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RESEARCH ARTICLE

# Identification of endangered Hawaiian ducks (*Anas wyvilliana*), introduced North American mallards (*A. platyrhynchos*) and their hybrids using multilocus genotypes

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**Abstract** Hawaiian ducks (*Anas wyvilliana*), or koloa, are endemic to the Hawaiian Islands and are listed as a federal and state endangered species. Hybridization between koloa and introduced mallards (*A. platyrhynchos*) is believed to be a primary threat to the recovery of koloa. We evaluated the utility of two sets of nuclear markers (microsatellite loci and amplified fragment length polymorphisms) and a variable portion of the mitochondrial DNA control region to distinguish among koloa, mallards, and hybrids. We show that microsatellite and AFLP markers can be used to distinguish between koloa and mallard-koloa hybrids with a high degree of confidence. For all but one of the putative koloa in our sample, the posterior probability of belonging to the koloa category was >0.90. Similarly all but one of the mallard-koloa hybrids were assigned to the hybrid category with posterior probabilities >0.98. Subsets of markers led to poorer resolution among koloa, mallard and hybrid categories. Among a sample of 61 koloa, hybrids and mallards, we found 25 different mtDNA haplotypes, belonging to two groups of haplotypes (A and B) identified previously in mallards and their relatives. All putative koloa samples exhibited group B haplotypes, of which 65% comprised one haplotype, while the rest were divided among four haplotypes. All Hawai'i mallard samples exhibited haplotypes that belonged to group A. Hybrids and California mallards exhibited haplotypes belonging to both groups, but a majority were of group A, suggesting that hybridization may more commonly involve mating between Hawai'i mallard females and koloa males.

**Keywords** Hawaiian ducks · *Anas wyvilliana* · Hybridization · Genetic divergence · Asymmetric introgression

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## Introduction

Introduction of non-native species continues to be one of the most pervasive and pressing global conservation concerns (Donlan et al. 2003; Clavero and García-Berthou 2005). This problem is compounded when non-native species hybridize with local taxa, particularly when those taxa are already rare or in decline. Hybridization can contribute to population declines in two ways (Wolf et al. 2001): (1) if hybrids exhibit reduced viability or fertility, the growth rate of the population of the taxon of concern will be negatively impacted; and (2) if hybrids are viable and fertile, and backcross to parental populations, mixing of gene pools of the formerly distinct taxon by introgression occurs, potentially leading to the loss of genetic distinctiveness (swamping of the gene pool) of the native taxon (Rhymer and Simberloff 1996). Extinction by introgression has become a widespread concern for conservation biologists (Rhymer and Simberloff 1996; Wolf et al. 2001). This problem has become particularly prevalent for several species of waterfowl (family Anatidae), a group for which interspecific hybridization is disproportionately common (Grant and Grant 1992) and hybrids are known to be fertile (Tubaro and Lijtmaer 2002). For example, several species of native ducks have been impacted by hybridization with feral mallards (*Anas*

*platyrhynchos*). Mallards have a holarctic distribution; however, because of their popularity, they have been introduced into other regions, especially in the southern hemisphere, where they have hybridized with closely related endemic species with limited distributions, resulting in subsequent introgression (Rhymer and Simberloff 1996). In several cases, the level of introgression is sufficiently high that it has become a major conservation concern (e.g., the grey duck *A. superciliosa superciliosa* in New Zealand, the African black duck *A. sparsa* and yellow-billed duck *A. undulata* in southern Africa, and the Florida mottled duck *A. fulvigula* and black duck *A. rubripes* in North America (Rhymer and Simberloff 1996)).

The Hawaiian duck (*A. wyvilliana*), known locally as the koloa maoli ("native duck") or simply koloa, is an endemic species found only on the Hawaiian Islands. Koloa were once common, but are now listed as an endangered species both federally and by the State of Hawai'i (Griffin et al. 1989). The koloa is also listed as endangered in the IUCN Red list (IUCN 2007) because "it is inferred to have a very small and fragmented range on a few islands, where wetlands are being lost and degraded, and where hybridization is slowly reducing the number of pure individuals". Historically, koloa inhabited many of the main Hawaiian Islands, but were extirpated from all islands except Kaua'i and Ni'ihau by 1962 (Engilis et al. 2002). This statewide decline has been attributed to the draining of wetlands, indiscriminate hunting, and introduced mammalian predators (Engilis et al. 2002). There has been some progress in these areas: hunting is no longer allowed, predators are being controlled, freshwater wetlands are better managed, and there are on-going efforts to protect and restore the remaining wetlands (Engilis et al. 2002). Hybridization between koloa and domesticated mallards is now considered by the United States Fish & Wildlife Service to be the primary threat to the recovery of the koloa (USFWS 2005; Engilis et al. 2002). Mallards were first brought to Hawai'i in the late 1800s, and large numbers of mallards were imported from North America in the 1950s and 1960s to stock hunting areas. Many of these populations are now feral. Koloa are known to hybridize readily with mallards and the hybrid offspring are viable and fertile. Recovery plans indicate that the integrity of the koloa as a species is in jeopardy if the hybridization problem is not addressed (USFWS 2005; Engilis et al. 2002).

There have been several attempts to re-introduce the koloa to parts of its former range. After they were extirpated from most of the islands, koloa were successfully re-established on the island of Hawai'i, mainly through captive propagation and release beginning in the 1950s (Engilis et al. 2002; Fig. 1). The origin of the captive flock was the island of Kaua'i which at the time had an estimated 3,000 koloa (Swedberg 1967). Reintroduction efforts also occurred on O'ahu and Maui, but these koloa were released into areas where feral domesticated mallards were common, and these populations now almost completely comprise mallard-koloa hybrid swarms (Engilis et al. 2002; Fig. 1). The state population has been estimated at around 2,500 koloa total, with around 2,000 on Kaua'i and Ni'ihau, 300 on O'ahu, 25 on Maui, and 200 on Hawai'i (Engilis and Pratt 1993; USFWS 2005). However, these estimates may be inaccurate. On one hand, the surveys did not include remote montane streams and wetlands where koloa reside, possibly leading to an underestimate of the number of birds; on the other hand, many of the birds counted during the surveys on O'ahu and Maui were likely hybrids, thus inflating the estimate (Engilis et al. 2002; Rhymer 2001). Mallard-koloa hybrids have now spread from the islands of O'ahu and Maui to both Kaua'i and Hawai'i, where pure populations were thought to have existed in the past (Engilis et al. 2002; Fig. 1).

