequencing

DNA was extracted from blood with a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) and sheared using a Qsonica Q800R (Newton, CT); libraries were constructed on sheared DNA using KAPA library preparation kits (Kapa Biosystems, Wilmington, MA). Each sample was dual indexed with unique adapters, and following library prep, low-cycle number PCRs were run in duplicate or triplicate and pooled to minimize carryover of PCR artifacts. Post-PCR libraries were subsequently pooled in groups of eight samples and then hybridized for 24–48 h to a custom-designed and filtered (Arbor Biosciences, Ann Arbor, MI) set of 40,000 oligonucleotide baits targeting single nucleotide polymorphisms (SNPs) distributed randomly across non-repetitive portions of the genome (Cassin-Sackett et al. 2019a). Baits were designed using the genome of the Hawaiʻi ʻamakihi (*Chlorodrepanis virens*; Callicrate et al. 2014), which diverged from ʻakekeʻe 2.47 million years ago (mya) and from ʻakikiki 4.73 mya (Lerner et al. 2011), corresponding to approximately 1.2 million generations for ʻakekeʻe and 2.3 million generations for ʻakikiki (Hammond et al. 2015). After hybridization, pools were combined and size-selected with a Pippin Prep (Sage Science, Beverly, MA) prior to sequencing. Libraries were sequenced on 150 bp paired-end runs on an Illumina HiSeq at Johns Hopkins University or Brigham Young University.

SNP filtering and processing

Reads were trimmed using Trimmomatic 0.36 (Bolger et al. 2014) with the following parameters: ILLUMINACLIP:NexteraPE-PE.fa:2:30:10, LEADING:3, TRAILING:3, SLIDINGWINDOW:4:20, MINLEN:36. Trimmed reads were subsequently aligned to the ʻamakihi genome (Callicrate et al. 2014) using BWA-MEM 0.7.17 (Li 2013). Reads with MAPQ < 20 were removed from the alignments using SAMtools 1.6. (Li et al. 2009)), and mismatch rates were calculated using the edit distance in SAMtools 1.11. PCR duplicates were marked using Picard 2.9.4 MarkDuplicates (Broad Institute, http://broadinstitute.github.io/picard). Reads were realigned around indels using the Genome Analysis Toolkit (GATK) 3.7.0 IndelRealigner (McKenna et al. 2010). Single nucleotide variants and small indels were called for each species using the GATK HaplotypeCaller; non-variant sites were not included in downstream analysis. Variants within the baited regions were extracted using VCFtools 0.1.15 (Danecek et al. 2011), and filtered for depth (DP ≥ 4) and quality in GATK (ReadPosRankSum ≥ -8.0, MQRankSum ≥ -12.5, FS ≤ 60.0, QD ≥ 2.0) and minor allele frequency (maf ≥ 0.01) in VCFtools 0.1.15. Removing sites with low minor allele frequency aims to eliminate SNPs generated from sequencing error;

the number of remaining SNPs after applying these filters was 16,778 for ʻakekeʻe and 22,482 for ʻakikiki. We also filtered for missingness in VCFtools (individuals missing data at > 95% of loci were removed; in the remaining dataset we retained loci genotyped in at least 80% or 100% of individuals, depending on whether the analysis allowed for missing data). Due to our interest in specific individuals, particularly in the conservation breeding populations, we prioritized retaining individuals over retaining loci even when samples from those individuals were of poor quality (leading to higher missing data). Nonetheless, to ensure our results were not artifactual, we also repeated some analyses with an idealized dataset containing more loci and fewer individuals (see Supplementary Materials for details).

Kinship

We removed Z chromosome variants using VCFtools 0.1.15; no baits targeted chromosome W. We included only biallelic SNPs for kinship estimation. Final datasets for kinship analysis consisted of matrices with individuals missing fewer than 95% of loci and loci genotyped in at least 80% of individuals. Bootstrapped kinship analysis (100 bootstrap replicates) using maximum likelihood estimation of identity by descent was performed in SNPRelate 1.14.0 (Zheng et al. 2012) in R 3.5.0 (R Core Team 2018) using kinshipUtils (Campana, M.G.: https://github.com/campanam/kinshipUtils) following Cortes-Rodriguez et al. (2019). We pruned SNPs in linkage disequilibrium using a threshold of 0.2 for the absolute value of the correlation coefficient (|r|).

Population structure and diversity

Population structure was assessed in several ways. First, we estimated the number of ancestral populations using three replicate runs in ADMIXTURE 1.3 (Alexander et al. 2009), allowing K to vary from 1 to 20. Second, we inferred ancestry coefficients of each individual in Structure 2.3.4 (Pritchard et al. 2000; Hubisz et al. 2009), assuming correlated allele frequencies and allowing admixture. We performed three replicate runs consisting of 250,000 burn-in and 1,000,000 iterations. We allowed K to vary from 1 to 8 and ensured convergence among runs with the same assumed K. Next, we tested for isolation by distance by performing a Mantel test on linearized F_{ST} against the log of geographic distance in the vegan package (Dixon 2003) for R. Finally, using a dataset with no missing data, we used the vegan package to perform a Principal Coordinate Analysis (PCoA) with Prevosti's Distance method. A PCoA was preferred over Principal Component Analysis because both datasets were characterized by more SNP sites than individuals (Rohlf 1972).

