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Genetic admixture supports an ancient hybrid origin of the endangered Hawaiian duck

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Abstract

Speciation is regarded primarily as a bifurcation from an ancestral species into two distinct taxonomic units, but gene flow can create complex signals of phylogenetic relationships, especially among different loci. We evaluated several hypotheses that could account for phylogenetic discord between mitochondrial DNA (mtDNA) and nuclear DNA (nuDNA) within Hawaiian duck (*Anas wyvilliana*), including stochastic lineage sorting, mtDNA capture and widespread genomic introgression. Our results best support the hypothesis that the contemporary Hawaiian duck is descended from an ancient hybridization event between the mallard (*Anas platyrhynchos*) and Laysan duck (*Anas laysanensis*). Whereas mtDNA clearly shows a sister relationship between Hawaiian duck and mallard, nuDNA is consistent with a genetic mosaic with nearly equal contributions from Laysan duck and mallard. In addition, coalescent analyses suggest that gene flow from either mallard or Laysan duck, depending on the predefined tree topology, is necessary to explain contemporary genetic diversity in Hawaiian ducks, and these estimates are more consistent with ancient, rather than contemporary, hybridization. Time since divergence estimates suggest that the genetic admixture event occurred around the Pleistocene–Holocene boundary, which is further supported by circumstantial evidence from the Hawaiian subfossil record. Although the extent of reproductive isolation from either putative parental taxon is not currently known, these species are phenotypically, genetically and ecologically different, and they meet primary criteria used in avian taxonomy for species designation. Thus, the available data are consistent with an admixed origin and support the hypothesis that the Hawaiian duck may represent a young hybrid species.

Introduction

Gene flow, or the exchange of genetic material between two populations, is an important and sometimes necessary process in speciation. Although interspecific interactions can test and reinforce species barriers (Dobzhansky, 1940; Hoskin *et al.*, 2005; Rundle & Nosil, 2005; Schluter, 2009), gene flow can also inhibit the completion of speciation (Mallet, 2005, 2007), cause a reversal of speciation (Seehausen, 2006; Webb *et al.*, 2011), create hybrid zone(s) (Barton & Hewitt, 1989), cause the extinction through introgressive hybridization (Rhymer, 2006) and lead to the creation of novel taxa via hybrid speciation (Mallet, 2007). Testing between these scenarios requires understanding the effects of gene flow on the genome, which can often appear heterogeneous across different regions (i.e. nuclear vs. mitochondrial) (Wu, 2001; Harrison, 2012). Under neutrality, gene flow can lead to strongly admixed genomes, but the extent of admixture will depend on the magnitude of gene flow and strength of selection (Mallet *et al.*, 1990; Rosenblum *et al.*, 2007). Specifically, selection can inhibit the introgression of

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maladaptive alleles, or cause favourable introgressed alleles to sweep through a population (Rieseberg *et al.*, 1999). The interactions between these forces can lead to discordant demographic or phylogenetic histories among different markers (e.g. mitochondrial DNA, nuclear DNA and morphology). Determining the cause for these discordances is an important goal in evolutionary biology (Toews & Brelsford, 2012).

Observing discordant phylogenetic relationships between different marker types can provide evidence of admixed histories, but it does not necessarily reveal the consequences of gene flow at the genomic level, such as mitochondrial without (or with little) nuclear introgression (i.e. mitochondrial capture; Ballard & Whitlock, 2004; Toews & Brelsford, 2012), nuclear without mitochondrial introgression or widespread admixture leading to a genomic mosaic. Furthermore, stochastic lineage sorting can also result in discordant phylogenies, and this process needs to be ruled out before concluding that gene flow has had a prominent influence on genetic diversity. Here, we aim to systematically test and distinguish between these alternative scenarios within the endangered, island endemic Hawaiian duck (*Anas wyvilliana*), in which mitochondrial (mt) DNA, nuclear (nu) DNA and morphological characteristics provide conflicting evidence of phylogenetic relationships (Lavretsky *et al.*, 2014b). We test *a priori* hypotheses to determine whether stochastic lineage sorting, mtDNA capture or widespread introgression can explain these discordances by examining evidence for a genetic mosaic (Jacobsen & Omland, 2011).

The Hawaiian duck is one of 14 taxa within the mallard complex (Livezey, 1991; Engilis *et al.*, 2002; Lavretsky *et al.*, 2014b). Recent phylogenetic reconstructions of this complex uncovered morphological–nuclear–mitochondrial discordances for several lineages within this group, including the Hawaiian duck (Lavretsky *et al.*, 2014b). Morphology (Livezey, 1991) and nuDNA (Lavretsky *et al.*, 2014b) suggest the Hawaiian duck is sister to the Laysan duck (*A. laysanensis*), a relationship that received 100% posterior support in species tree reconstructions (Lavretsky *et al.*, 2014b). In contrast, Hawaiian duck mtDNA supports a closer affinity to the mallard (*A. platyrhynchos*) (Lavretsky *et al.*, 2014b). Specifically, Hawaiian ducks possess a monophyletic lineage of mtDNA haplotypes that are species specific but nested within, and derived from, a polyphyletic clade consisting of mallard and other New World mallard-like ducks to the exclusion of Laysan ducks (Fowler *et al.*, 2009; Lavretsky *et al.*, 2014b). Although these results suggest an admixed origin, analyses conducted by Lavretsky *et al.* (2014b) were insufficient to discern whether the apparent absence of Laysan duck-like mtDNA haplotypes in Hawaiian ducks (Fowler *et al.*, 2009) is explained by New World mallard mtDNA capture (in the absence of nuDNA introgression) or widespread genetic admixture. Thus, we aim to test for signatures of a nuclear mosaic consisting of both mallard and Laysan duck alleles within Hawaiian ducks, which would be consistent with widespread introgression. Alternatively, a simple case of mitochondrial capture would be supported in the absence of an admixed nuclear genome, and we expect that Hawaiian ducks would be genetically more similar to Laysan ducks across nuclear loci as suggested by the nuDNA phylogeny (Lavretsky *et al.*, 2014b).

