Received: 5 August 2019 | Revised: 14 October 2019 | Accepted: 15 October 2019

DOI: 10.1111/mec.15286

ORIGINAL ARTICLE

MOLECULAR ECOLOGY WILEY

Persistence of an endangered native duck, feral mallards, and multiple hybrid swarms across the main Hawaiian Islands

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Funding information
U.S. Geological Survey; Selma Herr Endowment; Oregon State University; Dennis G. Raveling Endowment; U.S. Fish and Wildlife Service; Hawai`i Department of Land and Natural Resources; NIH S10 Instrumentation, Grant/Award Number: S10RR029668 and S10RR027303

Abstract

Interspecific hybridization is recognized as an important process in the evolutionary dynamics of both speciation and the reversal of speciation. However, our understanding of the spatial and temporal patterns of hybridization that erode versus promote species boundaries is incomplete. The endangered, endemic koloa maoli (or Hawaiian duck, *Anas wyvilliana*) is thought to be threatened with genetic extinction through ongoing hybridization with an introduced congener, the feral mallard (*A. platyrhynchos*). We investigated spatial and temporal variation in hybrid prevalence in populations throughout the main Hawaiian Islands, using genomic data to characterize population structure of koloa, quantify the extent of hybridization, and compare hybrid proportions over time. To accomplish this, we genotyped 3,308 double-digest restriction-site-associated DNA (ddRAD) loci in 425 putative koloa, mallards, and hybrids from populations across the main Hawaiian Islands. We found that despite a population decline in the last century, koloa genetic diversity is high. There were few hybrids on the island of Kaua'i, home to the largest population of koloa. By contrast, we report that sampled populations outside of Kaua'i can now be characterized as hybrid swarms, in that all individuals sampled were of mixed koloa × mallard ancestry. Further, there is some evidence that these swarms are stable over time. These findings demonstrate spatial variation in the extent and consequences of interspecific hybridization, and highlight how islands or island-like systems with small population sizes may be especially prone to genetic extinction when met with a congener that is not reproductively isolated.

KEYWORDS
conservation genetics, evolution, genetic extinction, Hawaiian duck, hybridization, island biogeography

Wells and Lavretsky contributed equally to this work.

1 | INTRODUCTION

Interspecific hybridization can play a role in both the generation and erosion of biodiversity (Green et al., 2010; Kearns et al., 2018; Mallet, 2005, 2007; Pennisi, 2016; Seehausen, 2006; vonHoldt, Kays, Pollinger, & Wayne, 2016). Studying the natural overlap of geographic ranges has yielded substantial insight into the hybridization-selection dynamics of stable hybrid zones (e.g., Baiz, Tucker, & Cortés-Ortiz, 2019; Barton & Hewitt, 1985; Brelsford & Irwin, 2009; Nürnberger, Barton, MacCallum, Gilchrist, & Appleby, 1995). Increasingly, however, human activity is introducing non-native species into new areas, where they come into contact with native species (Seebens et al., 2017). When formerly allopatric species are brought back into contact, the backcrossing of fertile hybrids can lead to the rapid invasion of genes from the introduced species into the genome of the native species (Fitzpatrick et al., 2010; Rhymer & Simberloff, 1996). At one extreme, widespread hybridization and subsequent introgression may produce a hybrid swarm in which all individuals are genetically admixed (Allendorf, Leary, Spruell, & Wenburg, 2001; Rhymer & Simberloff, 1996), resulting in the effective genetic extinction of one or both species. The formation of hybrid swarms may be more likely in species with restricted ranges and small population sizes where gene flow has a disproportionally larger effect, compared to species with larger ranges and population sizes (Levin, Francisco-Ortega, & Jansen, 1996; Randler, 2004), although few examples have been well documented.

Among birds, ducks and geese (Family *Anatidae*) exhibit some of the highest rates of natural hybridization (Grant & Grant, 1992; Scherer & Hilsberg, 1982), with hybrids reported between many interspecific, and even some intergeneric, crosses (Johnsgard, 1960; Ottenburghs et al., 2017; Ottenburghs, Ydenberg, Van Hooft, Van Wieren, & Prins, 2015). There has been particular interest in the dynamics and consequences of hybridization among members of the "mallard complex" (Rhymer, 2006) which comprises the widely distributed mallard (*Anas platyrhynchos*) and 12 other closely related, mallard-like species found around the world (Clements et al., 2018; Lavretsky, McCracken, & Peters, 2014). Although mallards are widely distributed across both Eurasia and North America, range expansion of *A. platyrhynchos* due to natural invasion, as well as the establishment of feral populations from the escape or intentional release of domesticated breeds, has brought mallards into secondary contact with other *Anas* species, where they readily hybridize (Champagnon et al., 2013; Guay & Tracey, 2009; Lavretsky, Hernández Baños, & Peters, 2014; USFWS, 2013). For example, the release of game-farm mallards – mallards reared in captivity but released for hunting stock into natural areas – on several tropical and temperate island systems is thought to have had significant genetic impacts on native species (e.g., Pacific black duck, *A. superciliosa*; Meller's duck, *A. melleri*; and the Hawaiian duck or koloa maoli, *A. wyvilliana*; Rhymer & Simberloff, 1996).

The endangered koloa is the only endemic duck remaining on the main Hawaiian Islands and is threatened with genetic extinction through ongoing hybridization with feral mallards (USFWS, 2012). Game-farm mallards were first imported to the Hawaiian Islands for food and hunting beginning in the 1800s (Engilis, Pyle, & David, 2004; Pyle & Pyle, 2017). Later, mallards were commercially farmed on Oʻahu during the 1930s and 1940s, and multiple feral populations became established on Kauaʻi, Oʻahu, Maui, and Hawaiʻi (Engilis & Pratt, 1993). Historically, koloa occurred on the main Hawaiian Islands of Kauaʻi, Niʻihau, Oʻahu, Maui, Molokaʻi, and Hawaiʻi, but were extirpated from all islands except Kauaʻi and Niʻihau by the 1960s (Engilis, Uyehara, & Giffin, 2002). Subsequently, koloa were captive-reared and reintroduced onto Oʻahu and Hawaiʻi until the late-1980s, when the release programme was halted due to funding cuts; remaining captive birds were released onto Maui in 1989. Earlier molecular work confirmed that hybridization between koloa and mallards was occurring on Oʻahu (Browne, Griffin, Chang, Hubley, & Martin, 1993), with subsequent work documenting that Oʻahu hybrids had predominantly mallard ancestry (Fowler, Eadie, & Engilis, 2009). Although feral mallards have been present in the main Hawaiian Islands for over 100 years, biannual waterbird surveys reporting more birds with intermediate koloa-mallard plumage suggest that the number of koloa-mallard hybrids has been recently increasing (USFWS, 2012). Additionally, there is some uncertainty about hybridization involving wild mallards which irregularly visit the Islands during the winter and could remain to breed (Paton, Taylor, & Ashman, 1984). Currently, both the source (i.e., from feral and/or wild mallards) and the extent of hybridization on the Hawaiian Islands remain poorly understood.