We used VCFtools to estimate several diversity metrics within species: (1) inbreeding coefficients for each individual, (2) mean relatedness of an individual to each other member of the population, and (3) nucleotide diversity (π, Nei and Li 1979) in non-overlapping 1-million base pair bins within species (these larger than typical bins were used because of the small number of genotyped loci). In addition, we performed an AMOVA on each species in the ade4 package (Dray and Dufour 2007) for R, assuming two hierarchical population clusters: the lowest-level cluster separated all sampling locations and the higher cluster grouped HPK separately from the remaining sampling sites, a division consistent with geography (Fig. 1). We calculated individual genome-wide observed heterozygosity (H_o) across sites using the adegenet package (Jombart 2008) for R and expected heterozygosity (H_e) as measured by gene diversity in Genepop (Rousset 2008). Finally, we calculated pairwise F_{ST} values between sampling sites using the Weir and Cockerham method in the adegenet package in R. Due to the observed distribution of sequencing depth in ʻakikiki loci (Figure S2), we repeated diversity analyses after removing loci with a depth greater than the mean plus two standard deviations (see Supplementary Materials).

Next, we aimed to assess whether the population in managed care adequately represents genomic variation in the wild. First, we compared the three diversity metrics above between wild and managed populations. Second, we constructed median joining networks to visualize the proportion of the network covered by managed individuals. To do so, we generated a fasta alignment file from each filtered vcf using SNiPlay (Dereeper et al. 2015) and constructed a median joining network (Bandelt et al. 1999) for each species in the pegas v. 0.14 (Paradis 2010) R package. We then plotted the networks to determine whether individuals in managed care were found throughout the network.

Detection of bottlenecks

Because both species have suffered dramatic population declines in recent years, we used several approaches to test whether we could detect genetic signatures of bottlenecks. First, we examined the degree of heterozygosity excess relative to expectations based on allelic diversity at each site (Cornuet and Luikart 1996). We calculated both H_e and H_o in the Genepop package for R and conducted a sign test with 95% confidence intervals (Cornuet and Luikart 1996) using the BSDA package (Arnholt and Evans 2017) for R. Second, we used VCFtools to calculate Tajima's D in 5 million base pair bins. Third, we used GADMA (Genetic Algorithm for Demographic Analysis, Noskova et al. 2020) to explore demographic history separately in each species, assuming a single population and allowing for up to four time intervals (i.e., where each interval was allowed a different

pattern of population growth). This approach generates a site frequency spectrum using SNPs, and performs simulations using the spectrum to infer demographic history beginning at the emergence of the species. Simulations were run using the *moments* scheme (Jouganous et al. 2017). Candidate models contained either three or four time intervals with linear, exponential or sudden population growth or decline in each interval. Models were run in triplicate and the best models (evaluated with log likelihood and Akaike Information Criterion) were visualized. The timing of the onset of each interval was inferred by the model, but because of the bias inherent in selecting sites for analysis that are known to be variable, we did not attempt to estimate precise timing of any demographic events (e.g., population declines); rather, we were solely interested in patterns of population growth and decline as well as relative timing (e.g., old vs. recent). We repeated the analyses with different values of theta to ensure our results were robust to changes in this parameter. Fourth, we estimated historical trends in effective population size (N_e) over time using SNeP (Barbato et al. 2015), which uses linkage disequilibrium to estimate N_e in the more recent past. Because changes in most run parameters did not appreciably change the numerical estimates of N_e, we used the default settings for all parameters except the minimum distance between SNPs (set to minimum of 1 base pair, thus using all SNPs in the calculations of LD) and the minimum allele frequency for inclusion in the analysis (set to minimum of 0.01). These parameter settings were designed to maximize the number of loci used in the calculations. Finally, because SNeP may underestimate very recent and current N_e (Barbato et al. 2015), we estimated current effective population sizes in both species using the molecular coancestry method implemented in NeEstimator v2.1 (Do et al. 2014) and generated jackknife confidence intervals. To validate these estimates, we repeated the analysis with the dataset containing no missing genotypes.

Results

Samples

Final post-filtering datasets included 37 ʻakekeʻe (29 wild, eight managed) and 64 ʻakikiki (36 wild, 28 managed) individuals (Supplementary Tables S1–S2). Mismatch rates against the ʻamakihi reference averaged 1.94% for ʻakikiki (SD = 0.00091) and 1.5% for ʻakekeʻe (SD = 0.00056), consistent with the closer phylogenetic relationship between ʻamakihi and ʻakekeʻe (Lerner et al. 2011). Because our aim was to maximize the number of individuals in the dataset, and several individuals had large proportions of missing data (e.g., six wild and three managed retained ʻakekeʻe, as well as four wild and eight managed retained ʻakikiki, were missing genotypes at > 90% of SNPs), datasets for kinship analyses and population structure (80% complete) contained 1021 (ʻakekeʻe) and 1439 (ʻakikiki) SNPs (Supplementary Tables S3–S5; resulting levels of missing data among individuals shown in Figure S1). The distribution of coverage depth of loci was similar before and after filtering was applied (Figure S2); mean coverage per site per individual in the filtered datasets was 65.8 in ʻakekeʻe and 62.0 in ʻakikiki. Final datasets for PCoA did not permit missing data and included 218 (ʻakekeʻe) and 246 (ʻakikiki) SNPs; this dataset was also used for estimating demographic history. Sequences are available on GenBank (BioProject PRJNA527134).