In addition, we tested *a priori* predictions under a hypothesis of an admixed origin by estimating gene flow rates under different evolutionary scenarios (Hawaiian duck sister to Laysan duck vs. Hawaiian duck sister to mallard). If the nuclear genome of the Hawaiian duck was consistent with a hybrid origin, then regardless of the predefined topology, gene flow from the nonsister taxon will be required to explain the observed genetic diversity in Hawaiian ducks. Specifically, we predict nonzero gene flow from the most diverged lineage into the Hawaiian duck or its ancestor. Alternatively, if an apparent genetic mosaic is driven by common ancestry and stochastic lineage sorting, then no gene flow will be necessary to explain the genetic variability observed in Hawaiian ducks. A potential complication is that Hawaiian ducks and mallards have recently come into secondary contact and hybridize (Fowler *et al.*, 2009). Under this scenario of contemporary introgression, we expect to detect gene flow from mallards into Hawaiian ducks, regardless of predefined topology, and we expect some Hawaiian ducks to receive assignment probabilities indicative of contemporary mallard introgression. We note that contemporary introgression leading to the observed mito-nuclear discord seems unlikely, because Hawaiian ducks are fixed for mallard-like mtDNA haplotypes but do not share haplotypes with mallards (Fowler *et al.*, 2009; Lavretsky *et al.*, 2014b), which suggests that sufficient time has passed since introgression for the evolution of a species-specific mtDNA lineage in Hawaiian ducks.

Materials and methods

Sample preparation and nuclear marker amplification

Genomic DNA was isolated from 21 Laysan ducks, 15 Hawaiian ducks and 25 mallards (see Table S1 for specimen information) using a Qiagen DNA extraction kit (Quagen, Venlo, the Netherlands) following the manufacturer's protocol. We note that to limit the effects of contemporary gene flow on inferences, all Hawaiian duck individuals were sampled from Kauai, which is thought to be free from contemporary mallard – Hawaiian duck hybridization, and chosen based on previous hybrid indices vetting these as 'pure' (Fowler *et al.*, 2009). Moreover, Kauai individuals are known to possess a derived mtDNA haplotype lineage

nested within the paraphyletic New World mallard clade (Fowler *et al.*, 2009; Lavretsky *et al.*, 2014b). This suggests that enough time has passed for a derived haplotype group to exist, which supports ancient rather than contemporary mitochondrial introgression.

Nineteen nuclear intronic loci, each from a different chromosome, that were previously optimized in gadwall (*Anas strepera*) (Table S2; Peters *et al.*, 2012) were used. Putatively neutral markers (i.e. introns) were used as these are expected to differ in allopatric systems as a result of stochasticity and population demography (Dobzhansky, 1940; Mayr, 1963) rather than selection, which can quickly drive favourable alleles to fixation and decrease the 'hybrid' signal (Seehausen, 2004; Nolte & Tautz, 2010). Amplification by PCR was carried out with 1.5 μL of an individual's DNA combined with 1 nM of both forward and reverse primers, and 2× GoTaq Green Master Mix (Promega, Madison, WI, USA) for a total of a 15-μL reaction per individual per locus. PCR was conducted using an Eppendorf Mastercycler (ep*gradient*) thermocycler under the following conditions: DNA denaturation at 94 °C for 7 min, followed by 45 cycles of DNA denaturation at 94 °C for 20 s, primer annealing at 58 °C for 20 s and DNA extension at 72 °C for 1 min, and a final DNA extension at 72 °C for 7 min. Amplification was verified using gel electrophoresis with a 1.5% agarose gel. PCR products were cleaned with AMPure XP beads, following Agencourt's protocol (Beckman Coulter Co., Fort Collins, CO, USA). Sanger sequencing was performed using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Waltham, MA, USA) following manufacturer's protocol. Sequenced products were sent to the DNA Analysis Facility at Yale University for automated sequencing on an ABI 3730. Sequences were aligned and edited using Sequencher v. 4.8 (Gene Codes, Corp., Ann Arbor, MI, USA). All sequences were submitted to GenBank (Table S1).

Gametic phases were resolved first for sequences with indels by methods outlined in Peters *et al.* (2007), which were then used as known alleles when resolving the remaining sequences with the program PHASE (Stephens & Donnelly, 2003). PHASE derives the most likely state of each allele algorithmically by comparing all known alleles. Additionally, all mallard sequences were previously resolved with > 95% confidence from a larger data set that included extensive allele-specific priming (Peters *et al.*, 2014a, b) and were also treated as known alleles. Linkage between loci was not considered as all markers are found on different chromosomes.

Identifying a genetic mosaic

Pairwise Φ_{ST} estimates for nuclear and mtDNA were calculated in Arlequin v. 3.5 (Excoffier & Lischer, 2010). In addition, a locus-by-locus AMOVA was used to analyse single nucleotide polymorphisms (SNPs) across nuclear markers as implemented in Arlequin v. 3.5.