The Revised Recovery Plan for Endangered Hawaiian Water birds (USFWS 2005) mandates the removal of all mallards and koloa-mallard hybrids from the islands to prevent further hybridization. This task is severely complicated by the fact that mallard-koloa hybrids are very difficult to distinguish from pure koloas in the field. Moreover, since hybrids are reproductively viable, backcrosses are likely, further blurring the distinction between pure koloa and hybrids. Koloa are a monochromatic species closely related to mallards but considerably smaller. Hybrids have intermediate sizes and intermediate plumage characteristics (Engilis et al. 2002; Engilis unpublished data). The recovery task includes development of field methods to enable managers to differentiate between hybrids and pure koloas in the wild so that all birds that are not pure koloa can be removed without inadvertently affecting the koloa population.

Developing a field key is a three-step process, requiring: (1) a method to accurately differentiate hybrid individuals from pure koloa stock; (2) an analysis of morphological or plumage characteristics that differ between hybrid and pure individuals, and (3) an assessment of whether these characteristics can be used to distinguish koloa from hybrids in the field. In the present study, we focus on the first step in this process; specifically, our objective is to develop genetic markers to evaluate and compare genetic differences among koloa, mallards and mallard-koloa hybrids, with the ultimate goal to identify hybrids. To do so, we use three sets of variable molecular markers: two nuclear markers (microsatellite loci and amplified fragment length polymorphisms, AFLPs) and a variable portion of the mitochondrial DNA (mtDNA) control region. We contrast the efficacy of each of these sets of markers alone and in combination, and we use a Bayesian statistical approach in the program NewHybrids (Anderson and Thompson 2002)

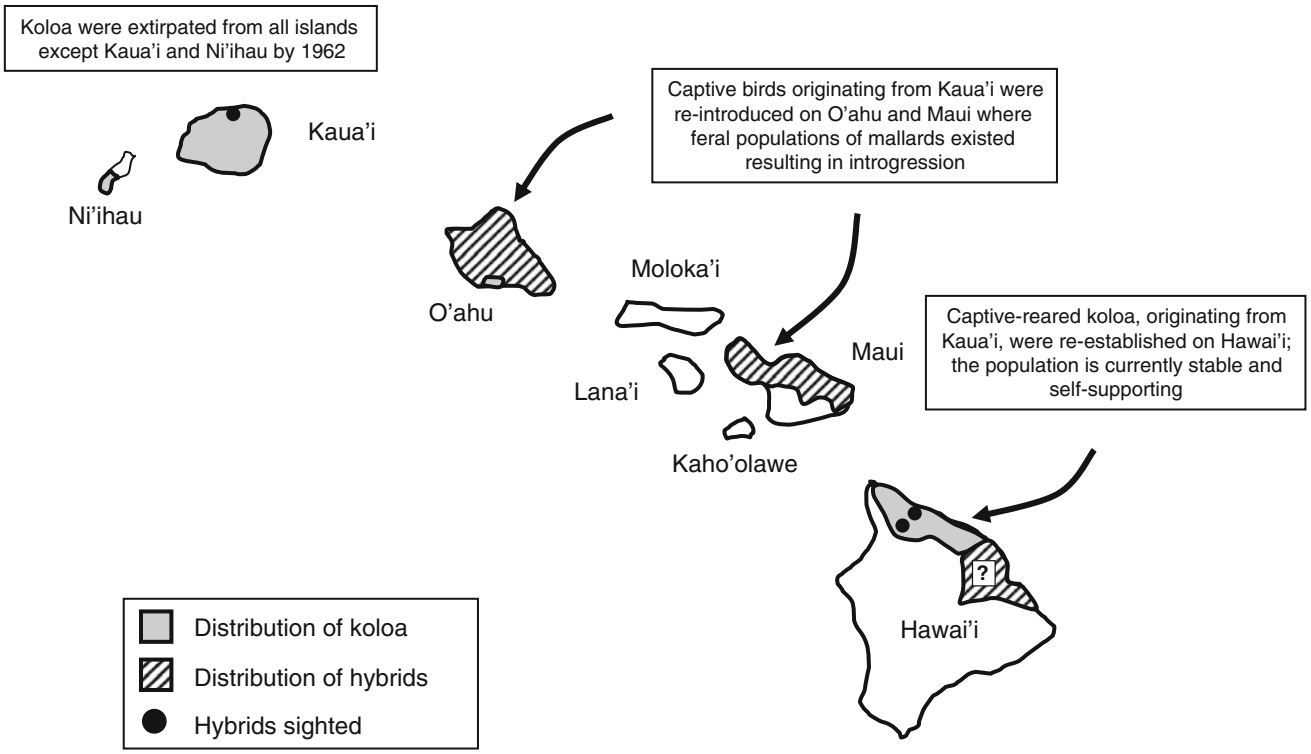


**Fig. 1** Map of the Hawaiian Islands showing the distribution of koloa (*light gray areas*) and mallard-koloa hybrids (*hatched areas*) and a brief description of the sequence of extirpation and reintroduction (after Engilis et al. 2002)

to determine the posterior probability that a sampled individual belongs to hybrid or pure categories.

## Methods

We obtained 23 specimens of putative koloa from Kaua'i, where they were picked up dead during botulism outbreaks between 2002 and 2005. All birds on Kaua'i have, until recently, been thought to be pure koloa (Engilis et al. 2002). Eleven birds that were thought to be mallard-koloa hybrids and 10 birds thought to be mallards (hereafter Hawai'i mallards) were collected from Maui and O'ahu, where they were either picked up dead during botulism outbreaks or shot by federal resource management USDA-APHIS officials. All specimens were classified initially using measurements of size and mass, and plumage characteristics (details in Engilis et al. 2002; Engilis unpublished data). However, our analysis of genetic differences among pure strains and hybrids is not based on these a priori morphological classifications—rather, we used these simply to screen genetic markers that might prove informative, and to compare the posterior probabilities derived from Bayesian statistical analysis with the putative identifications based on morphology.

We sampled muscle tissue and feathers from all specimens. We used tissue samples to extract nuclear DNA and feather samples to extract mtDNA (see next section for rationale of using feather samples and avoiding problems of amplifying nuclear sequences of mitochondrial origin). Muscle tissues and specimens (as vouchers) are archived at the Museum of Wildlife and Fish Biology, University of California, Davis, CA, USA. For an independent sample of known mallard stock, we collected 20 tissue samples from hunter-shot North American birds in Butte County, California during the winter of 2004 (hereafter California mallards).