In this study, we genotyped ducks collected from 1998 to 2015 at sites on Kauaʻi, Oʻahu, Molokaʻi, Maui, and Hawaiʻi to pursue three objectives: (a) Evaluate koloa molecular diversity (b) characterize population structure of koloa, mallards, and putative hybrids across the main Hawaiian Islands, and (c) determine if hybrid prevalence has changed over the past decade. Our first objective posits that the koloa may have low genetic diversity as a consequence of its population decline in the early-mid 1900s (Banko, 1987) and this may pose a challenge for future conservation efforts. To compare koloa diversity to another Hawaiian-island endemic, its endangered relative the Laysan duck (*A. laysanensis*), we also report genome-wide data for the Laysan duck, which has previously been shown to have little genetic variation (Reynolds, Pearce, Lavretsky, Seixas, & Courtot, 2015) due to a severe bottleneck in the last century (Moulton & Marshall, 1996). Our second objective tests the hypothesis that koloa populations are genetically similar across islands, a potential consequence of Kauaʻi being the source for all reintroductions on the other islands (Engilis et al., 2002). Alternatively, given genetic drift in small populations coupled with restricted interisland gene flow, particularly between Kauaʻi/Niʻihau and other islands C.P. Malachowski, B.D. Dugger, K.J. Uyehara & M.H. Reynolds; unpublished data (currently in prep). koloa populations on different islands may be genetically differentiated. This objective also tests the hypothesis that koloa × mallard hybridization is predominantly with feral, as opposed to wild, mallards. Our third objective tests for temporal changes in the occurrence of hybrids; given that count data from state waterbird surveys suggest an increasing prevalence of mallard-like koloa

on multiple islands (USFWS, 2012), we predicted that our genetic data would reflect an increase in hybrid prevalence over the survey period.

2 | MATERIALS AND METHODS

2.1 | Sampling, DNA extraction, and collection and processing of ddRAD-seq data

Blood or tissue was obtained from a total of 141 individuals from 1998 to 2009, including a single individual believed to be a wild, migratory mallard over-wintering on Kaua'i, and 284 individuals from 2011 to 2015 across the islands of Hawai'i, Maui, Moloka'i, O'ahu, and Kaua'i (Table S1). The current population size of koloa on Kaua'i is unknown, but in the past was estimated at N = 2,000 (Engilis et al., 2002); if this is correct, our sample size represents ~8.5% of the total population. In addition, we collected genetic data for 30 Laysan ducks and 30 wild North American mallards.

Blood was taken during banding efforts, during which birds were captured using baited swim-in traps, walk-in traps, mist nets, or net guns. Additionally, birds that were sick or had died of various causes (e.g., botulism, vehicle collision, predation) were sampled opportunistically. Tissues were also sampled from specimens in the Museum of Wildlife and Fish Biology at the University of California, Davis. Phenotypic (e.g., plumage coloration) and morphometric data (e.g., body mass, wing arc) were also obtained for sampled individuals at time of banding or collection. DNA extraction was performed using the DNeasy Blood and Tissue Kit (Qiagen), according to manufacturer's instructions. Extractions were quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific Inc.) to ensure a minimum concentration of 0.02 µg/µl. Samples were subsequently digested with two restriction-enzymes and fragment libraries were prepared for multiplexing and high-throughput sequencing following steps outlined in DaCosta and Sorenson (2014). Barcoded libraries were pooled in equimolar concentrations, and 100 or 150 bp, single-end sequencing was completed on an Illumina HiSeq 2500 at the UC Berkeley Genomics Sequencing Laboratory and the Tufts University Core Genomics Facility. Prior to analyses all 150 bp reads were cut to 100 bp. Raw Illumina reads have been deposited in NCBI's Sequence Read Archive (SRA; http://www.ncbi.nlm.nih.gov/sra; SRA BioProject PRJNA57758, SAMN13029772 - SAMN13030226).

Processing of raw Illumina reads was performed using the computational pipeline described by DaCosta and Sorenson (2014; Python scripts available at http://github.com/BU-RAD-seq/ddRAD-seq-Pipeline). Briefly, demultiplexed and quality-filtered reads were clustered into putative loci based on sequence similarity and genomic position as determined by BLAST. Reads were then aligned within each putative locus, and the alleles (or haplotypes) for each individual at each locus were called (see DaCosta & Sorenson, 2014 for additional details). Because of some variation in the quality of extracts and sequencing results, we focused on a subset of 375 higher quality samples for the purpose of selecting loci for analysis. Using these samples, we excluded loci with median per sample sequencing depth ≤15 and >10% missing data. We further excluded putative loci showing potential evidence of paralogous loci being clustered together, including extreme departures from Hardy-Weinberg expectations (e.g., all individuals heterozygous with the same two alleles), ≥2% of individual samples being "flagged" either for departure from the 50:50 read depth expectation for two alleles in heterozygotes or for evidence of >2 alleles per individual (see DaCosta & Sorenson, 2014), and exceptionally high read depth (median ≥683; representing the extreme tail of the distribution and a level at which most putative loci also failed the above tests). Finally, we excluded loci with potentially incorrect alignments due to polymorphic indels near the end of the sequence read. After adding back 110 lower quality samples, we further excluded any loci with >20% missing data in the overall sample of n = 485. This resulted in a final data set of 3,308 loci of which 194 were inferred to be on the Z-chromosome based on BLAST results and differences in sequencing depth and homozygosity between males and females (Figure S1; also see Lavretsky, Dacosta, et al., 2015). In birds, females are the heterogametic sex (i.e., ZW), and thus Z-linked loci should appear to be homozygous in females and, empirically, are recovered at about half the average sequencing depth as the same loci in males.

2.2 | Mitochondrial DNA

We used primers L78 and H774 to amplify and sequence ~ 650 bp of the mtDNA control region (Sorenson, Ast, Dimcheff, Yuri, & Mindell, 1999; Sorenson & Fleischer, 1996) for a subset of samples (n = 266), following PCR and Sanger sequencing methods described in Lavretsky, McCracken, et al. (2014). Final products were sequenced on an ABI 3,730 at the Yale University DNA Analysis Facility. Sequences were aligned and edited using Sequencher v. 4.8 (Gene Codes, Inc). All sequences have been submitted to GenBank (accession numbers MN563303-MN563571 and MN603671–MN603691). In addition to new sequences, 40 previously published sequences were also used (accession numbers EU399761–EU399785, Fowler et al., 2009; KF608499 & KF608500, Lavretsky et al. 2014; KF857646 & KF857649, Peters et al. 2014; KP856505–KP856508, Lavretsky, Engilis, Eadie, & Peters 2015; and MK425362–MK425493, Lavretsky, Janzen, & McKracken 2019; KF608512–KF608513, KP856504–KP56513, Lavretsky, Engilis, Eadie, & Peters, 2015). Sequences were cut to the 303 bp present in all samples, which included the hypervariable region.

Koloa mtDNA comprises a monophyletic lineage nested within the New World (NW) "B" haplo-group (Fowler et al., 2009), which is divergent from the Old World (OW) "A" haplo-group (Avise, Ankney, & Nelson, 1990; Lavretsky, Hernández Baños, et al., 2014), and from the Laysan duck mtDNA group (Lavretsky, Dacosta, et al., 2015; Lavretsky, Hernández Baños, et al., 2014). In general, Eurasian mallards possess OW A haplotypes, as do domesticated mallard breeds and game farm mallards, which are thought to be derived from Eurasian stock (Avise et al., 1990; Hou et al., 2012; Johnson

& Sorenson, 1999; Kulikova et al., 2005; Kulikova, Zhuravlev, & McCracken, 2004; Lavretsky, McCracken, et al., 2014). Thus, the occurrence of OW A mtDNA haplotypes in the Hawaiian Islands would most likely indicate OW ancestry from domesticated or feral mallards. We assigned the mtDNA sequence of each sample to either the OW (A) or NW (B) haplo-group, and evaluated proportions of the two haplo-groups present on each island.