A finer examination of overall genetic connectivity among individuals with linked nodes representing reticulate events (i.e. hybridization or recombination) was conducted in SplitsTree (Huson & Bryant, 2006). An unrooted phylogenetic network was reconstructed from 19 nuclear loci that were first concatenated for each individual with IUPAC nucleotide codes used for polymorphic sites. A neighbour-net analysis with character transformations based on an uncorrected P, and an equal angle for both splits and reticulate transformations was used. Under the hypothesis of an admixed genome, we predict that Hawaiian ducks will occupy intermediate positions between and share many reticulations with mallards and Laysan ducks in this network. Alternatively, under stochastic sorting or mitochondrial capture, we expect Hawaiian ducks to primarily group as a sister taxon to Laysan ducks (Lavretsky *et al.*, 2014b).

STRUCTURE v. 2.3.4 (Pritchard *et al.*, 2000) uses Bayesian clustering methods to estimate admixture proportions from molecular data and was used to estimate the optimum number of populations (K) and assignment probabilities to those populations within our nuclear data set. STRUCTURE attempts to maximize Hardy–Weinberg and linkage equilibrium within populations when assigning individuals. However, several of our loci contained high genetic diversity and many singletons, especially for the mallard (Peters *et al.*, 2014b), and these loci likely contributed little to these assignments. Therefore, we analysed three different data sets that likely varied in their noise to signal ratio: (i) the full sequences for each marker, (ii) truncated sequences consistent with no recombination and (iii) the most informative SNPs differentiating Laysan ducks and mallards – one SNP per locus was used to maintain marker independence (i.e. minimize biases caused by linkage disequilibrium).

STRUCTURE was run for 1 000 000 iterations after a burn-in of 250 000 for one to five populations (K) with ten replicates per K. All loci were considered independent, and the admixture model was used to determine per cent genome composition. The optimum K was determined by calculating ΔK in the program STRUCTURE HARVESTER (Earl & VonHoldt, 2012). Final STRUCTURE outputs were based on the optimal clustering alignment across all ten replicates for each optimum K using a FullSearch algorithm as implemented in the program CLUMPP (Jakobsson & Rosenberg, 2007). If introgression has been widespread in Hawaiian ducks, then we expect individuals to display admixture proportions relative to the contribution of its parental taxa at lower K populations.

Estimating gene flow and divergence time

IMa2 (Hey, 2010a, b) assigns posterior probability density estimates for population sizes and migration rates

from nonrecombinant sequence fragments for multiple populations using Bayesian Markov chain Monte Carlo (MCMC) algorithms (Nielsen & Wakeley, 2001). Specifically, IMa2 estimates six demographic parameters scaled to the neutral substitution rate per locus (u), including immigration rates (M, where $M = m/u$; m is the rate at which alleles enter the population through immigration and gene flow) for each of the populations, and time since divergence (t, where $t = Tu$; T is the number of years since the subspecies diverged). Loci were first tested for recombination using the program IMgc (Woerner *et al.*, 2007) and then manually truncated to retain polymorphic sites (> 2 states) that would have been automatically removed by IMgc (Table S2). Weight was given to maximize fragment length, unless sample size was decreased by > 10% of each population, in which case fragment lengths were reduced to maximize sample size. Phylogenetic relationships were manually entered into IMa2. Given the discord in sister relationships derived from mtDNA vs. nuDNA markers (Lavretsky *et al.*, 2014b), gene flow estimates were derived under two alternative tree topologies that included mtDNA-like (Hawaiian duck is sister to mallard) and nuDNA-like relationships (i.e. Hawaiian duck is sister to Laysan duck). Each analysis was run for 1 000 000 burn-in generations and ≥ 5 000 000 sampling generations so that the minimum ESS across parameters was ≥ 50 (Hey & Nielsen, 2004, 2007). Appropriate *priors* for splitting time ($t = 0.3$), maximum population size ($N_e = 20$) and migration rate ($M = 25$) were attained from pilot runs. We used Metropolis coupling with a geometric heating scheme for one cold chain and 59 heated chains and replicated each run with a different random number seed; the results converged on the same stationary distributions.

Years since divergence (T) was derived as $T = t/\mu$, t being the time since divergence parameter in IMa2. A mutation rate (μ) of 2.67×10^{-7} substitutions/locus/year was derived from the geometric mean number of base pairs (222.32 bp) and previously calculated average mutation rate of $\mu = 1.2 \times 10^{-9}$ substitutions/site/year (s/s/y) (Peters *et al.*, 2008; Table S2). Additional divergence time estimates were obtained from species tree branch lengths (obtained from Lavretsky *et al.*, 2014b) that were reconstructed in *Beast v. 1.7.4 (Drummond *et al.*, 2012) using nuDNA (mean $\mu_{nuDNA} = 1.2 \times 10^{-9}$ s/s/y) or mtDNA ($\mu_{mtDNA} = 4.8 \times 10^{-8}$ s/s/y; Peters *et al.*, 2008). Given the discord in sister relationships between the two trees, estimates derived from these branch lengths provide divergence time between Hawaiian duck and mallard (mtDNA) or Laysan duck (nuDNA). Under an ancient admixture scenario, we expect the Hawaiian duck to simultaneously split from both putative parental taxa and to be represented by significant overlap across independently derived time estimates from mtDNA branch length, nuDNA branch length and IMa2 nuDNA derived t0 (i.e. split between Hawaiian and Laysan ducks). Alternatively, under a bifurcation and recent contemporary introgression scenario, we expect to observe a significantly shallower time divergence between Hawaiian ducks and the introgressing mallards (i.e. time estimates from mtDNA branch length) as compared to divergence from Laysan ducks (i.e. nuDNA branch length and IMa2 nuDNA derived t0). We acknowledge that the latter scenario would be difficult to differentiate from one in which secondary contact occurs on the heels of bifurcation where a budding population is still largely genetically indistinguishable from their source population.