### DNA analysis

We extracted DNA from the feather and tissue samples using Qiagen DNeasy Tissue Kits. To increase the quantity of DNA from feather quills, we added 20 µl of 1 M dithiothreitol to the digestion buffer, incubated the mixture overnight, and then proceeded with the extraction. DNA was quantified and diluted to 50 ng/ml for use in all the PCR reactions.

Initially, we screened six koloa, four putative mallard-koloa hybrids, and two California mallards for allelic variation at 55 biparentally inherited microsatellite loci (Aph1–Aph25 (Maak et al. 2000, 2003), Aal$\mu$1 and Sfi$\mu$1, Sfi$\mu$3, Sfi$\mu$4, Sfi$\mu$5, Sfi$\mu$7, Sfi$\mu$8 (Fields and Scribner 1997), Bca$\mu$10, Bca$\mu$11 and Hhi$\mu$5 (Buchholz et al. 1998), Apl2, Apl12, Apl13, Apl14, Apl23, Apl26, Apl36 (Denk et al.

2004), cmAAT16, cmAAT28 cmAAT35 cmAAT38 (Stai and Hughes 2003), Smo4, Smo6–Smo9, Smo10, Smo11, Smo13, (Paulus and Tiedemann 2003), Anas (Cathey et al. 1996)); a subset of loci were further screened with 10 koloa, 6 hybrids, 6 Hawaiian mallards and 10 California mallards. For each primer, 50 ng of template DNA was amplified in 10 μl reaction volumes with PCR buffer (20 mM Tris–HCl pH 8.4, 50 mM KCl), 1.5–2.5 mM $MgCl_2$, 200 μM dNTPs, 0.4 μM of each forward and reverse primer pair, and 0.5 U Invitrogen Taq DNA polymerase. Thermocycler conditions for PCR consisted of one cycle of 94°C denaturation for 2 min; 35 cycles of locus-specific annealing temperatures for 45 s, 72°C extension for 45 s, and 94°C denaturation for 45 s; followed by one cycle of locus-specific annealing for 1 min and 72°C extension for 5 min.

Similar to the microsatellite loci, we screened three koloa, three mallard-koloa hybrids, and three California mallards with 40 pairs of AFLP primers. We tested all combinations of five Eco primers (AAC, ACC, AGG, AAG, and AA) and eight Mse primers (CAG, CGC, CGG, CTA, CTG, CAT, CAA, and CTC). EcoA adapters were produced by annealing the primers EcoA1 [ctcgta gactgcgtacc] and EcoA2 [aattggtacgcagtctac], and MseA adapters were produced by annealing MseA1 [gacgatgagtc ctgag] and MseA2 [tactcaggactcat] primers. Lyophilized primers were first diluted in 10 mM Tris pH 8.0; 50 mM NaCl; 1 mM EDTA to a concentration of 650 μM. Equimolar concentrations (650 μM) of each set of primers (EcoA1 with EcoA2 and MseA1 with MseA2) were combined and denatured at 95°C for 3 min and annealed by gradual cooling to room temperature. Annealed EcoA adapters were then diluted to 5 μM and MseA adapters to 50 μM with TLE (10 mM Tris–HCl pH 8.0, 0.1 mM EDTA).

The digestion and ligation reactions were carried out simultaneously in volumes of 20 μl, containing 250 ng genomic DNA, 1× T4 ligase buffer, 1 U MseI, 5 U EcoRI, 1 U T4 DNA ligase, 0.05 mg/mL BSA (all products from New England BioLabs), 50 mM NaCl, 156 nM EcoA adapter, and 15.6 nM MseA adapter. After digestion overnight at room temperature, the product was diluted tenfold with 180 μl of TLE. Preselective PCR was performed in volumes of 20 μl, containing 1× PCR Buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.3 μM of each of the Eco and Mse primers with a one-base extension (A and C for the Eco and Mse primers, respectively), and 0.16 U of Taq DNA polymerase (all products from Promega). The thermocycler conditions were 94°C for 2 min; 19 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 2 min; and a terminal step of 60°C for 30 min. The product was again diluted to 200 μl with TLE and kept at 4°C. PCRs were performed in total volumes of 10 μl, containing 3 μl of the diluted preselective product, 1× PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.5 μM each of the Eco and Mse primers (with two- or three-base extensions), and 0.16 U Taq DNA polymerase (all products from Promega). The Eco primers were 5′-labeled with fluorescein (Invitrogen). Thermocycler conditions for selective PCR were 94°C for 2 min; 9 cycles of 94°C for 20 s, 66°C for 30 s, 72°C for 2 min; 19 cycles of 94°C for 20 s, 56°C for 30 s, 72°C for 2 min; and a terminal step of 60°C for 30 min.

Amplification products were separated on 5.5% denaturing polyacrylamide gels, and scanned with a Molecular Dynamics Fluorimager 595 (Molecular Dynamics, Sunnyvale, CA, USA). Base-pair designations were assigned using a 10 bp ladder (The Gel Company, San Francisco, CA, USA). We manually scored both microsatellite loci and AFLP markers from the fluorimager output. For both microsatellite loci and AFLP markers, samples were amplified and run multiple times to check for band repeatability and for scoring errors. For microsatellite markers, roughly 1/5 or 15 individuals (different individuals for each locus) were amplified 2–4 times and used as standards on gels; no errors were recorded. For AFLP markers, 15 individuals were amplified at least twice and 6 of these were used as standards on every gel (3 gels per primer pair); again no errors were recorded at the selected bands. For this study, similar sized bands were assumed to be homologous.

During the initial screening, we focused our search for loci and bands that appeared to differ in frequency between groups. Our intent was to find a subset of markers that might prove most effective and efficient (time and expense) in identifying pure koloa and hybrids. Thus, our study was not intended to be a systematic survey of genetic variation among koloa, mallards and hybrids; rather, our goal was to find markers that would prove most useful to conservation managers. Microsatellite markers were considered for further screening if there appeared to be little overlap of alleles between koloa and hybrids. For the AFLP markers, we selected primer pairs with bands that were dark and repeatable in individuals from one run to another and, similar to the microsatellite loci, appeared to exhibit differences in frequency among groups.