Kinship

Kinship values are based on the genetic similarity of individuals, which usually reflects a close kin relationship (identity by descent). Mean empirical kinship of all ʻakekeʻe individuals was 0.088 (range 0.003–0.215; Fig. 2, top left; Supplementary Tables S6–S7), and was similar in wild (0.095; 95% CI 0.092–0.098) and managed (0.075; 95% CI 0.060–0.090) individuals. Mean kinship of all ʻakikiki individuals was 0.065 (range 0.003–0.362; Fig. 2, bottom right; Supplementary Tables S8–S9), and was nearly identical in wild (0.066; 95% CI 0.063–0.068) and managed (0.066; 95% CI 0.062–0.069) individuals.

We examined eggs removed from the same nest, which we expected to be siblings or half siblings in the case of extra-pair mating (n = nine ʻakikiki pairs, one ʻakekeʻe pair and one ʻakekeʻe trio). In ʻakikiki, these pairings showed expected kinship levels (~ 0.25 for siblings), but in ʻakekeʻe the values were lower than expected for full or half siblings (i.e., ʻakekeʻe nestmates SB1, SB2 and SB3 all had pairwise kinships below 0.14). These low kinship values were not related to the quality or coverage of the sequence data—instead, they may reflect extra-pair mating, intraspecific brood parasitism, or simply independent assortment (but likely a lower level than we found). The nestmate pairing kinship value was 0.141 for the single remaining ʻakekeʻe nest and averaged 0.204 (range 0.178–0.244) for ʻakikiki nests, a range expected for half or full siblings (especially given that independent assortment can cause high variation in sibling kinship estimates relative to parent–offspring values).

Population structure and genetic diversity

For both species, coancestry analysis in ADMIXTURE 1.3 indicated the highest support for a single ancestral population (K) containing all extant individuals (estimated by the lowest cross-validation error), with decreasing support for each increase in number of ancestral populations.