Results

Identifying a genetic mosaic

There were no fixed SNPs between Hawaiian ducks and either putative parental taxa (Fig. 1). The only SNP that was fixed within the Hawaiian duck, and diagnostic

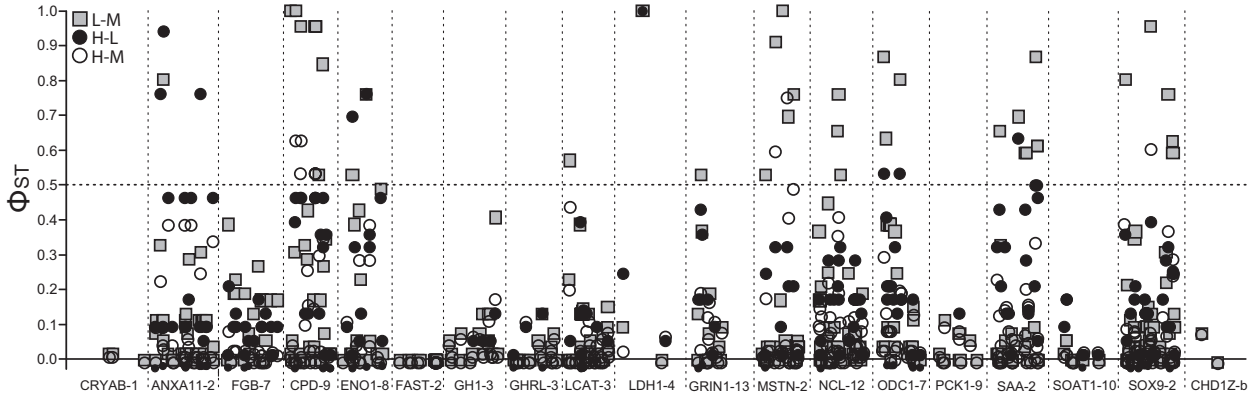

Fig. 1 Pairwise Φ_{ST} estimates between Laysan ducks (L), Hawaiian ducks (H) and mallards (M) for single nucleotide polymorphisms (SNPs) across assayed markers.

between Laysan ducks and mallards, was shared with mallards at LDH1–4. The remaining SNPs had frequencies that were intermediate between the two putative parental species as expected under widespread, heterogeneous gene flow.

For nuclear DNA, Hawaiian ducks clustered at intermediate positions between mallards and Laysan ducks, and shared many reticulations with both species that is consistent with a genetic mosaic (Fig. 2a). However, Hawaiian ducks were genetically differentiated from both mallards (mean $\Phi_{ST} = 0.12$) and Laysan ducks (mean $\Phi_{ST} = 0.34$), clustering as a distinct group in the tree. Under a classical bifurcating history, we would have expected Hawaiian ducks to cluster more closely and share more reticulations (resulting from incomplete lineage sorting) with its sister species, as observed in the mtDNA neighbour-net tree, which shows Hawaiian ducks to be deeply nested within mallards (Fig. 2b). The close affinity to mallards in mtDNA was also reflected by the relatively lower, although significant, Φ_{ST} between Hawaiian ducks and mallards ($\Phi_{ST} = 0.33$) relative to Hawaiian and Laysan ducks ($\Phi_{ST} = 0.98$).

Across all three data sets that varied in the length of sequences used in STRUCTURE analyses, the optimum number of populations was two. When using the full sequences (presumably, high noise to signal ratio), STRUCTURE assigned all Laysan and Hawaiian ducks to population A with an average probability of 99% (± 0.0% SD) and 99% (± 0.22% SD), respectively, and all mallards to population B with an average probability of 99% (± 1.2% SD) (Fig. 3). Higher values of K likewise supported a single population comprising Hawaiian and Laysan ducks that were distinguishable from mallards. For data sets with loci truncated to the largest nonrecombinant fragment, STRUCTURE assigned all Laysan ducks to population A with an average probability of 99% (± 0.0% SD) and all mallards to population B with an average probability of 97% (± 3.8% SD). Consistent with a genetic mosaic, all Hawaiian duck individuals were recovered as being admixed with an average assignment of 47% (± 16.2% SD) to population A and 53% (± 16.2% SD) to population B (Fig. 3). This admixed ancestry was also recovered at higher values of K. Finally, using the most diagnostic SNP per locus that differentiated Laysan ducks and mallards (Fig. 1), STRUCTURE assigned all Laysan ducks to population A with an average probability of 99% (± 0.0% SD) and all mallards to population B with an average probability of 98% (± 1.3% SD), whereas all Hawaiian duck individuals were admixed with an average assignment of 41% (± 9.9% SD) to population A and 59% (± 9.9% SD) to population B (Fig. 3). Unlike the other two analyses, analysing SNPs with a $K = 3$ population model, Laysan ducks, Hawaiian ducks and mallards were each assigned to independent populations with an average probability of 99% (± 0.0% SD), 94% (± 8.3% SD), 98% (± 3.1% SD), respectively (Fig. 3). In general, assignment probabilities from the recombination-filtered and SNP data sets recovered significantly similar ($R^2 = 55$; $P < 0.001$) admixture proportions across Hawaiian ducks that are consistent with a nearly 50 : 50 mosaic. Moreover, this suggests that recombination and/or high genetic diversity does influence assignment probabilities.