A fragment of the 5′ end of the mtDNA control region was amplified using template DNA with control region primers L78 and H493 (Sorenson and Fleischer 1996). PCR conditions were modified from Sorenson et al. (1999) and optimized use in our laboratory. PCR was carried out in 50 μl reactions containing 150 ng of template DNA, 1× GeneAmp Applied BioSciences PCR Gold Buffer (15 mM Tris–HCl pH 8.0, 50 mM KCl), 1.25 mM $MgCl_2$, 200 μM dNTPs, 0.5 μM of each primer, and 1.0 U Applied BioSciences AmpliTaq Gold. Thermocycler conditions for PCR consisted of one cycle of 95°C denaturation for

10 min; 35 cycles of 58°C annealing temperature for 1 min, 72°C extension for 1.5 min, and 94°C denaturation for 45 s; followed by one cycle of 58°C annealing for 1 min and 72°C extension for 10 min. The PCR products were gel-purified in a 1.0% agarose gel at 100 volts for about 1 h, excised and recovered using a Qiagen QIAquick Gel Extraction Kit. Products were cycle-sequenced on an ABI 3730 Capillary Electrophoresis Genetic Analyzer using BigDye, version 3, at the Department of Biological Sciences Automated Sequencing Laboratory, University of California, Davis, CA, USA. For each sample, the heavy and light strands were sequenced and aligned using ClustalX (Thompson et al. 1997). Sequences have been submitted to GenBank (Accession Numbers: EU399761–EU399785).

Waterfowl are well-known to exhibit transpositions of copies of mitochondrial sequences to the nuclear genome (nuclear sequences of mitochondrial origin or "Numts"; Sorenson and Quinn 1998) and it is possible to co-amplify the nuclear and mitochondrial copies of mtDNA sequences. This problem is particularly apparent when analyzing blood samples, since mature avian red blood cells are nucleated, but are relatively deficient in mtDNA, increasing the likelihood that a nuclear copy of the sequence will be amplified. Feather samples are less problematic than blood samples with respect to nuclear contamination (Sorenson and Quinn 1998), therefore, we used only feather samples to extract and sequence mtDNA. We examined all mtDNA sequence data derived from feather samples for ambiguous sequences and found no evidence to suggest that nuclear copy sequences were amplified—in all samples, mtDNA sequences were resolved unambiguously and corresponded closely with similar control region sequences reported for mallards by Kulikova et al. (2005), see "Results".

## Data analysis

For the microsatellite analyses, we calculated observed heterozygosity and tested for linkage disequilibrium and deviations from Hardy–Weinberg equilibrium (HWE) in GENEPOP (Raymond and Rousset 1995). Because our groups are not true populations per se, but rather species and hybrids, we would not necessarily expect them to meet Hardy–Weinberg expectation. We tested for HWE to evaluate the possibility that null alleles or linkage among loci might exist within or among groups. We conducted these calculations separately for each group (putative pure koloa, putative hybrids and putative Hawai'i, and California mallards). We were also interested in the degree of genetic differentiation among groups. Again, our goal was not a systematic assessment of genetic variation among groups, but rather to examine the degree of difference detectable using the markers thought, a priori, to be most effective in distinguishing among these groups. For microsatellite and AFLP markers, we used TFPGA version 1.3 (Miller 1997) to calculate the theta ($\theta$) estimator of Wrights $F_{ST}$ between all pairs of groups. TFPGA uses a bootstrapping procedure (1,000 permutations) to build the 95% confidence intervals to evaluate the level of significance. We used ARLEQUIN version 2.0 (Schneider et al. 2000) to test for group differentiation using AMOVA in microsatellite loci, AFLP markers, and mtDNA haplotypes and to calculate pairwise group comparisons of $\Phi_{ST}$ for mtDNA. We used TCS (Clement et al. 2000) to illustrate unrooted haplotype networks.

Several methods exist to identify hybrid individuals using genetic data, including methods based on alleles that are unique to each species and methods that derive the conditional probability of an individual's multilocus genotype, given parental allele frequencies (reviewed in Anderson and Thompson 2002). A problem for many studies of hybridization is such methods require that allele frequencies be known for the parental species and/or separate, pure (usually allopatric) samples of the parental species be available (Anderson and Thompson 2002; Munoz-Fuentes et al. 2007). Anderson and Thompson (2002) developed a new Bayesian statistical method that instead computes the posterior probability that an individual belongs to a hybrid category. This method has several advantages, including: (1) it does not require that parental classes be sampled separately; (2) it does not require that loci be diagnostic or even that species possess unique alleles, instead making use of information in frequency difference of alleles that are not fixed in either species; (3) it incorporates uncertainty, given that allele frequencies are always estimated; and (4) the posterior probability reflects the level of certainty that an individual belongs to a hybrid category and so provides an explicit probability of misclassification. This method is similar to the Bayesian approach adopted by Pritchard et al. (2000) to analyze structured populations, but does so using an explicit inheritance model for populations known to consist of pure individuals and recent hybrids of two species (Anderson and Thompson 2002).

We used NewHybrids (Anderson and Thompson 2002) to evaluate the reliability of microsatellite and AFLP markers in assigning individuals to hybrid and pure categories based on their multilocus genotype. An additional advantage of the program NewHybrids is that it is capable of including both AFLP and microsatellite loci in a single analysis. The program computes a posterior probability that an individual belongs in each of several possible categories, including pure strains of either species, various generations of hybrids, or backcrosses to either parental species. Without large numbers of genetic markers, it is often difficult to distinguish between all hybrid categories

after even 2 or 3 generations of potential interbreeding (Anderson and Thompson 2002). Accordingly, we set the program to compute posterior probabilities for only six genealogical classes (pure koloa, pure mallard, F1 and F2 hybrids, and F1 backcrosses to each parental species). We then pooled F1, F2 and F1- backcross categories into a single category ('hybrids'), such that our final comparisons focused on assignments to the two pure parental categories and one category of various types of hybrids. We assumed that California mallards were pure mallards and designated them as such using the *Z* option, implemented in NewHybrids. As recommended, we used a burn-in run of at least 5,000 sweeps and ran >125,000 sweeps.

## Results

### Microsatellites and AFLPs

Of the 55 microsatellite primer pairs screened for a subset of koloa and hybrid samples, 37 (67%) did not amplify, exhibited a fixed pattern (monomorphic, uninformative), or the alleles overlapped extensively between species (polymorphic, uninformative). Eighteen possibly informative primer pairs (i.e., little overlap between koloa and mallard) were screened further with a larger sample of ducks and 10 primer pairs were selected (Table 1). As with the microsatellite screening, we screened 40 pairs of AFLP primers in a subset of ducks and selected primer pairs with bands that were dark and repeatable in individuals from one run to another and those that appeared to have frequency differences between hybrids and pure individuals; by these criteria, we found 29 fragments with 13 primer pairs (Table 1). No single marker for either the microsatellite loci or the AFLP fragments was diagnostic (i.e., present in only one group).