2.3 | Population structure and genetic diversity

For analyses of population structure, we extracted biallelic polymorphisms including both SNPs and unique indels (i.e., each multibase indel treated as one polymorphism) from the 3,114 autosomal RAD-seq loci, 2,958 of which had one or more polymorphisms in the overall sample. For both STRUCTURE and PCA analyses, we limited the data set to polymorphisms with minor allele frequency >0.01 (see Linck & Battey, 2019); 8,492/17,391 SNPs and 474/969 indels met this minimum frequency threshold. We ran STRUCTURE v. 2.3.4 (Falush, Stephens, & Pritchard, 2003) using the autosomal data set for all samples, implementing the admixture model and assuming correlated allele frequencies among populations. We ran nine replicates each for each value of *K* (= number of populations) from one to nine, with 10,000 burnin iterations followed by 20,000 sampling iterations. We used the Evanno, Regnaut, and Goudet (2005) method in StructureHarvester (Earl & VonHoldt, 2012) to determine the number of populations that best represented the data, but also examined the results for a larger number of assumed populations because the Evanno method may be too conservative (Janes et al., 2017). Given highly consistent results among replicate runs, we used results from the first replicate for $K = 3$ and $K = 4$, respectively, for plotting and summarizing the results. To test for a change in the prevalence of hybrid ancestry between the 2000s and the 2010s, we used *t* tests to compare the ancestry coefficients of individual koloa between time periods.

As an additional means of visualizing population structure, we analyzed a comparable data set using principal components analysis (PCA). Laysan ducks, which were clearly distinct from all other populations, were excluded from the PCA, resulting in a set of 8,419 biallelic SNPs and 469 biallelic indels meeting the >0.01 frequency threshold. We scored each genotype as having 0, one or two copies of the reference allele following the approach of Novembre and Stephens (2008). For missing genotypes (~3.2% of the data matrix), individuals were assigned a value equal to two times the population-specific allele frequency. Results were nearly identical when replacing missing data with global allele frequencies.

We also analyzed the full set of 2,958 variable autosomal loci in fineRADstructure (Malinsky, Trucchi, Lawson, & Falush, 2018), which emphasizes patterns of recent shared ancestry by leveraging the information available in the linkage of SNPs within each RAD-seq locus. More specifically, each individual's ancestry at a given locus is allocated to other individuals that carry an identical haplotype (or nearest neighbour haplotype if the focal individual has a unique allele). Rare alleles, which are on average of more recent origin and which are defined by the rare SNPs excluded from the above analyses, make the greatest contribution to the resulting pairwise coancestry coefficients. Thus, this analysis emphasizes different information in the data than STRUCTURE or PCA.

Finally, we calculated composite pairwise estimates of relative divergence (Φ_{ST}), nucleotide diversity (π), and Watterson's θ for mtDNA, autosomal and Z-linked ddRAD-seq loci, respectively, in the R package PopGenome (Pfeifer, Wittelsbürger, Ramos-Onsins, & Lercher, 2014) using concatenated data sets for each data type and with indel positions treated as missing. After exclusion of sites with indels, these three data sets comprised 300; 278,034; and 17,407 alignment positions, respectively.

3 | RESULTS

After filtering for quality, our final ddRAD-seq data set included 3,114 autosomal and 194 Z-linked loci (Figure S1). Median sequencing depth for these loci was 73 reads per locus per individual, and 98.2% and 85.2% of individual genotypes were scored unambiguously for the 375 higher quality and 110 lower quality samples, respectively. For an additional 1.2% and 10.3% of genotypes for the two groups of samples, respectively, we scored one allele and treated the other as missing when sequencing depth was low (<5 reads) or when a second allele accounted for <29% of reads for a given sample and locus (DaCosta & Sorenson, 2014). Careful examination of results for each of the analyses indicated no systematic effects of missing data on inferences for lower quality samples; for example, PCA scores for lower quality samples were similar to those of higher quality samples from the same populations.

3.1 | Genetic diversity

All data sets indicate that Laysan duck is well-differentiated from the other populations we sampled (Table 1). Relative differentiation (Φ_{ST}) was high in all comparisons involving Laysan duck, but with the smallest values observed in comparisons with the Kaua'i population of koloa: 0.41 and 0.38 for autosomal and Z-linked loci, respectively (Table 1). The population from Kaua'i and a small sample of birds from Moloka'i ($n = 6$) were relatively divergent from each other and from both mallards and the other Hawaiian populations, whereas Φ_{ST} values were generally much lower for comparisons among North American mallards and the samples from Hawai'i, Maui and O'ahu (Table 1), all of which had relatively greater genetic diversity (Figure 1). Estimates of autosomal and Z-linked differentiation were strongly correlated and the ratio of Φ_{ST} estimates for Z-linked versus autosomal loci (ranging between 0.67 and 1.57 across pairwise comparisons, Table 1) were within the expected range under a model of neutral divergence (Lavretsky, Dacosta, et al., 2015).

TABLE 1 Pairwise Φ_{ST} estimates between Laysan ducks (LADU), wild North American mallards (MALL), and the combined samples of ducks from each of five Hawaiian Islands for ddRAD autosomal loci, ddRAD Z-linked loci, and the mitochondrial control region (mtDNA)

	Autosomal	Z-Chromosome	mtDNA	Z:Aut Ratio	mt:Aut Ratio
LADU					
MALL	0.52	0.51	0.71	0.99	1.37
Hawai'i	0.48	0.44	0.73	0.91	1.52
Maui	0.51	0.48	0.80	0.94	1.55
Moloka'i	0.63	0.65	–	1.03	–
O'ahu	0.48	0.43	0.80	0.89	1.69
Kaua'i	0.41	0.38	0.95	0.92	2.31
MALL					
Hawai'i	0.06	0.08	0.08	1.31	1.26
Maui	0.07	0.06	0.14	0.86	2.02
Moloka'i	0.22	0.22	–	1.02	–
O'ahu	0.08	0.11	0.26	1.38	3.37
Kaua'i	0.15	0.24	0.28	1.57	1.88
Hawai'i					
Maui	0.06	0.04	0.00	0.67	−0.05
Moloka'i	0.20	0.20	–	0.97	–
O'ahu	0.03	0.02	0.06	0.90	2.13
Kaua'i	0.10	0.13	0.49	1.36	5.13
Maui					
Moloka'i	0.16	0.15	–	0.90	–
O'ahu	0.06	0.05	0.04	0.82	0.62
Kaua'i	0.13	0.18	0.60	1.34	4.49
Moloka'i					
O'ahu	0.21	0.18	–	0.87	–
Kaua'i	0.25	0.31	–	1.25	–
O'ahu					
Kaua'i	0.09	0.09	0.68	0.94	7.43