If the apparent mosaic was due to stochastic lineage sorting, then we expected other species within the mallard complex to show assignment probabilities similar to the Hawaiian duck when analysed with the same set of SNPs. However, we found no evidence of this when assigning other mallard-like species to a two- or

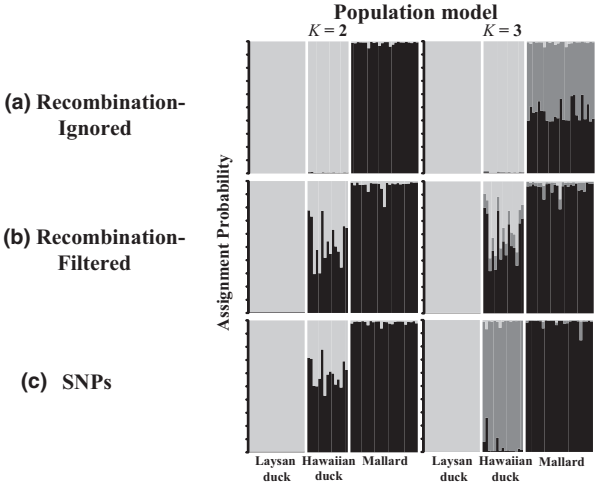

Fig. 3 Assignment probabilities obtained from data sets where (a) recombination was ignored, (b) loci were filtered for recombination, and (c) 17 diagnostic SNPs ascertained by comparing 21 Laysan ducks and 25 mallards (Table S2) and assayed in 15 Hawaiian ducks. Population models include $K = 2$ (optimum across analyses) and $K = 3$. A genetic mosaic was identified in (b) and (c), suggesting that recombination is influencing structure results.

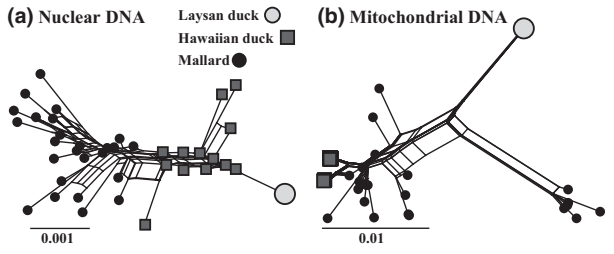

Fig. 2 Neighbour-net trees for (a) nuclear DNA (6682 aligned nucleotides) showing Hawaiian ducks as being intermediate between mallards and Laysan ducks and (b) mitochondrial DNA control region (645 bp) showing Hawaiian ducks to be deeply nested within mallard and distinct from Laysan ducks. We note that Laysan ducks appear as a 'single' individual in SplitsTree analyses because they did not possess any diversity across markers.

three-population model (Fig. S1). In a two-population model, all other species were assigned with strong posterior support to the same population as mallards. We note that clustering of mallard-like ducks within a 'mallard' population does not necessarily reflect introgression, but rather retention of the mallard instead of the Laysan SNP at these particular loci via stochastic processes.

Gene flow

We found nonzero nuclear gene flow under the mtDNA-like topology (Hawaiian duck sister to mallard; Fig. 4a) from Laysan ducks into the Hawaiian duck ($2Nm$ = 1.58; 95% CI 0.52–8.8), whereas under the nuDNA-like topology (Hawaiian duck sister to Laysan duck; Fig. 4b), nonzero gene flow from mallards into the ancestor of Hawaiian and Laysan duck was supported ($2Nm$ = 1.37; 95% CI 0.87–26.11). Thus, a simple bifurcating history was insufficient to explain the evolution of this group. Although the highest posterior probability under each topology supported gene flow from both parental species into Hawaiian ducks, we could not reject zero gene flow (i.e. complete isolation) between Hawaiian ducks and their putative sister species under either topology (Fig. 4). Moreover, the nonzero gene flow from mallards into the Hawaiian–Laysan duck ancestor supported ancient rather than recent introgression, suggesting that contemporary gene flow from mallards (Fowler *et al.*, 2009) is unlikely to explain the apparent genetic mosaic.

Divergence time

In IMa2 analyses, divergence times from nuDNA were reliably obtained (ESS ≥ 50) under the nuDNA-like topology only (Fig. S2). The inability to obtain a divergence time estimate under the mtDNA-like topology is likely due to forcing nuDNA to fit a demographic scenario that is inconsistent with the data in which Hawaiian ducks are sister to mallards (Fig. 4). Defining a nuDNA-like topology, divergence time between the Laysan–Hawaiian duck ancestor and mallard (t1) was estimated to be ~650 000 years before present (YBP) (95% highest posterior density (HPD) = 364 000–1 100 000 YBP; Fig. 5), which is consistent with the older mtDNA divergence between Laysan ducks and other mallard-like ducks (Johnson & Sorenson, 1999; Lavretsky *et al.*, 2014b). Divergence time between Hawaiian ducks and Laysan ducks (t0) was estimated at ~3000 YBP (95% HPD = 0–207 000 YBP). Similar time estimates were derived from species tree branch lengths from mtDNA (i.e. 29 000 YBP, 95% HPD = 0–105 000, between Hawaiian duck and mallard) and nuDNA (i.e. 60 000 YBP, 95% HPD = 18 000–109 000 YBP, between Hawaiian duck and Laysan duck). The broadly overlapping HPDs among the three independent time estimates derived from IMa2 and species tree analysis (Fig. 5) are consistent with a simultaneous split for mallard–Hawaiian duck mtDNA and Laysan–Hawaiian nuDNA. Note that none of the HPDs overlapped the estimated divergence time between mallards and Laysan ducks from IMa2.