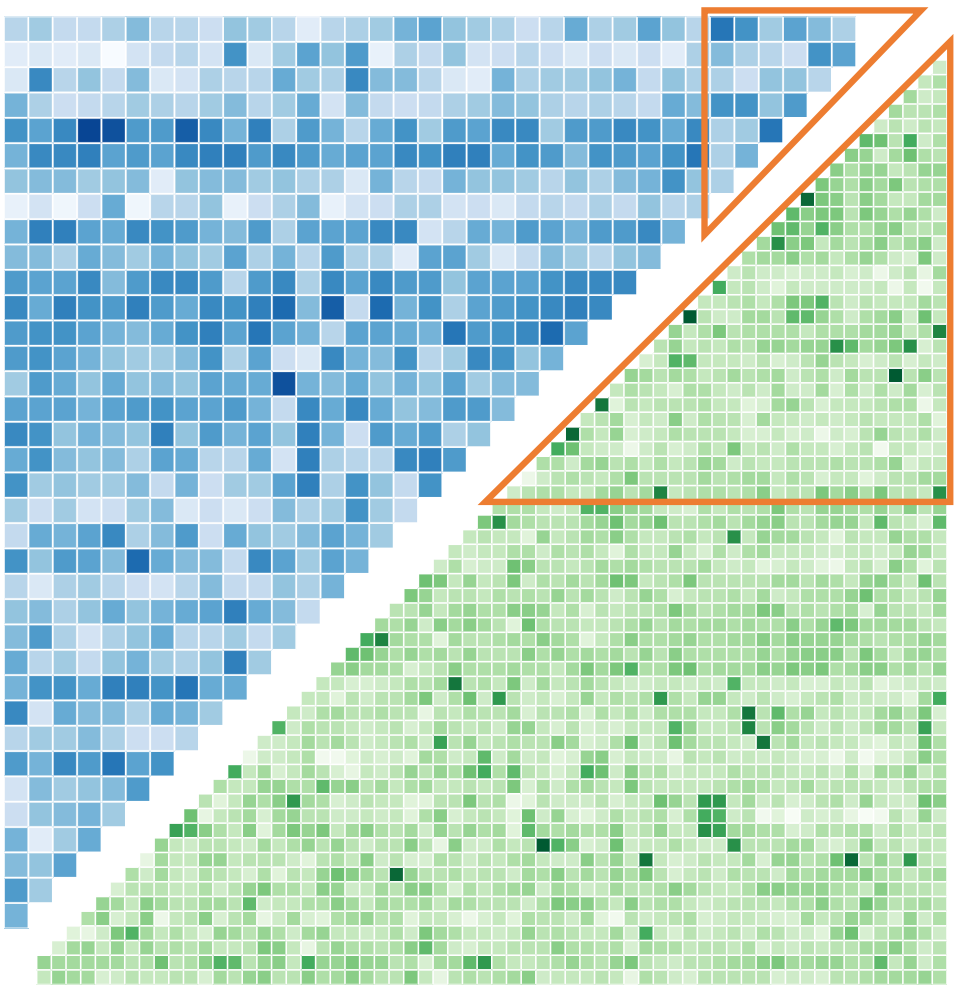

Fig. 2 Kinship matrixes showing estimated pairwise relatedness among ‘akeke‘e (top left) and ‘akikiki (bottom right) individuals. Darker shading represents higher kinship; triangles surround individuals in the conservation breeding population. The primarily light colors in pairwise relationships among individuals in the conservation breeding program (triangles) indicate the low degree of relatedness among individuals

Log-likelihood scores also supported a single population. However, for ‘akikiki, cross-validation errors were similar for one, two and three ancestral populations. Structure results also indicated support for a single population in both species.

The principal coordinates analysis recovered groupings that were related to sampled locations, with one site, HPK, encompassing nearly all of the variation and most other sampling locations containing genetic diversity that also existed in HPK (Fig. 3). A second site, MOH, also contained some unique variation in both species, as did KWK in ‘akeke‘e. Mantel tests detected no isolation by distance in either species ($p \geq 0.5$, Figure S3). The AMOVA did not recover significant genetic variance partitioned among sampling locations in ‘akeke‘e (0.33% of the variation, $p = 0.19$). However, in ‘akikiki, there was a small but significant amount of genetic variance partitioned among sampling locations (0.70% of the variation, $p = 0.04$). In both species, the remaining explained variation existed not among individuals but within individuals. In other words, individuals did not contain many unique SNPs that were not present in other individuals; instead, individuals were heterozygous at many SNPs. In line with this result, pairwise F_{ST} values between sites were low, particularly in akeke‘e (Table 1; ‘akeke‘e in bold text). No private alleles were detected within any sampling location in ‘akeke‘e; the frequency of private alleles in ‘akikiki was 0.17.

The mean nucleotide diversity (using all genotyped loci in 1 million base pair bins) in ‘akeke‘e was 1.61×10^{-6} and in ‘akikiki was 1.07×10^{-6}, and was not significantly different between managed and wild individuals (Tables 2, S1). The mean inbreeding coefficient was negative in both species (‘akeke‘e − 0.478, ‘akikiki − 0.373, Table S1), and all individuals were characterized by negative inbreeding coefficients. Managed individuals had a slightly less-negative (i.e., higher) inbreeding coefficient than wild individuals, particularly in ‘akeke‘e (Table 2). Observed heterozygosity within individuals was high: 0.623 in ‘akeke‘e and 0.543 in ‘akikiki.

Median joining networks showed that the populations in managed care largely represent existing genomic variation (Fig. 4). Nonetheless, if additional egg collections are