Alleles at the 10 microsatellite markers ranged from 3 to 18 per locus, with the largest number of alleles per locus in California mallards (Table 2). We found few deviations from Hardy–Weinberg equilibrium (HWE) for the microsatellite loci; only Apl11 deviated significantly ($P = 0.006$) due to a heterozygote deficiency in California mallards ($P = 0.003$). None of the overall tests for each group revealed deviations from HWE (Table 2) and no loci deviated from linkage equilibrium after correcting for multiple comparisons (analyses conducted separately for each group). We were not able to amplify products for all samples for all markers, and our final sample sizes for microsatellite and AFLP analyses were 19 putative pure koloa, 10 mallard-koloa hybrids, 10 putative Hawai'i mallards, and 20 California mallards.

For both the microsatellite and AFLP markers, calculations of $\theta$ (Table 3) indicated that there was significant structuring of genetic diversity among most of the sampled groups, based on the set of markers analyzed. In pairwise group comparisons of $\theta$, only the 95% confidence intervals from the mallard-koloa hybrid and Hawai'i mallard comparison overlapped with 0, indicating differences among most groups. Similarly, AMOVA revealed highly significant differences among groups for both microsatellite ($\Phi_{ST} = 0.12$, $P < 0.001$) and AFLP markers ($\Phi_{ST} = 0.36$, $P < 0.001$).

**Table 1** A list of microsatellite loci and AFLP primer pairs used for identifying koloa, hybrids and mallards

| Marker type | Primers |
|---|---|
| Microsatellite loci | Aph9 (12), Aph13 (12), Aph15 (3), Aph16 (7), Aph18 (4), Aph21 (18), Aph22 (6), Smo7 (8), cmAAT16 (14), Apl11 (13) |
| AFLP primer pairs | ACC-CAG (4), ACC-CGC (1), ACC-CGG (3), ACC-CTA (1), ACC-CTC (1), ACC-CTG (3), AA-CGG (2)[b], ACC-CGG (1), ACC-CTA (2), AGG-CGC (3), AGG-CAT (2), AAG-CGG (5), AAG-CAT (1) |

Number of alleles found at each microsatellite locus or the number of bands scored per fragment from each AFLP primer pair is in parentheses

[a] Primer sources: Aph (Maak et al. 2000, 2003), Apl (Denk et al. 2004), cmAAT (Stai and Hughes 2003), Smo (Paulus and Tiedemann 2003)

[b] Only unique bands (those not found in 3 bp motifs) were used from this 2-basepair extension of Eco

**Table 2** Sample sizes (*N*) and population genetic diversity indices calculated from 10 microsatellite loci (first four columns) and haplotype diversity (*H*) and nucleotide diversity ($\pi$) calculated from mtDNA (last four columns) for koloa, mallard-koloa hybrids and Hawaiian and California mallards

| | *N* | Average no. alleles | Observed/Expected heterozygosity | Hardy–Weinberg probability test | *N* | Number of haplotypes | *H* | $\pi$ |
|---|---|---|---|---|---|---|---|---|
| Koloa | 25 | 5.2 | 0.50/0.52 | 0.13 | 23 | 5 | 0.56 ± 0.11 | 0.04 ± 0.03 |
| Hybrids | 12 | 4.4 | 0.59/0.63 | 0.13 | 11 | 4 | 0.78 ± 0.07 | 0.21 ± 0.12 |
| Hawaiian mallards | 12 | 4.8 | 0.66/0.65 | 0.21 | 8 | 4 | 0.82 ± 0.10 | 0.03 ± 0.02 |
| California mallards | 19 | 8.0 | 0.65/0.69 | 0.11 | 19 | 17 | 0.98 ± 0.03 | 0.26 ± 0.14 |

**Table 3** Pairwise group comparisons of $\theta$ (for microsatellite and AFLP) or $\Phi_{ST}$ (for mtDNA) for genetic variation among putative pure koloa, mallard-koloa hybrids and Hawaiian and California mallards

| Groups | Koloa | Hybrids | Hawaiian mallard |
|---|---|---|---|
| Microsatellites loci | | | |
| Hybrids | 0.11* | | |
| Hawaiian mallard | 0.17* | 0.02 | |
| California mallard | 0.15* | 0.09* | 0.10* |
| AFLP markers | | | |
| Hybrids | 0.33* | – | |
| Hawaiian mallard | 0.49* | 0.11 | – |
| California mallard | 0.49* | 0.14* | 0.12* |
| Mitochondrial DNA | | | |
| Hybrids | 0.66* | | |
| Hawaiian mallard | 0.91* | 0.20 | |
| California mallard | 0.39* | 0.03 | 0.34* |

All significant comparisons (*) are those whose 95% confidence intervals do not overlap with zero

## Bayesian analysis of hybrid categories

Using the program NewHybrids, we found that microsatellite and AFLP markers were effective at distinguishing between pure koloa and hybrid individuals. For 18 of the 19 putative koloa, the posterior probability that an individual belonged to the pure koloa category was very high: 11 were >0.99, 16 were >0.95 and 18 were >0.90 (Fig. 2a). The posterior probability for a single bird that was thought to be a pure koloa based on plumage and morphological measurements (Ko 5887) was instead highest for the hybrid category (>0.99, Fig. 2a). Most (9 of 10) of the mallard-koloa hybrids were assigned to the hybrid category with posterior probabilities >0.98 (Fig. 2a). Assignments of Hawai'i mallards were mixed; posterior probabilities for over half of these birds (6 of 10) were highest (>0.99) for the hybrid category. For the remaining four birds, the posterior probability was highest for the pure mallard category, although confidence was