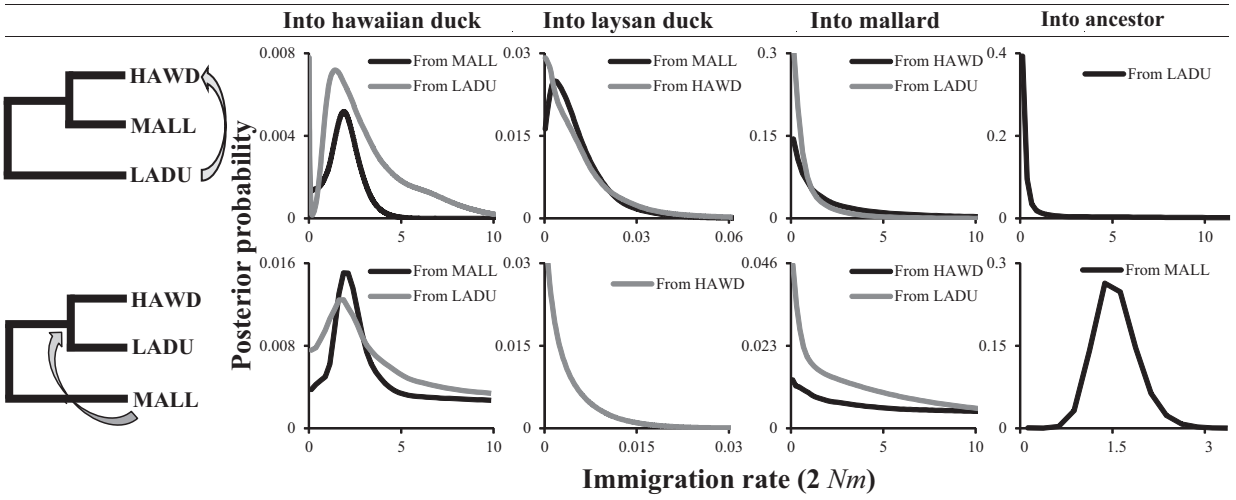

Fig. 4 Population migration rates ($2Nm$) estimated from 19 nuclear loci and defining a mtDNA-like topology (above) or nuDNA-like toplogy (below) (Lavretsky *et al.*, 2014b; HAWD = Hawaiian Duck; LADU = Laysan Duck; MALL = Mallard). The 95% highest posterior distributions that did not include zero gene flow (i.e. rejected complete isolation) was from Laysan ducks into Hawaiian ducks under the mtDNA-like topology and from mallards into the Hawaiian–Laysan duck ancestor under the nuDNA-like topology. Thus, consistent with the hybrid origin hypothesis, gene flow from the nonsister species is necessary to explain the genetic variability within Hawaiian ducks.

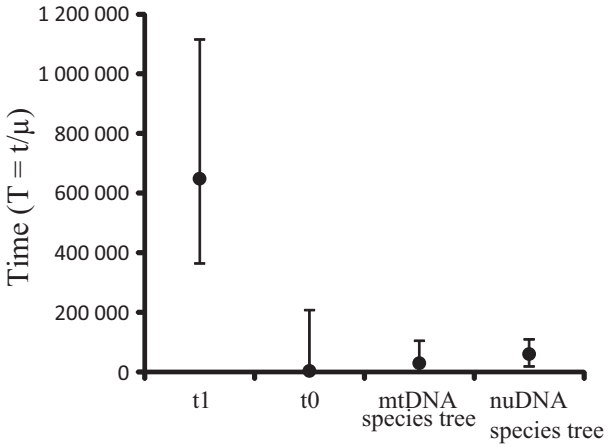

Fig. 5 IMa2 (Nielsen & Wakeley, 2001) time since divergence (T = t/μ; with 95% CI) estimates derived from 19 nuclear introns under the nuDNA-like topology (see Fig. 3) for t1 (i.e. divergence between mallards and Hawaiian–Laysan duck ancestor) and t0 (i.e. divergence between Hawaiian and Laysan ducks). Additionally, divergence estimates derived from species trees (nuDNA branch lengths obtained from species tree reconstructed with recombination-filtered data; Lavretsky *et al.*, 2014b) reconstructed with mtDNA (i.e. divergence between Hawaiian duck and mallard) or nuDNA (i.e. divergence between Hawaiian duck and Laysan duck) in *Beast v. 1.7.4 (Drummond *et al.*, 2012) are presented – the estimated demographic time (DMT) is DMT (± 95% HPD) divided by their respective mutation rate (Materials and Methods; also see μ in Peters *et al.*, 2008).

Discussion

In this study, we used multiple nuclear and mitochondrial markers to test several hypotheses (i.e. lineage sorting, mitochondrial capture, genomic admixture) that could explain the mito-nuclear discordance observed among three species of ducks. Rather than a simple case of mitochondrial capture, our results support widespread, heterogeneous nuclear admixture within the Hawaiian duck's genome. Specifically, the Hawaiian duck appears to comprise a genetic mosaic consisting of a high frequency of both mallard and Laysan duck ancestry. First, with the exception of LDHB, for which SNPs were shared between mallards and Hawaiian ducks, SNP frequencies that differed significantly between Laysan ducks and mallards were intermediate in the Hawaiian duck (Table S2). Second, Bayesian assignment probabilities support a nearly 50 : 50 (Laysan duck: mallard) admixed genome within Hawaiian ducks at the optimum *K* for recombination-filtered and SNP data sets (Fig. 3). Similar assessments across five additional mallard-like taxa revealed that these admixture proportions are unique to Hawaiian ducks and are unlikely to be explained by recent ancestry and stochastic lineage sorting (Fig. S1). Third, coalescent results suggest that gene flow from the nonsister taxon is required to explain the genetic variation observed within Hawaiian ducks (Fig. 4). We conclude that the observed mito-nuclear discord (Lavretsky *et al.*, 2014b) is attributable to among-locus heterogeneity in the extent of introgression coupled with the fixation of mallard-like mtDNA (and perhaps mallard-like LDHB alleles) within the Hawaiian duck's genome.