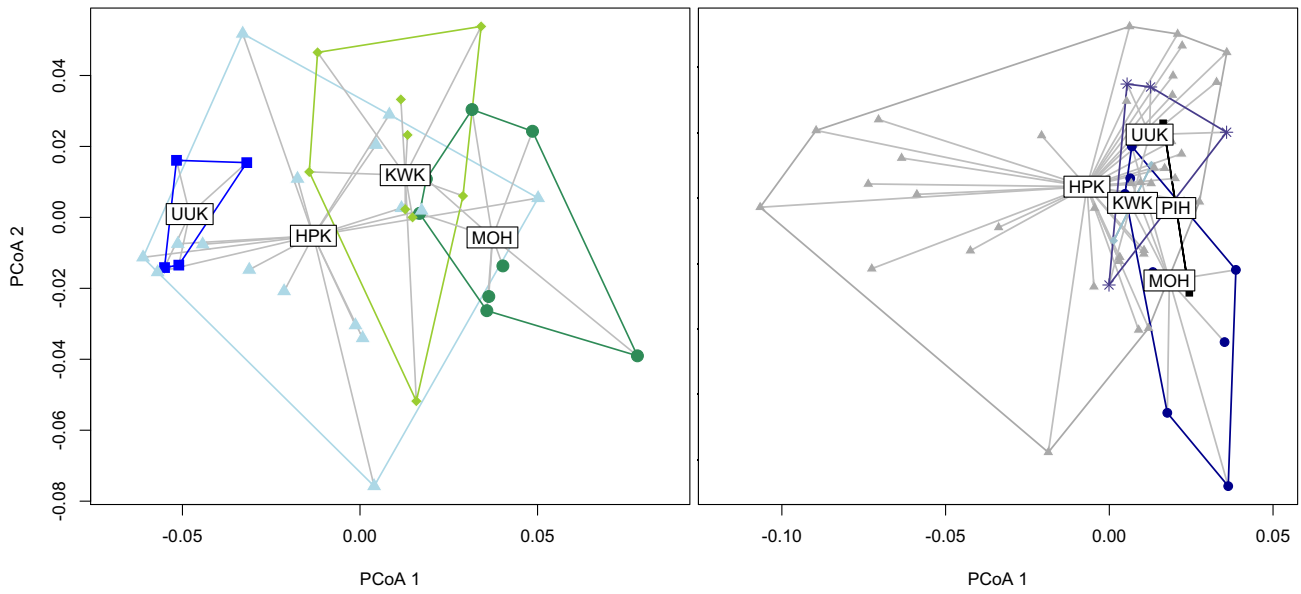

Fig. 3 Principal Coordinates Analysis of (left) ‘akeke‘e genotypes from four sampled sites and (right) ‘akikiki genotypes from five sampled sites on the Alaka‘i Plateau

deemed necessary, the networks indicate slightly under-represented regions of the genomic space that could be targeted to augment diversity in the managed population

Table 1 Pairwise F_{ST} between sampling sites of ‘akeke‘e (upper triangle, in bold) and ‘akikiki (lower triangle)

	HPK	KWK	UUK	PIH	MOH
HPK		**0.00440**	**0.00549**		**0.01413**
KWK	− 0.00260		**0.01207**		**0.00650**
UUK	0.02069	0.04210			**0.02285**
PIH	− 0.00133	− 0.01336	0.03814		
MOH	0.01889	0.01918	0.03014	0.01661	

The bold text is for ‘akeke‘e

(e.g., left side of the ‘akeke‘e network). When plotting the median joining networks that included samples collected in the 1990s (insets in Fig. 4), the managed population of ‘akeke‘e appears to miss a notable proportion of diversity. However, when plotting only the individuals that may still be alive, this pattern disappears. This suggests that extant wild ‘akeke‘e populations are missing some diversity that was present in the 1990s.

Detection of bottlenecks

Both species (with individuals pooled across sampling locations) exhibited a marked excess of heterozygosity relative to expectations based on allelic diversity. In ‘akeke‘e, 89.5% of loci were characterized by higher heterozygosity than

Table 2 Mean relatedness among and diversity within wild individuals and managed individuals; statistics were calculated in VCFtools

Statistic	Species	Wild (all years)	Wild (current)	Managed
Relatedness	AKEK	0.0161	0.0334	0.0519
	AKIK	0.0194	0.0236	0.0192
Nucleotide diversity (π)	AKEK	1.64×10^{-6}	1.75×10^{-6}	1.55×10^{-6}
	AKIK	1.16×10^{-6}	1.17×10^{-6}	1.03×10^{-6}
Inbreeding coefficient (F)	AKEK	− 0.4853	− 0.4818	− 0.3631
	AKIK	− 0.3644	− 0.3652	− 0.3605

‘All years’ refers to all sampled wild individuals including those sampled in the 1990s; ‘current’ refers to individuals sampled recently enough that the birds may still be alive (i.e., since 2014). This distinction was designed to evaluate the recent loss of diversity. Estimates may differ from whole-species estimates in Table S1 due to the automatic exclusion of non-informative loci in data subsets (e.g., quality filtered managed AKEK comprised a dataset of only nine individuals). Sample sizes are as follows: AKEK wild all N = 29, AKEK wild current N = 12, AKEK managed N = 9, AKIK wild all N = 31, AKIK wild current N = 25, and AKIK managed N = 30