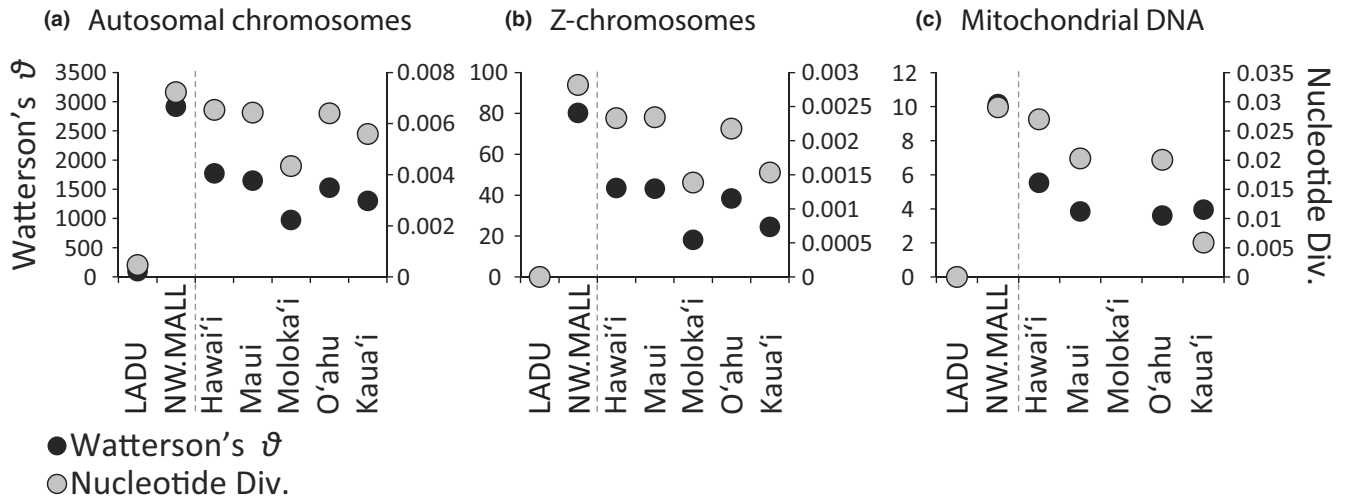

FIGURE 1 Estimates of nucleotide diversity and Watterson's θ across 3,114 ddRAD autosomal markers, 194 Z-chromosome-linked markers, and the mitochondrial control region. Hawaii-sampled ducks are grouped by island. No mtDNA was sequenced from Moloka'i ducks

As expected, genetic diversity was minimal in Laysan duck; autosomal estimates of Watterson's θ and nucleotide diversity (π) were just 3% and 6%, respectively, of comparable estimates for wild mallards, and 7%–8% of comparable estimates for koloa. In fact, for Laysan ducks, we detected no variation in any Z-linked loci or in mitochondrial DNA (Figure 1). Koloa and wild mallards were more

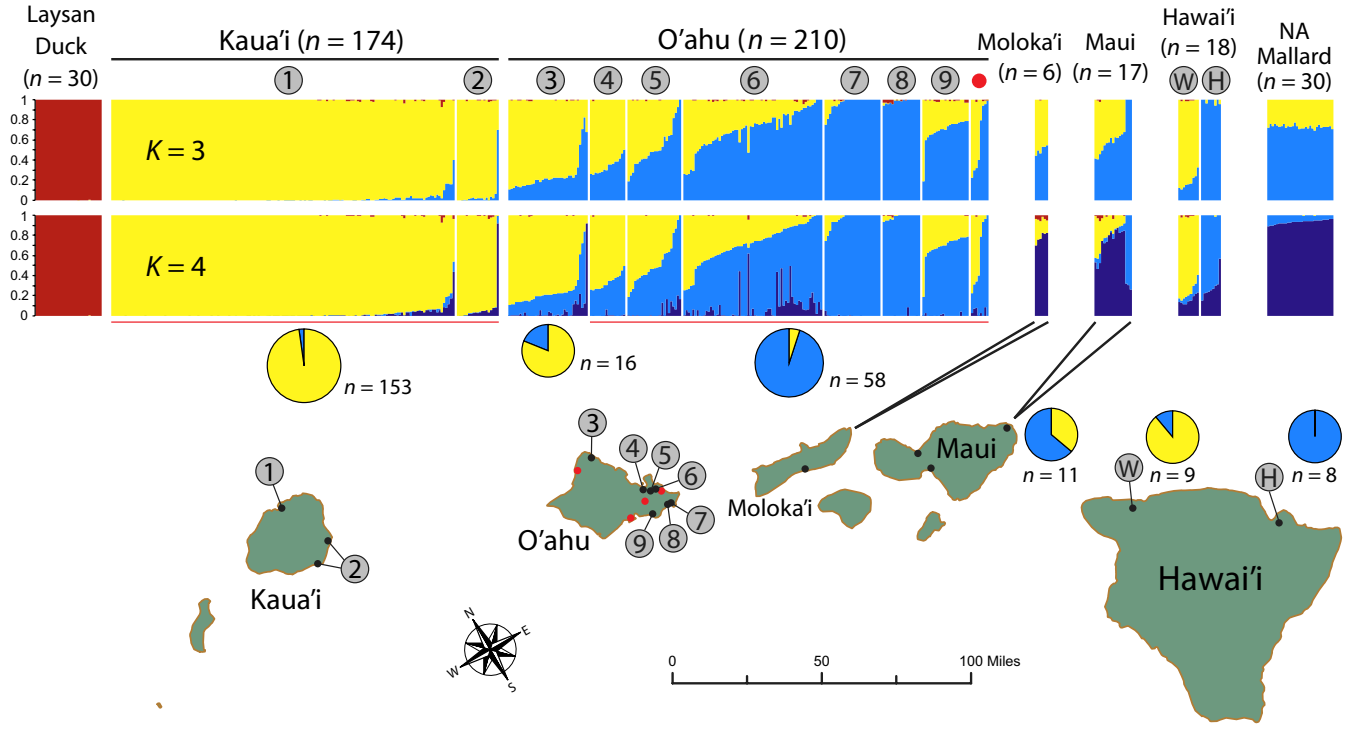

FIGURE 2 Results from STRUCTURE along with map of sample locations within the Hawaiian Islands (also see Table S1; $n = 425$) and summaries of mtDNA haplotype data. Individual ancestry proportions from STRUCTURE are shown for analyses assuming both $K = 3$ and $K = 4$ populations, including Laysan ducks and North American mallards. Within each island or sampling location (in the case of Kaua'i, O'ahu and Hawai'i), individuals are sorted from greater to lesser Hawaiian duck ancestry to facilitate visualization of differences in admixture proportions among locations. Pie charts represent the proportion of samples with the putative New World "B" mtDNA lineage (yellow) and the putative Old World "A" lineage (blue) for a subset of ducks on each island [Colour figure can be viewed at wileyonlinelibrary.com]

similar in estimates of genetic variation, with koloa populations averaging approximately three-quarters the diversity of wild mallards.

3.2 | Population structure and individual ancestry

Analysis of STRUCTURE results using the Evanno method as implemented in STRUCTURE HARVESTER (Earl & VonHoldt, 2012; Evanno et al., 2005) indicated $K = 3$ as the number of distinct populations represented in the data (Figure S2), but additional structure was clearly evident and interpretable at $K = 4$ (Figure 2). At $K = 3$, we interpreted the three genetic clusters as corresponding to Laysan duck, koloa, and mallard, with phenotypically-mallard ducks from Paikō Lagoon and Hawai'i Kai on O'ahu (locations 7 and 8 in Figure 2) showing nearly 100% mallard ancestry. Also at $K = 3$, wild mallards from North America show mixed ancestry between the putative mallard and koloa clusters, a result we interpret as a signature of relatively ancient mallard ancestry within koloa (Lavretsky, Engilis, et al., 2015). Results for $K = 4$ were broadly similar except that the "mallard" cluster is split in two, with one cluster corresponding to wild North American mallards, which no longer show any evidence of koloa ancestry. As exemplars of the other mallard cluster, the birds from Paikō Lagoon and Hawai'i Kai exhibited traits consistent with domestic mallards such as mallard plumage coloration and large body size (average mass of adult males = 1,024 g, compared to 694 g for Kaua'i koloa adult males), and were genetically distinct from North American mallards. Thus, we interpret this cluster as representing domesticated and/or the feral mallard lineages, which are thought to have a primarily Eurasian origin (Hou et al., 2012). Moreover, we find the $K = 4$ results to be both biologically meaningful and more informative than $K = 3$, providing insight into the sources of mallard introgression (feral vs. wild) in different populations across the island chain.