Determining the timing of DNA introgression is important when inferring mechanisms of speciation. However, conclusively distinguishing between contemporary and ancient gene flow can be difficult (Becquet & Przeworski, 2009; Strasburg & Rieseberg, 2011), particularly in systems that have come into secondary contact. In this study, we provide three lines of evidence that support ancient admixture rather than ongoing, contemporary gene flow as the cause of the apparent genetic mosaic within sampled Hawaiian ducks. First, gene flow from the nonsister taxon, regardless of tree topology, is required to explain the genetic diversity observed within Hawaiian ducks, and that ancient gene flow from mallards into the Hawaiian/Laysan ancestor is better supported than contemporary gene flow (Fig. 4). Second, under a hypothesis of contemporary gene flow, we expected putative contemporary hybrids to cluster with mallards, but they do not in SplitsTree (Fig. 2a) or STRUCTURE (Fig. 3). Third, although Hawaiian duck mtDNA haplotypes are most similar to those found in the New World, and particularly to those of Florida mottled ducks (*Anas fulvigula fulvigula*; Lavretsky *et al.*, 2014b), they are a monophyletic cluster (Fig. 2b) and no haplotypes are shared between Hawaiian ducks and other species. Thus, a sufficient amount of time has passed since divergence or admixture for Hawaiian ducks to accumulate novel mtDNA haplotypes and to sort to monophyly. Although any single test can be sensitive to a number of assumptions (Becquet & Przeworski, 2009), collectively, these multiple lines of evidence support ancient hybridization over contemporary gene flow. These inferences demonstrate the need to define and test multiple *a priori* hypotheses when determining the timing of gene flow.

In general, studies that have argued for admixed genomes have relied solely on genetic evidence. However, in the case of the Hawaiian duck, phenotypic-based studies and the Hawaiian subfossil record further support an admixed genome. Hawaiian ducks have intra-appendicular skeletal and sternal dimensions that are intermediate between Laysan ducks and continental mallards (Livezey, 1993). Moreover, ongoing studies note high variation in plumage characteristics within Hawaiian ducks that appear to be intermediate between mallards and Laysan ducks (Engilis *et al.*, 2002) and corroborate a morphology-based *Anas* phylogeny that placed the Hawaiian duck as intermediate between the Laysan duck and mallard (Livezey, 1991). Furthermore, the temporal distribution of Hawaiian subfossils is con-

sistent with a broad-scale mixing event; the subfossil record contains Laysan-like duck forms dating to the mid-Pleistocene, intermediate Laysan–Hawaiian duck forms dating to the Holocene (Olson & James, 1991; Cooper *et al.*, 1996; Burney *et al.*, 2001), but only a few recent bones attributable to modern Hawaiian ducks (H. James, pers. comm.). Although circumstantial, these data strengthen the conclusion that the Hawaiian duck has had a history of genomic admixture.

The timing associated with the presence of the various forms of Hawaiian subfossils closely corresponds with our molecular estimates of divergence times, which suggest a Pleistocene–Holocene split for Hawaiian–Laysan nuDNA and a late Pleistocene split for Hawaiian–mallard mtDNA (Fig. 5). Thus, both molecular and subfossil data support an ancestral hybridization event near the Pleistocene–Holocene transition between the once widespread Laysan duck (Cooper *et al.*, 1996) and mallards that probably arrived on the Hawaiian Islands by happenstance during migration. Dispersal by 'migratory dropouts' of several species of Holarctic waterbirds, including mallards, continues to be documented on the islands (Engilis *et al.*, 2004). However, we acknowledge that whether the proposed admixture event was a result of mallard introgression into the Hawaiian duck on the heels of a speciation event (i.e. bifurcation from Laysan ducks followed by secondary contact with mallards and widespread introgression) or whether hybridization was the catalyst for speciation (i.e. hybrid speciation; Mallet, 2007) requires additional evidence. For example, examining the temporal and spatial distributions of available subfossil morphotypes, coupled with ancient DNA analyses (Huynen *et al.*, 2003; Willerslev & Cooper, 2005), would provide a unique opportunity to determine when mallard-derived alleles became present within the Hawaiian Islands. If the presence of mallard alleles corresponds with the emergence of Hawaiian–Laysan and Hawaiian duck-like forms, with only Laysan genotypes present prior to this in the fossil record, then a coupling of admixture and divergence leading to speciation would be supported. Moreover, the Laysan–Hawaiian–mallard study system affords a unique opportunity to study the influence of gene flow at different temporal scales by comparing genomes that are putatively derived via ancestral (i.e. Hawaiian ducks from Kauai) or contemporary gene flow (i.e. Hawaiian ducks and mallards that have more recently come into secondary contact on other Hawaiian Islands (Engilis & Pratt, 1993; Engilis *et al.*, 2002; Fowler *et al.*, 2009)). Specifically, future work will benefit from genomic assessments to determine the architecture (e.g. parent-specific haplotype blocks vs. random distributions) of Laysan duck and mallard genes across 'pure' and contemporary Hawaiian duck hybrid genomes, as well as provide the opportunity to understand how selection and genetic drift influence these processes.