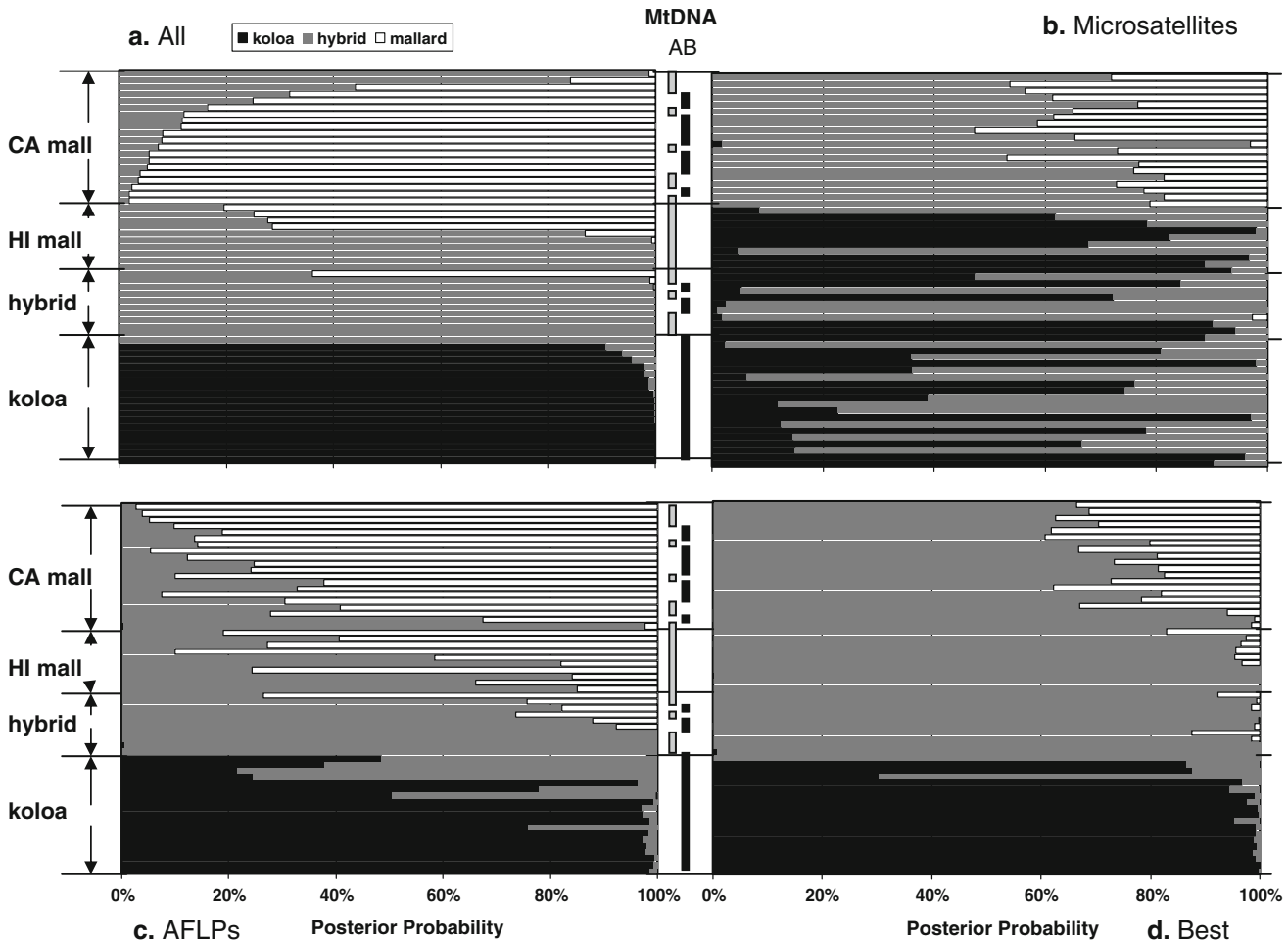


**Fig. 2** The posterior probability that individuals from sampled groups belong to either pure koloa (*black*), pure mallard (*white*), or hybrid (*shaded*) categories as determined using all markers (**a**), only microsatellite markers (**b**), only AFLPs (**c**) and the best subset of markers (**d**). Center panel shows mtDNA haplotype group for each individual

lower (>0.70, Fig. 2a). All but two of the 20 California mallards were assigned to the mallard category, although posterior probabilities were lower (Fig. 2a). Those two California mallards were assigned to the hybrid category, possibly indicating retained poylmorphisms rather than evidence of hybridization. Most importantly, none of the mallard-koloa hybrids, Hawai'i mallards, or California mallards had posterior probabilities of belonging to the pure koloa category >0.0001 (Fig. 2a). Consequently, the probability of a bird being identified genetically as a pure koloa when it appears morphologically to be a hybrid or mallard is very small.

Conservation managers may desire a smaller subset of markers to classify birds in the field, saving time and expense. To determine whether a smaller number of markers would prove as effective in assigning birds to pure or hybrid categories, we repeated the Bayesian analysis of hybrid categories using program NewHybrids under the following conditions: (1) using only the microsatellite markers (Fig. 2b); (2) using only the AFLP markers (Fig. 2c); and (3) using the best subset of each (Fig. 2d). In the later case, we chose the five most informative microsatellite loci and 10 most informative AFLP markers, based on their Kullback–Leibler divergence calculated by NewHybrids. Analyses based on only microsatellite markers served to differentiate California mallards from all other groups; however the posterior probabilities were rather low (most were <80%). The microsatellite marker analysis did not differentiate between putative koloa and hybrids or Hawai'i mallards (Fig. 2b). In contrast, AFLP markers were generally better at separating pure koloas from all other categories (Fig. 2c). The subset of "best" microsatellite and AFLP markers (Fig. 2d) increased the level of resolution by which pure koloa could be distinguished, although the resolution between hybrids and pure mallards was poor. None of the subsets of markers resolved koloa and hybrids as effectively as did the analysis using all the markers combined (Fig. 2a).

## mtDNA

We sequenced a 350 bp portion of the control region of mtDNA from most samples. We found 39 variable sites, corresponding to 25 different haplotypes (Fig. 3). Of the 39 variant positions, 37 were transitions (>75% between C and T), one was a transition with a transversion, and one was a deletion. The deletion represents the break between group A and B haplotypes as first described by Avise et al. (1990) in mallards. Most of the koloa samples (>65%) exhibited a single mtDNA haplotype (J, Fig. 3). The koloa, hybrid, and Hawai'i mallard groups had fewer haplotypes than did California mallards and mtDNA diversity indices appeared to be lower (Table 2).