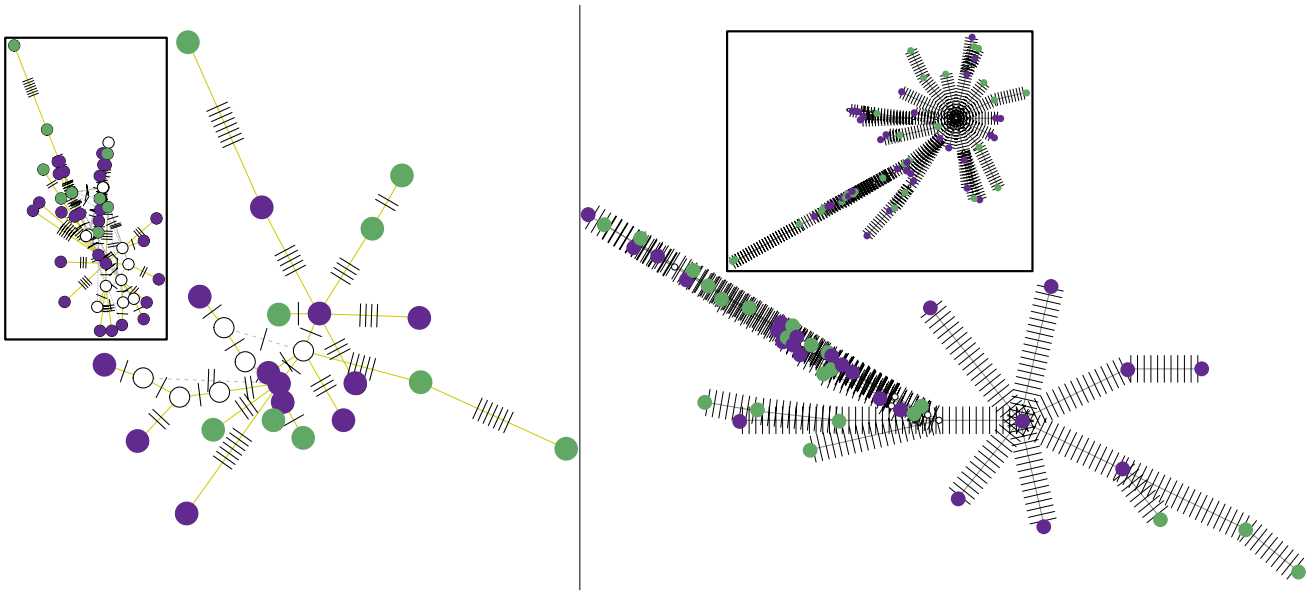

Fig. 4 Median joining networks of ‘akeke‘e (left) and ‘akikiki (right) genomic variation. Wild individuals are denoted in dark/purple circles, managed individuals in light/green circles, and median vectors (unobserved intermediate branching nodes) in white circles; hash marks represent mutations. In both species, the main figure comprises potentially extant individuals, while the inset figure also contains individuals sampled in the 1990s. In ‘akeke‘e, the managed population captures most extant diversity but misses a small portion of existing wild diversity (left side of network), while in ‘akikiki, managed individuals are present throughout the network. In ‘akeke‘e, some diversity was present in the 1990s that is not contained among extant individuals

expected (sign test $p < 2.2e\text{-}^{16}$; median $H_e - H_o = -0.199$), while in ‘akikiki, 73.9% of loci displayed higher heterozygosity than expected (sign test $p < 2.2e\text{-}^{16}$; median $H_e - H_o = -0.065$). Tajima’s D was strongly positive in both species (mean 1.81 in ‘akeke‘e, mean 2.15 in ‘akikiki; Table S1), consistent with population bottlenecks (Nei et al. 1975; Gattepaille et al. 2013). GADMA indicated consistent support for exponential population decline in both species (Figure S4), with similar patterns in the models for each replicate run. Within replicates, visualizations indicated ostensibly identical patterns of decline among all well-supported models, differing only in the relative timing of the onset of population size reduction. Replicate runs resulted in 1–15 statistically indistinguishable ($\Delta AIC < 2$) models; when there were multiple indistinguishable models, at least three were visualized to ensure inferences were consistent. In all ‘akeke‘e models, the declines occurred over relatively long time intervals (estimated not in actual timing but duration of the existence of the species, on the order of the last 20–25% of the species’ existence; Figure S4). ‘Akikiki population decline occurred more rapidly and more recently (on the order of the last 4–9% of the species’ existence; Figure S4). Effective population sizes estimated in SNeP historically numbered in the tens of thousands, but exhibited trends of linear decline during the past several hundred generations in both species (Figs. 5, S4). Approximately 150 generations ago (generation time ~ 2 years), effective population sizes numbered in the thousands; in the last five generations, N_e fell from 79.5 to 10 in ‘akeke‘e and from 158 to 15.3 in ‘akikiki (Figure S5). Because the number of bins used can influence the magnitude of inferred N_e in very recent time periods (Barbato et al. 2015), this number should be interpreted with caution. Estimates of current effective population size from NeEstimator were similarly small: for datasets allowing up to 20% missing data, N_e was estimated to be 18.5 (95% CI 15.4–21.8) in ‘akeke‘e and 13.4 (95% CI 11.4–15.5) in ‘akikiki. Estimates were slightly higher with larger confidence intervals for datasets with no missing data (‘akeke‘e: 25.3, CI 13.0–41.4; ‘akikiki: 16.5, CI 11.0–23.1).

Discussion

Using multiple approaches, we explored the distribution of genetic variation in wild and founding conservation breeding populations of two endangered Hawaiian honeycreepers. We detected high heterozygosity, little to no spatial structure among sampled locations, and genetic signatures of severe population declines in both species. We also found that individuals were not inbred, with a high proportion of variation contained within individuals and universally negative inbreeding coefficients. The high levels of observed relative to expected heterozygosity in both species (e.g., 5–10 × higher than in Hawai‘i ‘amakihi; Cassin-Sackett et al. 2019a) are likely indicative of both recent severe population bottlenecks, which appear coincident with human settlement