Most samples from Kaua'i appear to be pure or nearly pure koloa, with 117 of 174 samples (67%) having >99% koloa ancestry, and another 31 samples (18%) having >95% ancestry in the $K = 4$ STRUCTURE analysis. Among the remaining Kaua'i samples, one is the suspected overwintering mallard from Hulē'ia NWR, which is confirmed as a North American wild mallard in all our analyses (92% mallard ancestry in the $K = 4$ analysis), and another appears to have approximately 50:50 koloa and mallard ancestry, as expected for an F1 hybrid. Excluding these two samples, mallard ancestry ranges from zero to 22.2% among the Kaua'i samples and averages just 1.6%. Moreover, mallard ancestry on Kaua'i appears to derive primarily from wild mallards rather than domesticated/feral mallards (Figure 2). The predominance on Kaua'i of the New World mitochondrial "B" lineage (Figure 2) is also consistent with a lack of female-mediated introgression from feral or domesticated mallards, which generally carry the Old World "A" lineage (Hou et al., 2012).

In contrast to Kaua'i, all ducks from the remaining islands show evidence of variable levels of mixed ancestry, with feral and/or wild

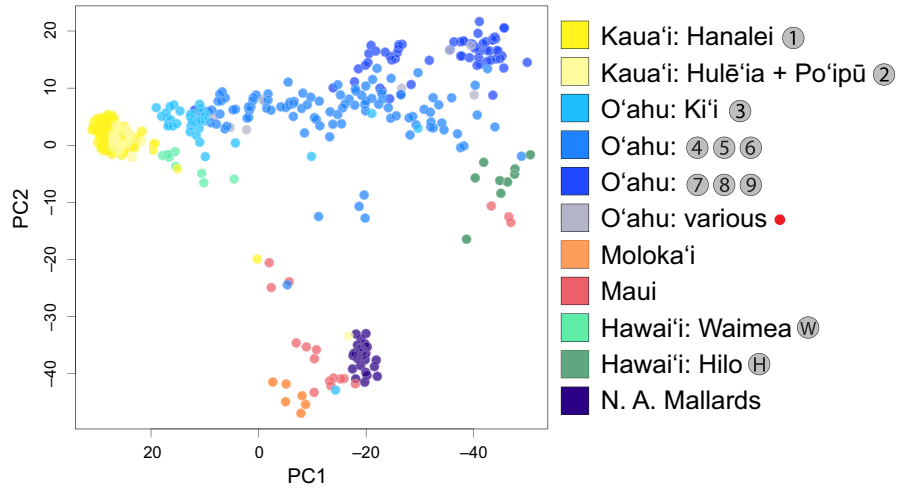

FIGURE 3 Principal component analysis of genetic diversity excluding Laysan ducks, which were clearly divergent from all other populations in all analyses (e.g., Table 1). Analysis is based on a set of 8,419 biallelic SNPs and 469 biallelic indels meeting the >0.01 minor allele frequency threshold (extracted from 2,515 autosomal ddRAD-seq loci with qualifying polymorphisms). Each dot represents one individual, colour-coded by sampling location (see legend with numbered locations corresponding to Figure 2). Results for the first two principal components are shown. The third axis further separates different populations on Oʻahu, whereas the fourth axis separates mallards and birds from Maui and Molokaʻi, respectively, into three distinct clusters [Colour figure can be viewed at wileyonlinelibrary.com]

mallard ancestry at 10% or more in all samples. Populations across Oʻahu varied substantially in koloa ancestry, with averages ranging from a high of 74% in the Kiʻi Unit of James Campbell NWR in northern Oʻahu (location 3 in Figure 2) to nearly zero in southeastern Oʻahu (locations 7 and 8 in Figure 2), where the birds were phenotypically identified as mallards. Other Oʻahu populations were decidedly admixed, with 76% of samples (95 of 125) from locations 4, 5, 6 and 9 in Figure 2 ranging from 20% to 80% in koloa ancestry. Samples from Molokaʻi and Maui were similarly admixed, but with mallard ancestry apparently derived primarily from wild mallards. The birds from Waimea in the northern end of Hawaiʻi had greater koloa ancestry ($\bar{x}$ = 75%) than most other populations outside of Kauaʻi, but with appreciable wild mallard ancestry evident in all samples ($\bar{x}$ = 16%). Also in contrast to Kauaʻi, the Old World mitochondrial "A" lineage, suggesting maternal ancestry from feral or domesticated lineages, was generally most common on the other islands (Figure 2). Considering Oʻahu samples for which both ddRAD-seq and mtDNA haplotype data were available, inferred autosomal ancestry was strongly correlated with mtDNA haplotype. Oʻahu A haplotype birds showed a significantly lower proportion of koloa ancestry ($\bar{x}$ = 28%, n = 58) than B haplotype birds ($\bar{x}$ = 68%, n = 16; t = −6.23, p < .00001), and a significantly higher proportion of feral mallard ancestry (62% vs. 23%, t = 6.19, p < .0001), but no difference in the proportion of wild mallard ancestry (10% vs. 8%, t = 0.27, p = .39; all tests one-tailed). On Kauaʻi, only three of 151 birds with mtDNA data carried an A haplotype, and despite the small sample, these birds had a significantly lower proportion of koloa ancestry ($\bar{x}$ = 91%) as compared to B haplotype birds ($\bar{x}$ = 98%, t = −2.04, p = .02). Similarly, the two sampling locations on Hawaiʻi differed markedly in both autosomal and mtDNA ancestry (Figure 2).

Principal components analysis of the autosomal RAD-seq data, for which we excluded the relatively divergent Laysan duck, yielded broadly consistent results (Figure 3). Three divergent clusters correspond to wild mallards from North America, feral mallards from southeastern Oʻahu, and koloa on Kauaʻi. The remaining samples are distributed between these three clusters in a manner that is fully consistent with the STRUCTURE results, including: (a) samples from Oʻahu show a broad range of mixed ancestry spanning from feral mallards to birds that have predominantly koloa ancestry; (b) samples from the Kiʻi Unit of James Campbell NWR in northern Oʻahu and from Waimea in Hawaiʻi show the greatest similarity to koloa on Kauaʻi; and (c) birds from Molokaʻi and Maui are generally more similar to wild mallards than to the feral mallards in Oʻahu.

Analysis of the haplotypic data at each ddRAD-seq locus in fineRADstructure further illustrates the complex patterns of shared and mixed ancestry among the samples in our analysis (Figure 4). One important insight not evident in the STRUCTURE or PCA analyses above is substantially greater coancestry between Laysan ducks and koloa from Kauaʻi than between Laysan ducks and mallards, either feral or wild. Different clusters of admixed samples from Oʻahu show variable levels of coancestry with Kauaʻi koloa and Laysan ducks, respectively, but in a correlated fashion in accord with their level of koloa ancestry. In contrast, wild mallards from North America have very low coancestry with Laysan ducks, but have greater coancestry with Kauaʻi koloa than do the feral mallards on Oʻahu. These results are consistent with the hypothesis of a relatively ancient hybrid origin for koloa (Lavretsky, Engilis, et al., 2015). Clustering of several Kauaʻi birds with populations on other islands could indicate either very recent shared ancestry or the derivation of similar ancestry proportions arising from independent instances of hybridization and admixture on different islands. As movement among these islands has not been documented for banded, radio-transmitter, or satellite-transmitter