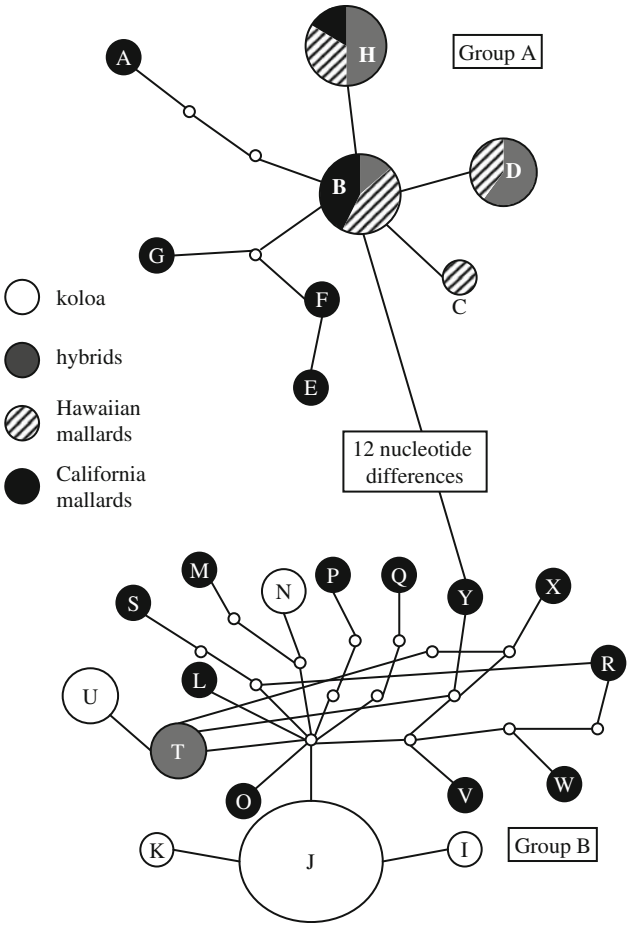


**Fig. 3** An unrooted network illustrating the relationship between mtDNA control region haplotypes found in koloa, hybrids, and Hawaiian and California mallards. Letters are unique haplotypes and the size of each *circle* is roughly proportional to the number of individuals with that haplotype. The *smaller circles* indicate possible intermediate haplotypes that were not sampled

Within groups A and B, many of the haplotypes were closely related, as depicted by the unrooted haplotype network (Fig. 3). There was significant divergence between the haplotypes in groups A and B, with 12 nucleotide differences (Fig. 3). Within each group, haplotypes differed by one to three base pairs, with the mallard haplotypes being most divergent. As seen with the nuclear markers, we found large amounts of genetic variation between groups, and the AMOVA was highly significant for the mtDNA control region ($\Phi_{ST} = 0.49$, $P < 0.001$). All pairwise group comparisons of $\Phi_{ST}$ were significant except those between the mallard-koloa hybrids, which did not differ from either Hawai'i or California mallards (Table 3).

All putative koloa samples exhibited group B haplotypes, whereas Hawai'i mallard samples were all from group A (Fig. 3). The mallard-koloa hybrids and California mallards were split between groups A and B (Fig. 3).

However, 7 of the 11 putative hybrids exhibited mtDNA haplotypes belonging to the A group, suggesting hybridization between female Hawai'i mallards (group A) and male koloas. Likewise, the six Hawai'i mallards with a high (>0.99) posterior probability of being a hybrid also exhibited mtDNA haplotypes belonging to the A group, further suggesting asymmetric hybridization events involving female mallards and male koloas.

## Discussion

Our goals in this study were twofold: first, we sought to identify molecular markers to distinguish between hybrids and koloas as a first step in providing conservation managers with a way to identify hybrids in the field; and second, we wished to evaluate the relative genetic contributions of mallards and koloas to the hybrid genotypes. We successfully used AFLP and microsatellite markers to distinguish between pure koloa and mallard-koloa hybrids with a high degree of confidence. All but one of the putative koloas had posterior probabilities >0.90 of being assigned to the pure koloa category, and based on more detailed examination of metrics and plumage features, the one outlier was likely a hybrid. Similarly, birds that were thought to be hybrids or mallards, based on morphology, had the highest posterior probabilities of belonging to those categories, and, most importantly, their probability of being assigned to the pure koloa category was <0.0001. These results indicate that koloa can be distinguished reliably from hybrids and Hawai'i mallards using the genetic markers we describe.

One of the advantages of the Bayesian analysis in the program NewHybrids (Anderson and Thompson 2002) was that there was no assumption about parental origins or hybrid status of birds in our sample; posterior probabilities of individuals belonging to each group are computed without prior information on group membership. This is critical in a study such as ours, where one cannot be certain that birds derived from a given locality represent pure stock. For example, although birds collected from Kaua'i were thought to be pure koloa, a small number of hybrids have been recorded previously on Kaua'i (Fig. 1). Our independent analysis using NewHybirds confirms that at least one of the putative koloa from Kaua'i may indeed have been a hybrid. Similarly, several of the birds identified as Hawai'i mallards had higher posterior probabilities of being hybrids. Accordingly, analyses based on genetic differences between putative morphotypes would have been inaccurate and could have misclassified birds. We note that we choose informative markers a priori, although this is not required for the NewHybrids analyses and other loci could have been used. Our intent was to find a small number of markers that would be of greatest utility and least expense for researchers and conservation managers in the field.

We further explored the possibility of using a smaller subset of markers to classify birds, an option that might be appealing to conservation mangers with limited access to the resources necessary to genotype birds using multiple marker systems. However, we found that including more markers in the analysis was the best option for resolving pure koloa from other categories. When we used only microsatellite or only AFLP markers in the analyses, or selected a subset of the 'best' markers from both, the degree of resolution was not as high as when all markers were included. Our results support Fallon's (2007) recommendation to use multiple markers when employing genetic data to identify species for conservation protection. Given that koloa are an endangered species and there may be a need, at some point, for even greater confidence in the identification of koloas, further resolution might be obtained by increasing the number of markers in the analyses and increasing samples from a broader geographic representation of the Hawaiian Islands.

Genetic divergence was significant among our sampled groups at AFLP and microsatellite markers. These results are not unexpected, given that we screened markers before hand to identify those that would be most informative in separating koloa from hybrids. Accordingly, we caution that our estimates of the degree of differentiation between koloa and mallards are undoubtedly exaggerated. Interestingly, even with these highly selective markers, we failed to find any significant genetic difference between the mallard-koloa hybrids and the so-called Hawai'i mallards using either AFLP or microsatellite markers. This suggests that many of the Hawai'i mallards may indeed be hybrids that have backcrossed with mostly mallard-like birds so they have a strong resemblance to mallards. The fact that several of the putative Hawai'i mallards had highest posterior probabilities of being hybrids supports this conjecture.