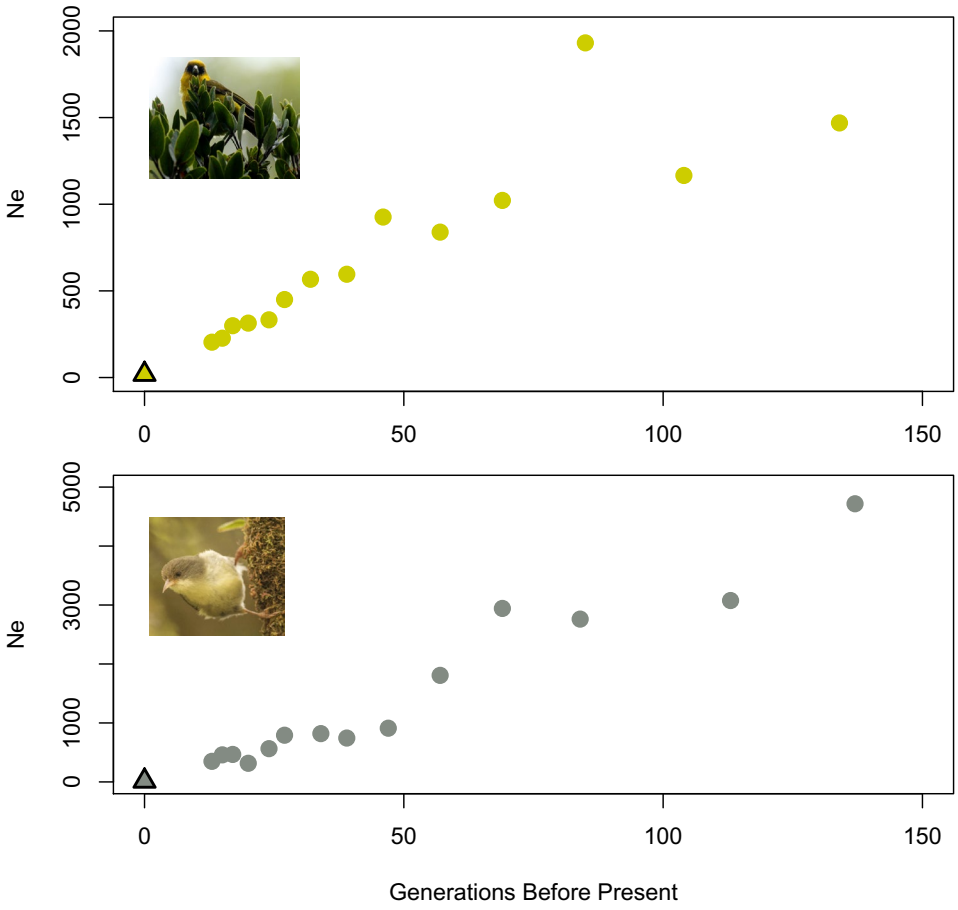

Fig. 5 Effective population size (N_e) over time as estimated in SNeP (Barbato et al. 2015); plots show only the most recent 150 generations to facilitate visualization of recent trends. Triangle point is the estimate of current effective size inferred from NeEstimator (Do et al. 2014). Longer time scales are shown in Figure S5. Top: ʻakekeʻe, photo by Lucas Behnke; bottom: ʻakikiki, photo by Justin Hite

of the island, and of linkage disequilibrium between variable sites (as LD increases after bottlenecks). If disassortative mating occurs based on a few loci (e.g., the major histocompatibility complex, Juola and Dearborn 2012) in high LD with other loci, then high heterozygosity across the genome can persist for multiple generations after bottlenecks. Both species also showed evidence of long-term population declines (potentially due to climatic fluctuations or changes in island size; Figs. 4, S5). Kinship between nestmates was generally in line with predictions, although a few hypothesized ʻakekeʻe siblings demonstrated lower relatedness than expected, suggesting potential intraspecific brood parasitism or multiple paternity. These scenarios support recent observations of multiple adults attending nests (L.H. Crampton, written communication, 2020). Finally, for both species the genetic diversity of founding individuals for the conservation breeding population is largely representative of what remains in nature.

The lack of genetic structure among sampled sites, along with high heterozygosity and negative inbreeding coefficients, suggests that wild ʻakekeʻe and ʻakikiki move relatively freely among sampling locations and either avoid inbreeding, experience selection against inbred individuals, or both (Keller et al. 1994; Hemmings et al. 2012). As a result of the species' apparent inbreeding avoidance and movement among sites, the egg collections to establish the conservation breeding populations appear to encompass existing genetic diversity (Sutton 2014) well for ʻakikiki and reasonably well for ʻakekeʻe, even without sampling all sites harboring individuals of these species. This conclusion is supported by the similarity in diversity measures between wild and managed individuals in both species (Table 2). Representation of wild genomic variation in captive ʻakekeʻe may be lower because the species occupies a larger portion of its range on the Alakaʻi Plateau relative to ʻakikiki (Behnke et al. 2016; Fricker et al. in press). Therefore, if additional egg collections are undertaken for ʻakekeʻe, attempts to sample eggs from individuals containing diversity that is not encompassed in the managed population (e.g., Fig. 4), if such nests can be found and accessed, will increase overall genetic diversity in the conservation breeding population. This strategy would amplify the probability of encapsulating all unique genetic variation and adding new unrelated founders to the managed population, thus maximizing the long-term viability of both species. The strategy of avoiding pairings of individuals from the same nest can be used in conjunction with the kinship and network data presented here to maximize the amount of genetic variation

within managed individuals relative to the available pool of diversity. Thus, managers can use genomic data to guide future breeding efforts (Galla et al. 2020), and these data may enable capturing a higher proportion of genetic variation than strategies not informed by genomics. The high proportion of genetic diversity both within and among individuals highlights the need to protect as many wild individuals as possible (Muya et al. 2011).