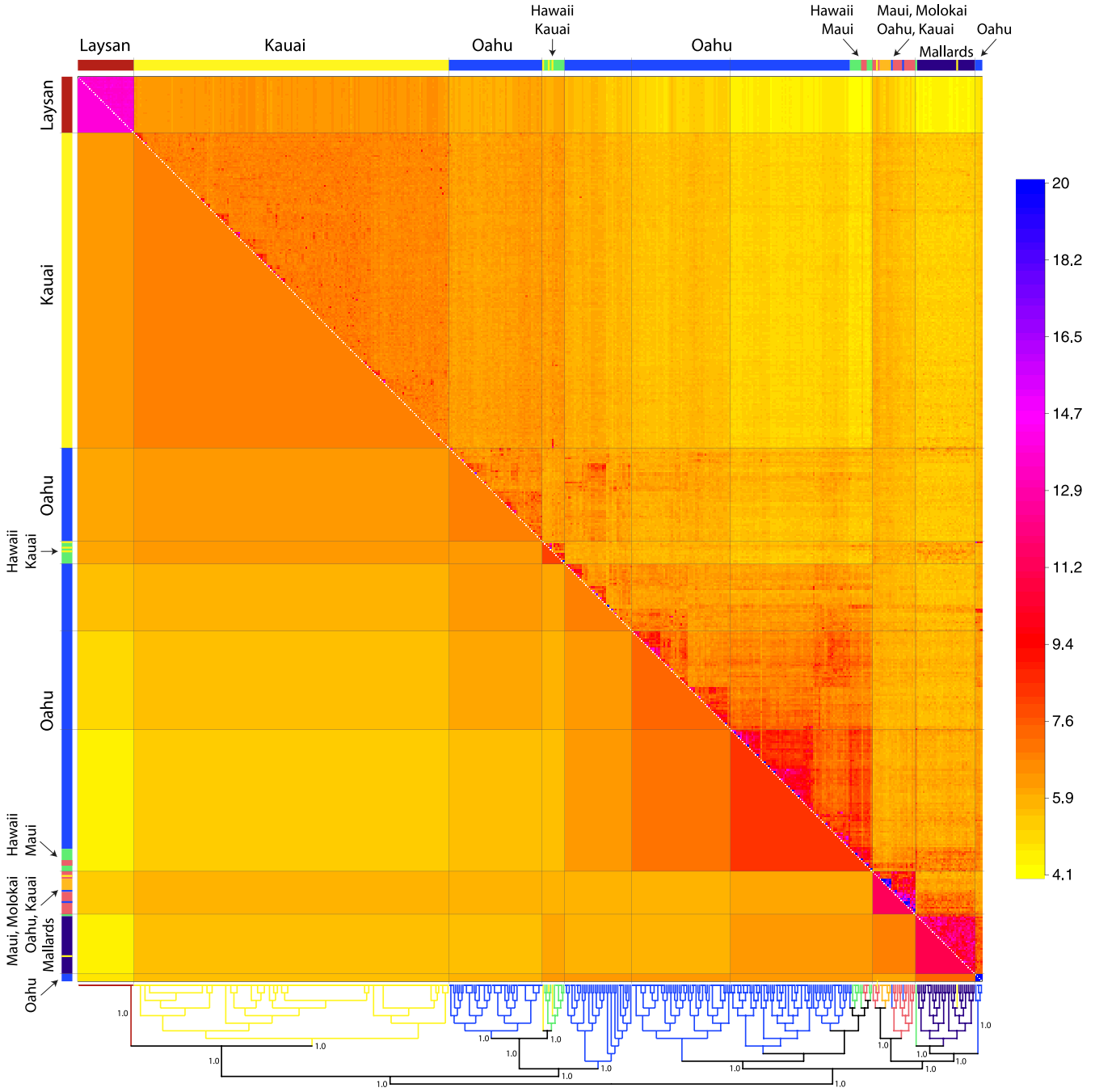

FIGURE 4 Matrix of individual (above the diagonal) and average (below the diagonal) co-ancestry coefficients along with the resulting dendrogram from fineRADstructure. Coancestry ranges from low (yellow) to high (blue) as indicated by the colour scale (note: the range of absolute values increases with the number of loci in the analysis). Individuals are ordered in the matrix on the basis of the dendrogram, which is generated from the matrix of pairwise co-ancestry coefficients. Coloured bars at top and left indicate the island or population of origin for each individual. Grey lines separate different clusters of individuals, as defined by major branches in the dendrogram (the number of which was somewhat arbitrarily determined), and average coancestry coefficients were calculated within each of these clusters [Colour figure can be viewed at wileyonlinelibrary.com]

equipped birds (C. Malachowski, unpublished data), we favour the latter explanation. Finally, within Kauaʻi, birds from Hulēʻia and Poipu clustered together along with a subset of the Hanalei birds on one branch of the fineRADstructure dendrogram, suggesting slightly elevated recent coancestry for birds from southeastern Kauaʻi but also a lack of significant population structure between these populations and the larger sample from Hanalei.

3.3 | Past versus present rates of hybridization

Four populations on three islands (Kauaʻi-Hanalei, Maui-Kanahā, Oʻahu-Kiʻi, and Oʻahu-Hāmākua) were sampled first in 1998–2007, and again in 2011–2015, allowing a comparison of hybrid prevalence and ancestry proportions over time. On Kauaʻi, hybrid individuals,

defined as having <95% koloa ancestry, were detected in both time periods. At Hanalei, there was a proportional decline in the prevalence of hybrids from the 2000s (eight of 29 samples = 28% hybrid prevalence) to the 2010s (10 of 126 samples = 8% hybrid prevalence), which coincided with a slight increase in the average proportion of koloa ancestry (from $\bar{x}$ = 0.95 to $\bar{x}$ = 0.99, t = 2.02, p = .05) (Figure S3a). Admixture was prevalent in both the O'ahu-Ki'i (n_{2000s} = 17, n_{2010s} = 19) and Maui populations (n_{2000s} = 6, n_{2010s} = 6), and neither of these populations showed a significant change in koloa ancestry over time (Figure S3c–d; Ki'i from $\bar{x}$ = 0.71 to $\bar{x}$ = 0.76, t = 0.75, p = .46; Maui from $\bar{x}$ = 0.20 to $\bar{x}$ = 0.15, t = 0.93, p = .39). Only the O'ahu-Hāmākua population (n_{2000s} = 57, n_{2010s} = 6), where there was substantial targeting and removal of male feral mallards between the two sampling periods, showed a large reduction in feral mallard ancestry in the recent samples (Figure S3b), and a significant increase in the proportion of koloa ancestry (from $\bar{x}$ = 0.25 to $\bar{x}$ = 0.53, t = 3.71, p < .001).

4 | DISCUSSION

Our study represents the most comprehensive phylogeographic study to date of the endangered koloa maoli, including assessment of the extent and sources of hybridization with mallards across islands and over time. Perhaps our most important result is the confirmation that pure koloa persist on Kaua'i in large numbers, and with relatively little evidence of hybridization during the past decade (Figure 1). Our finding of low hybrid prevalence on Kaua'i should allay concerns raised by waterbird survey data that the frequency of hybrids is increasing (USFWS, 2012). Although mallards have been present on Kaua'i (USFWS, 2012), Hanalei and Hulē'ia National Wildlife Refuges support large numbers of koloa (USFWS, 2012), where individual birds should encounter an abundance of conspecific mates. Hubbs's principal states that females should prefer to mate with a male of their own species, but in their absence mating with a male from a different species is better than not mating at all (Randler, 2002). Thus, interspecific hybridization may be more likely when conspecific mates are rare (van Dongen, Lazzoni, Winkler, Vásquez, & Estades, 2013; McCracken & Wilson, 2011; McCracken, Wilson, & Martin, 2013; Steeves, Maloney, Hale, Tylianakis, & Gemmell, 2010; White & Clausen, 2002). Additionally, banding data (n = 1,093 birds; B. Dugger and C. Malachowski, unpublished data), diurnal behavioural studies (n = 984; Malachowksi, Dugger, & Uyehara, 2019) and botulism records (n = 78; K. Uyehara, unpublished data) all indicate a male-biased sex ratio of over 3:1 at Hanalei, suggesting that breeding females are likely able to find conspecific mates (Steeves et al., 2010). Hence, the low hybrid prevalence on Kaua'i may be attributable to the relatively large and apparently male-biased population of koloa on that island.