We found that koloa mtDNA, similar to other monochromatic North American mallard species (i.e., mottled, American black, and Mexican ducks *A. p. diazi*), most likely diverged from the group B mallard lineage (Rhymer 2001; McCracken et al. 2001; Kulikova et al. 2004, 2005). Both of the haplotype lineage types are found in the California mallards—the A group, which has a holarctic distribution (McCracken et al. 2001; Kulikova et al. 2004, 2005) and the B group, which was thought be confined to North America but has recently been found in eastern spot-billed ducks (*A. zonorhyncha*) and mallards in the Russian far east (Kulikova et al. 2004, 2005). Mallards are much more variable than the other groups, as expected given their larger population sizes. Comparing our sequences

with mallards from Kulikova et al. (2005), we found only 3 mtDNA sequences that were similar between the two studies, mallard haplotypes B and F and a hybrid haplotype T. Therefore, the variation we found in mallards represents only a very small portion of their haplotype diversity.

An AMOVA using mtDNA revealed highly significant differentiation between sampled groups, especially between koloa and all the other groups. No Hawaiian mallard had group B haplotypes and only a single group B haplotype (*T*) was found in hybrids. Even though this haplotype differed from a koloa haplotype by just a single nucleotide, this separation of haplotypes resulted in very large $\Phi_{ST}$ values, suggesting that these are very different groups of birds. Our limited sampling of both hybrids and koloa makes it likely that we missed haplotypes, and further sampling is needed to address this question.

However, the unequal distribution of group A and B haplotypes in hybrids is similar to the bias found by Kulikova et al. (2004) for spot-bills and mallards in the Russian far east, and Rhymer et al. (1994) for grey ducks and mallards in New Zealand. These data suggest that hybridization between the two species has been asymmetrical. If this were driven by greater competitive success of male mallards for access to, or choice by, female koloa (as seen in black ducks; Brodsky and Weatherhead 1984; Brodsky et al. 1988; D'Eon et al. 1994), we would expect most of the hybrids to have koloa mtDNA haplotypes (Group B). In direct contrast to this hypothesis, we found that most of the hybrids had haplotypes from group A, suggesting an opposite effect, namely that the maternal lineage in these birds is mallard and not koloa. Rhymer (2001) likewise reported an observation of a koloa-mallard hybrid with mtDNA from group A. The higher proportion of hybrids having haplotypes from group A indicates that the crosses between koloa males and mallard females must have occurred more frequently; indeed, such pairings have been observed in the wild (A. E. Jr., Personal observation). Behavioral mechanisms may favor male koloa success with female mallards and might help explain asymmetric introgression as seen in other areas where mallard stock is the result of domesticated mallards becoming feral. It is well documented that female domesticated mallards exhibit relaxed mate preference and selection behavior (Kear 1990; Oring and Sayler 1992). The active and vigorous courtship behavior of the nonmigratory male koloa might offset the smaller males' monochromatic coloration, and act as a strong signal to female domesticated mallards, possibly overriding visual cues (bright male plumage and size) that otherwise influence mate selection.

Asymmetric hybridization may alternatively be explained by the fact that mallards far outnumbered koloas when the latter were released onto O'ahu, as was suggested by Kulikova et al. (2004) for spot-bills. From 1968 to 1982, fewer than 25 koloa per year (350 total) were released into areas with substantial feral populations of mallard (Engilis and Pratt 1993). Consequently, most initial matings of koloas would have been with the much more abundant mallard. Hybrids likely backcrossed with mallards, leading to a loss of the koloa mtDNA haplotypes via males (due to maternal inheritance of mtDNA), resulting in a hybrid population comprised predominantly of mallard (group A) mtDNA haplotypes. Hybrids were not out of HWE, unlike the spot-bills examined by Kulikova et al. (2004), but this may be a result of their more extensive mating with mallards (Browne et al. 1993).

Our results suggest that the hybrids on O'ahu are more similar to mallards than to koloa. This assertion is further supported by the work of Browne et al. (1993) on allozymes in koloa and mallards. However, the hybrids identified in our study are not similar genetically to California mallards (Table 3), suggesting that North American birds (or at least those that gave rise to the California population) were not the same stock as those that originally hybridized with koloa. This leaves open the question of the origin of the domesticated mallard stock in Hawai'i. There are no records of the first introductions of mallards to Hawai'i, but mallards were first domesticated in Europe (Kear 1990) and their introduction to the islands dates back to at least the mid-1800s (Engilis et al. 2004). During that period several European species were brought to the main Hawaiian Islands (Berger 1971). In addition to feral birds, migrant mallards from the Holarctic annually stray to the islands, and as with other waterfowl, may drop out of migration and remain on the islands, potentially to breed (Udvardy and Engilis 2001; Engilis et al. 2002).

One of the last strongholds of putatively pure populations of koloa was thought to exist on Kaua'i. However, even there hybridization and introgression from introduced mallards may be occurring, as suggested by the presence of birds that, at least phenotypically, have been suspected to be hybrids (Engilis et al. 2002). Our results further substantiate that the population of koloa on Kaua'i may contain hybrids. One of the putative "pure" koloa in our sample collected from Kaua'i had a high (>0.90) posterior probability of belonging to the hybrid category.

Without efforts to prevent hybridization of mallards and koloa on Kaua'i and the other Hawaiian Islands, koloa may face a similar situation as the New Zealand grey duck, whose entire population has hybridized with introduced mallards, creating a 'hybrid swarm' (Gillespie 1985; Rhymer et al. 1994). The Revised Recovery Plan for Endangered Hawaiian Waterbirds mandates the removal of all mallards and koloa-mallard hybrids from the Hawaiian Islands (USFWS 2005). Eradication of hybrids will be difficult, and some authors have questioned whether such efforts are always advisable (see review in Allendorf et al.

2001). In cases where only hybrids remain, their protection might offer an opportunity to capture some of the genetic variation that existed in the original population (Allendorf et al. 2001). Efforts to remove hybrids will be most effective where populations of the pure strain remain, and where further hybridization can be prevented by removal or control of the hybridizing taxon. This may be the situation for koloa, particularly on Kaua'i (although the discovery of possible hybrids on Kaua'i suggests that these opportunities may be time-limited). To fulfill the mandate of the Recovery Plan requires that a reliable field key be developed to enable conservation personnel to distinguish koloa from hybrids in the field. Our study represents a first step in this process—we show that hybrids and pure koloa can be identified with a high degree of confidence using molecular genotyping. Having developed methods by which birds can be independently categorized, we can now begin the process of developing field keys based on morphological characteristics to reliably identify hybrids in the wild.

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