Species concepts and admixed species

Speciation is a continuous process that is difficult to categorize into discrete measurements (Nosil *et al.*, 2009a, b; Hohenlohe *et al.*, 2010). This ambiguity has resulted in extensive debate over what criteria are appropriate to use when distinguishing between the various phases of speciation (Mayr, 1942, 1943; Cracraft, 1983; Green, 2005; Haig *et al.*, 2006; Phillimore & Owens, 2006; Gill, 2014; Sangster, 2014), and these same arguments apply to identifying hybrid species. Several criteria in support of a hybrid origin, and specifically of a hybrid species, have been proposed. First, criteria set by Jacobsen & Omland (2011) include the existence of (i) three identifiable taxa [i.e. hybrid speciation effectively increased biodiversity (Schwarz *et al.*, 2005)] in which a complex evolutionary history within the putative hybrid species is supported by (ii) a mito-nuclear discord and (iii) a genetic mosaic consisting of parental alleles from both species within the putative hybrid species. More recently, Abbott *et al.* (2013) and Schumer *et al.* (2014) included the necessity to demonstrate that a hybridization event resulted in hybrid speciation, and with the later arguing that in the absence of complete reproductive isolation from the parental taxa [as under the Biological Species Concept (BSC); Mayr, 1942], descendant populations are better considered as a hybrid swarm than a hybrid species. However, reproductive isolation in birds may require millions of years to achieve (Grant & Grant, 1992; Gill, 2014; Sangster, 2014), owing to their strong dispersal ability (Greenwood, 1980), chromosomal stasis (Ellegren, 2010) and relatively low levels of reinforcement (Grant & Grant, 1997), and therefore, reproductive isolation is often considered to be an inappropriate criterion for species delimitation (Cracraft, 1983; Gill, 2014). Rather than adhering to the BSC, diagnosability, degree of differentiation and monophyly have been the primary criteria used in avian taxonomy (see table 1 in Sangster, 2014). Given that the Hawaiian duck hybridizes with introduced mallards (Fowler *et al.*, 2009), demonstrating the lack of complete reproductive isolation, what is the evidence for recognizing the Hawaiian duck as a hybrid species?

Our analyses provide evidence that meet the criteria for an admixed/hybrid origin put forth by Jacobsen & Omland (2011), including strong support for ancient admixture during the history of the Hawaiian duck. Although secondary contact between mallards and many of the monochromatic forms has resulted in the production of viable offspring (Avise *et al.*, 1990), a form of isolation via Haldane's rule (Haldane, 1922) has been noted for other species within this young radiation (Kirby *et al.*, 2004). However, given that the mallard clade presumably radiated from Africa around 1 million years ago (Palmer, 1976; Johnson & Sorenson, 1999), with the most recent divergences within

the New World taxa dating to ~300 000 years ago (Lavretsky *et al.*, 2014a), complete reproductive isolation has not been reached in these ducks (criteria of Schumer *et al.*, 2014). Nevertheless, the Hawaiian duck is diagnosable at both molecular and phenotypic markers, with strong posterior support for monophyly at mtDNA (Lavretsky *et al.*, 2014b). Moreover, regardless of whether the Hawaiian duck's genome resulted from hybridization between the two putative parental taxa or introgression of mallard DNA on the heels of divergence between the Laysan duck and a proto-Hawaiian duck, both lineages contributed to the gene pool of what we recognize as the Hawaiian duck today. Given that the genetic and phenotypic diagnosability of the Hawaiian duck satisfies all primary criteria used in avian taxonomy for species designations (see table 1 in Sangster, 2014), as well as it being ecologically distinct (Engilis *et al.*, 2002; Uyehara *et al.*, 2008), we suggest that the Hawaiian duck may represent a young hybrid species (Gill, 2014). In conjunction with molecular data, future studies on behavioural and morphological work should focus on mate preference in areas where Hawaiian ducks and feral mallards currently coexist to test for the presence of assortative mating within Hawaiian ducks. If Hawaiian ducks display strong assortative mating, then we could conclude that they are at least partly isolated from both parental taxa. Moreover, assortative mating would suggest that concern over widespread hybridization with mallards (Engilis *et al.*, 2002; Fowler *et al.*, 2009) may not be warranted and that plumage variability, and specifically 'mallard-like' phenotypes, might be a result of retention of ancestral traits rather than contemporary hybridization.

Conclusion

There is growing evidence that speciation is not simply bifurcating lineages (Rieseberg *et al.*, 2003; Nolte *et al.*, 2005; Brelsford *et al.*, 2011; Consortium, 2012; Keller *et al.*, 2012). Specifically, studies are increasingly inferring a role of gene flow on species' evolutionary trajectories (Mallet, 2005, 2007; Abbott *et al.*, 2013). In the case of the Hawaiian duck, a simple bifurcating history was supported within species trees reconstructed with either mitochondrial or nuclear markers (Lavretsky *et al.*, 2014b); however, finer scale analyses revealed that their genome was most consistent with widespread, heterogeneous admixture of two closely related taxa. Additionally, by defining and testing several *a priori* hypotheses, we were able to establish the evolutionary time frame of the gene flow event(s) as ancestral, rather than contemporary. Thus, we conclude that gene flow has played an integral role, rather than an amalgamating force due to recent secondary contact, in the evolution of the Hawaiian duck. Future studies focusing on systems affected by gene flow should consider these alternative scenarios by establishing and testing several *a priori* hypotheses to provide independent lines of evidence regarding the effect of gene flow on the speciation process.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 *Structure* results using 16 SNPs across 8 taxonomic units of the mallard complex under (A) $K = 2$ and (B) $K = 3$ scenarios.

Figure S2 Posterior distribution of t1 (i.e. basal lineage divergence) and t0 (i.e. divergence within the sister relationship) divergence estimates under the (A) nuDNA-like or (B) mtDNA-like topology (see Fig. 3).

Table S1 Sample ID, origins, sex, and list of GenBank accession numbers.

Table S2 Characteristics of 19 nuclear loci.

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