In marked contrast, we find that all reintroduced populations on Hawai'i, Maui, and O'ahu now constitute hybrid swarms. Koloa from Kaua'i were the source for the 1960s-era captive breeding programme that supplied all reintroductions on other islands, yet it appears that no sampled individuals from O'ahu, Maui, or Hawai'i were unaffected by hybridization (Figures 2–4). Founding populations from captive breeding programmes, reintroductions, and translocations can exhibit significant shifts in allele frequencies from their source population due to genetic drift (Jamieson, 2011). Our results, however, support an alternative hypothesis in which extensive hybridization, primarily with nonwild mallards (i.e., feral and/or domesticated), explains the range of genetic variation seen across sampling sites outside of Kaua'i (Figure 2; Figure S3). We did not identify a single sample among the O'ahu, Maui and Hawai'i sites (n = 245 samples) with ≥95% Kaua'i koloa ancestry (Figure 1). Instead, there was clear evidence of variable levels of admixture between koloa and mallards across both sampling locations and individual birds. Relatively high proportions of Kaua'i koloa ancestry were found in the Waimea (average 75%) and Ki'i (average 74%) locations on Hawai'i and O'ahu, respectively. However, all non-Kaua'i samples were estimated to have ≥10% mallard ancestry. The wide and continuous spread of the O'ahu samples in the PCA analysis (Figure 3) reflects the highly variable genomic composition of individual birds and supports our characterization of the birds on O'ahu as a hybrid swarm. Finally, we found that a substantial fraction of individual birds in most locations outside of Kaua'i had mtDNA from the putative Old World "A" lineage (Figure 2), whereas almost all birds from Kaua'i had koloa-specific mtDNA haplotypes falling within the New World "B" lineage (Fowler et al., 2009; Lavretsky, Engilis, et al., 2015; Lavretsky, McCracken, et al., 2014). Notably, the "B" lineage was also common at Waimea on Hawai'i and Ki'i on O'ahu, where koloa ancestry was relatively high. These results strongly support the inference that hybridization has primarily involved feral and/or domesticated mallard lineages, which are believed to have an Old World origin (Avise et al., 1990; Hou et al., 2012; Johnson & Sorenson, 1999).

4.1 | Genetics of mallards in the Hawaiian Islands

There is a long history of introducing farmed mallards to the Hawaiian Islands for food and hunting, with the earliest records dating to the 1800s (Engilis et al., 2004; Pyle & Pyle, 2017). Later, mallards were farmed on O'ahu during the 1930s and 1940s, and multiple feral populations became established on Kaua'i, O'ahu, Maui, and Hawai'i (Engilis & Pratt, 1993). This history of mallard presence, combined with a high probability of interaction with native ducks in the state's limited wetland habitats (USFWS, 2012; Uyehara, Engilis, & Dugger, 2008), created conditions in which introgression into the congeneric koloa was highly probable. Although our dataset does not include known game-farm mallards, several lines of evidence indicate that admixture in the Hawaiian Islands is largely with feral mallards. First, current O'ahu populations that include phenotypically mallard-like ducks appear to be genetically differentiated both from wild North American mallards and Kaua'i koloa, clustering separately in both STRUCTURE and PCA

analyses (Figures 2 and 3). This pattern of genetic structure is consistent with Fowler et al. (2009), who found that mallards in Hawai'i were genetically distinct from California mallards. Likewise, the fineRADstructure analysis indicates that the O'ahu ducks least like Kaua'i koloa also have relatively low co-ancestry with wild mallards from North America. Thus, using a threshold of > 95% ancestry in STRUCTURE, 34 (16%) of the 210 samples from O'ahu represent feral mallards, and another 89 (42%) have >50% feral mallard ancestry (Figure 2). Further, similar ancestry profiles and close clustering of samples from Paikō (O'ahu), Hāna (Maui), and Hilo (Hawai'i) (Figures 3 and 4) suggests a similar source for feral mallards across the Hawaiian Islands. The finding that ducks with the mitochondrial "A" lineage typical of domestic mallard breeds also had higher proportions of feral mallard autosomal ancestry suggests that interbreeding between koloa and feral mallards is relatively recent. This is consistent with koloa encountering feral mallards during reintroduction efforts in the 1960s–1980s, and hybridizing soon after (Engilis & Pratt, 1993).

Notably, only a single duck sampled on Kaua'i had ancestry proportions consistent with and consistently clustered with wild mallards from North America (Figures 2–4; Figure S3). This individual confirms that wild mallards continue to arrive in the Hawaiian Islands, as do several other species of migratory waterfowl (Engilis et al., 2004; Pyle & Pyle, 2017; Richardson & Bowles, 1964), and hence could potentially interbreed with local populations. Indeed, ducks from Kaua'i with mallard ancestry had greater co-ancestry with wild than feral mallards (Figures 2 and 4). These samples included an apparent F1 hybrid with ~50:50 ancestry as well as a number of individuals with smaller proportions of mallard ancestry, as expected after multiple generations of backcrossing. Samples from Maui and Moloka'i – the only other Hawaiian samples with substantial wild mallard ancestry (Figure 1) – may represent cases of introgression in which a few wild mallards had a far greater influence by breeding on islands with very few koloa (Pyle & Pyle, 2017). For example, Moloka'i had mallards but no koloa until c. 2010, when an apparent koloa-mallard pair established on the island and began breeding (Pyle & Pyle, 2017; A. Dibbins-Young, personal observation).

Thus, our results suggest differential contributions of wild and feral mallards both within and among islands. The distinction between wild and feral mallard introgression is potentially important, because introgressed alleles from these two sources may have different consequences for koloa fitness. Compared to wild mallards, domestic breeds may exhibit higher fertility (Stunden, Bluhm, Cheng, & Rajamahendran, 1998), higher growth rates (Kenyon, Watkins, & Butler, 2004), smaller digestive organs (Kenyon et al., 2004), lower lamellar density (Champagnon, Guillemain, Elmberg, Folkesson, & Gauthier-Clerc, 2010), and lower survival (Champagnon et al., 2012). Hence, as descendants of game-farm mallards selected for traits such as larger clutch sizes (Cheng, Shoffner, Phillips, & Lee, 1980; Prince, Siegel, & Cornwell, 1970), feral mallard introgression may be more detrimental than wild mallard introgression to the survival of the island-adapted koloa.

4.2 | Characterization of hybrid swarms

Examples of true hybrid swarms may be rare simply because the backcrossing of hybrids back into large parental populations makes the persistence of large numbers of admixed individuals unlikely (e.g., Lavretsky et al., 2016). However, island populations and those that have recently declined are more susceptible to genetic swamping by an introduced species (Childs, Echelle, & Dowling, 1996; Rhymer, 2006). Here, we present evidence that all reintroduced koloa populations (i.e., those outside of Kaua'i) have to a greater or lesser extent been affected by introgression from mallards. Approximately, we would characterize 78% of the ducks we sampled outside of Kaua'i as koloa × mallard hybrids, 8% as admixed wild × feral mallards, and the remaining 14% as feral mallards. Given that not a single sample on O'ahu, Maui, Moloka'i, or Hawai'i had nonadmixed koloa ancestry (Figure 2) and that the level of mallard ancestry was highly variable among locations and individuals, we characterize these populations as contemporary hybrid swarms. Consequently, our results provide several examples on O'ahu, Maui, and Hawai'i of localized extinction by introgressive hybridization (Rhymer, 2006; Rhymer & Simberloff, 1996; Todesco et al., 2016).