The range of both ʻakekeʻe and ʻakikiki has been drastically restricted in recent years, due to the combined forces of introduced predators, habitat disturbance from humans (Behnke et al. 2016), and the arrival of introduced mosquitoes (Glad and Crampton 2015) and avian malaria (Atkinson et al. 2014) to high elevation forests as a result of climate change and the introduction of cold-adapted mosquitoes (Fonseca et al. 2006). With this range contraction, ongoing loss of genetic variation is expected in the absence of intervention (Frankham et al. 2002). In line with this prediction, we observed evidence of bottlenecks in both species, including heterozygosity excess and strongly positive Tajima's D. Conservation actions should aim to protect and restore the wild populations in the way that best eliminates the current threats, which may include selecting existing or novel reintroduction and translocation destination sites (Fortini et al. 2017) that have high quality forest habitat and low abundance of mosquitoes. Large-scale mosquito and predator control efforts should be considered (Liao et al. 2017), as reintroduction programs cannot succeed until the original threats are eliminated. Because these species each exist as single functional populations, they are vulnerable to stochastic extinction (Griffen and Drake 2008); thus, intervention measures such as establishing novel sites (e.g., Warren et al. 2019) on higher elevation islands, such as Maui or Hawaiʻi, may be warranted as a last resort to prevent extinction (Fricker et al. in press). Finally, continuing ongoing efforts to prioritize pairings of managed individuals from different nests and the least-related individuals (Fig. 2) will help to maintain maximum within-individual variation and reduce the risk of inbreeding depression. The extremely small effective population sizes (< 20 birds) in both species reveal their vulnerability to mutational meltdown (Lynch et al. 1995; Bank et al. 2016) and underscore the importance of ongoing management to preserve existing genomic variation and to prevent these forest bird species from going extinct.

Population bottlenecks caused by species introductions, climate change, and habitat modification can lead to diversity loss among populations due to genetic drift, which can erode adaptive variation—including alleles that may confer adaptation to these very selection pressures (Cassin-Sackett et al. 2019a). The high heterozygosity observed in ʻakekeʻe and ʻakikiki likely has both biological and technical origins. For instance, the combination of ascertainment bias (selecting only variable sites) and linkage disequilibrium resulting from bottlenecks results in high average heterozygosity. In addition, these species appear to avoid inbreeding (consistent with observations in many other species, e.g., Clutton-Brock 1989; Brouwer et al. 2011), which may slow the loss of genetic diversity within individuals. Despite the high levels of measured diversity, a global loss of allelic variation is likely inevitable in populations that have experienced severe bottlenecks. Nonetheless, the high heterozygosity within ʻakekeʻe and ʻakikiki suggests that inbreeding depression and homozygosity at lethal alleles are not imminent threats to these species; more pressing concerns are introduced avian malaria, introduced predators, and the possibility of environmental catastrophes (e.g., hurricanes). Thus, unless specific alleles conferring enhanced survival from malaria can be identified, the best breeding strategy is likely to continue to pair the least-related individuals.

Under novel selection regimes, such as those imposed by introduced species, native species may be pushed to the brink of extinction (Fortini et al. 2015). Other species on Kauaʻi, such as the ʻanianiau (*Magumma parva*), Kauaʻi ʻamakihi (*Chlorodrepanis stejnegeri*), ʻapapane (*Himatione sanguinea*) and iʻiwi (*Drepanis coccinea*), have also experienced declines (Paxton et al., 2016) as the temperature warms and mosquito-free refugia are lost (Fortini et al. 2015). The changes on Kauaʻi hint at future similar scenarios on other Hawaiian Islands (Liao et al. 2015) if large-scale integrative conservation efforts to reduce malaria transmission are not undertaken (Liao et al. 2017).

Islands contain some of the world's most imperiled species, which often face heightened pressure from introduced species and human-induced environmental change. In addition, their populations are often small owing to small geographic distributions, and thus are subject to elevated demographic stochasticity. Nonetheless, many of these at-risk species may demonstrate genetic resilience that can be leveraged in conservation. Island species can serve as models for species around the globe whose habitats are becoming increasingly fragmented, causing their populations to operate as functional islands.

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Author contributions This study was conceived and designed by LHC, BM, EHP, RCF, LCS and MGC. Material preparation, data collection, and data analysis were performed by LCS, MGC, NRM, HCL, NASP, BM, RTC, EHP, JTF, LHC and RCF. The first draft of the manuscript was written by LCS with MGC and RCF, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials Sequence reads have been published on GenBank as individual fastq files for each individual under BioProject PRJNA527134.

Code availability All software is publicly and freely available, and all custom scripts are published on GitHub.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Consent to participate All authors participated in the project and have seen this manuscript.

Consent for publication All authors consent to publication of this manuscript.

Ethical approval Blood sample collection protocols from individuals in the conservation breeding population were approved by San Diego Zoo Wildlife Alliance IACUC 18-022.

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