The absence of large native populations of koloa on O'ahu, Maui, Moloka'i, and Hawai'i likely precipitated the formation of hybrid swarms on these islands. Koloa reintroductions involved relatively few individuals, and captive-reared koloa were introduced on islands with established populations of feral mallards (Engilis et al., 2002). Concerns about hybridization were voiced during reintroduction efforts in the 1980s, and hence these populations may have already been hybrid swarms prior to our first sampling efforts in the late 1990s (Engilis & Pratt, 1993; Engilis et al., 2002); this may explain the limited changes detected in our comparison of ancestry proportions in samples from the same populations obtained a decade apart (Figure S3). Encouragingly, efforts to remove mallard-like males from one population of hybrids may have been successful in increasing the average proportion of koloa ancestry (Figure S3d). However, given the lack of large, pure populations of koloa into which hybrids can backcross, hybrid swarms will likely persist on these islands. Continued interbreeding or backcrossing with feral mallards risks the complete loss of koloa genetics on these islands (Abbott, Barton, & Good, 2016; Allendorf et al., 2001).

4.3 | Conservation implications and the unique case of the koloa maoli

Efforts to conserve many endangered species are hindered by a lack of genetic variation (Willoughby et al., 2015). Though not the focus of this study, we report the first genomic data for the endangered Laysan duck, which yield exceptionally low estimates of standing genetic variation (π = 0.00046 for autosomal ddRAD-seq loci; Figure 1), consistent with the results of earlier studies based on different kinds of genetic data (Browne et al., 1993; Lavretsky, Engilis, et al., 2015; Lavretsky, Engilis, & Peters, 2014; Reynolds et

al., 2015; Rhymer, 2001) and with records of a severe population decline (to n = 7) in 1912 (Warner, 1963). In contrast, autosomal genetic diversity in koloa on Kaua'i is roughly comparable to that of North American mallards, whereas Z-chromosome and mtDNA diversity are somewhat lower (Figure 3). This is unusual for an endangered species and perhaps unexpected as koloa likely number fewer than ~2,000 individuals in total (Engilis et al., 2002), as compared to a global census of 19 million for mallards (Wetlands International, 2018). Somewhat higher genetic diversity estimates on O'ahu, Maui, and Hawai'i are likely due to contemporary hybridization with feral and/or wild mallards. However, the relative lack of contemporary hybrids on Kaua'i suggests that recent hybridization with mallards does not explain the relatively high genetic diversity of Kaua'i koloa. Instead, high genetic diversity in koloa may reflect a somewhat more ancient hybrid origin; based on analyses of 19 nuclear introns, Lavretsky, Engilis, et al. (2015) concluded that koloa originated near the Pleistocene-Holocene boundary as a consequence of interbreeding and introgression between Laysan ducks and mallards. The results of this study are potentially consistent with this conclusion; though only a few Kaua'i koloa were inferred to have mallard ancestry in the STRUCTURE analysis (Figure 2), all show a consistent pattern of relatively high coancestry with Laysan ducks along with a lower, but relatively constant level of coancestry with wild mallards in the fineRADstructure analysis (Figure 4). Thus, the relatively high genetic diversity of the Kaua'i koloa population may reflect the somewhat older contributions of mallards from its hybrid origin, whereas the genetic composition and diversity of populations on O'ahu, Maui, Moloka'i, and Hawai'i represent the consequences of contemporary secondary contact between these closely related species. Consequently, koloa represent an interesting case in which gene flow and introgression likely played a critical role in their origin and evolution, but now threatens their status as a distinct species.

Island-restricted species are vulnerable to both demographic and environmental stochasticity. Hence, the establishment of self-sustaining breeding populations on multiple islands could improve prospects for the long-term persistence of koloa (USFWS, 2012). Our analyses point to the persistence of pure koloa, feral mallards, and multiple hybrid swarms in different locations across the main Hawaiian Islands (Figure 2; Figure S3), with little evidence of changes in genetic composition over the past decade. Thus, we predict that within hybrid swarms, in the absence of additional genetic contributions from Kaua'i koloa, time alone is unlikely to reduce hybrid prevalence or admixture proportions. Importantly, our results suggest that the removal of feral mallards is critical and should be considered a management priority to limit the chance that remaining koloa will be lost to hybridization; this approach of removing the non-native species has effectively limited hybridization between introduced ruddy ducks (*Oxyura jamaicensis*) and native white-headed ducks (*O. leucocephala*) in Spain (Muñoz-Fuentes, Green, & Negro, 2013), and between self-introduced pied stilt (*Himantopus himantopus leucocephalus*) and endemic black stilts (*H. novaezelandiae*) in New Zealand (Steeves et al., 2010). The same approach should be feasible in the Hawaiian Islands now that non-native mallards have been genetically identified.

ACKNOWLEDGEMENTS

We thank Oregon State University, USFWS (Region 1 Migratory Birds; Ecological Services, Pacific Islands Fish and Wildlife Office; Inventory and Monitoring; Invasive Species Control Program), USGS, the Hawai'i Department of Land and Natural Resources, the Selma Herr Endowment, and the Dennis G. Raveling Endowment for funding. We greatly appreciate the support provided by Kaua'i NWR Complex, particularly S. N. Smith, M W. Mitchell, and C. C. Smith, and James Campbell NWR. We thank Michelle Reynolds, KUPU/Americorps, and the numerous field assistants and volunteers who assisted with collecting samples. We thank I. E. Engilis and numerous undergraduate assistants of the UC Davis Museum of Wildlife and Fish Biology for technical support in obtaining genomic samples and access to specimens. We thank UC Davis Genomic Variation Lab and Wright State University for lab space. This work used the Vincent J. Coates Genomics Sequencing Laboratory at UC Berkeley, supported by NIH S10 Instrumentation Grants S10RR029668 and S10RR027303. Thank you to Helen James and Matthew Kweskin for access to the HYDRA computing cluster at the Smithsonian Institution.

AUTHOR CONTRIBUTIONS

C.P.W., K.J.U., B.D.D., J.M.E., A.E. designed research; C.P.W., P.L., J.L.P., S.T., K.J.U., C.M., and B.D.D. performed research; C.P.W., P.L., J.L.P., J.M.D., and M.D.S. analyzed data; C.P.W., P.L., and M.D.S. drafted the manuscript; all authors contributed to the manuscript.

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DATA AVAILABILITY STATEMENT

mtDNA: GenBank Accession Num. MN563303–MN563571 and MN603671–MN603691 ddRAD:SRA BioProject PRJNA577581, Sample accession IDs SAMN13029772–SAMN13030226 Other data files (e.g. FASTA files; ADMIXTURE input files; Table of GenBank accession numbers): Dryad accession https://doi.org/10.5061/dryad.ffbg79cqf (Wells et al., 2019).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Wells CP, Lavretsky P, Sorenson MD, et al. Persistence of an endangered native duck, feral mallards, and multiple hybrid swarms across the main Hawaiian Islands. *Mol Ecol*. 2019;28:5203–5216. https://doi.org/10.1111/mec.15286