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EVOLUTIONARY RELATIONSHIPS AND CONSERVATION OF THE HAWAIIAN ANATIDS

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Abstract. The Hawaiian Duck or Koloa Maoli (*Anas wyvilliana*), hereafter referred to as Koloa, and Laysan Duck (*A. laysanensis*) are two endangered species of waterfowl in the mallard complex that are endemic to the Hawaiian Islands. These nonmigratory, nondimorphic species were thought to be derived from stray migratory, sexually dimorphic common Mallards (*A. platyrhynchos*), that subsequently lost the dimorphic plumage character. Laysan Ducks currently occur only on the tiny island of Laysan, while Koloa are found on O'ahu, Hawai'i, and, primarily, Kaua'i. Recent ancient DNA analysis shows that subfossil bones in deposits on the Big Island, Hawai'i, belong to the extant Laysan Duck. Similar fossils have been found on many of the major Hawaiian Islands, indicating that the species was formerly more widespread. Because of extensive hybridization between introduced Mallards and Koloa and the superficial morphological similarity between the Hawaiian taxa, their taxonomic status and phylogenetic relationships have been controversial. The perception that they may be subspecies of the Mallard, or even conspecific, has influenced their recovery programs. Molecular analyses indicate that Koloa and Mallard are distinct but very closely related species, whereas the Laysan Duck is very distinct from either. Some of the nondimorphic species in the mallard complex, such as the Laysan Duck, may have evolved from a nondimorphic ancestor rather than the common Mallard. Repeated bottlenecks, inbreeding, and small population size have likely contributed to a loss of genetic variation in the Laysan Duck, but it is now possible to plan a captive breeding program to preserve remaining variation for possible reintroduction of the species to other previously occupied Hawaiian Islands. Hybridization with Mallards is one of the factors contributing to the decline of Koloa on O'ahu and Hawai'i. The Kaua'i population represents a stronghold for the species, but thorough census data and basic information on the ecology of Koloa on Kaua'i, essential for developing a specific conservation plan, are not available.

Key Words: *Anas laysanensis*; *Anas platyrhynchos*; *Anas wyvilliana*; ancient DNA; hybridization; molecular phylogeny; reduced genetic variation; species limits.

The Laysan Duck (*Anas laysanensis*) and Hawaiian Duck or Koloa Maoli (*A. wyvilliana*), hereafter referred to as Koloa, are endangered species of waterbirds endemic to the Hawaiian Islands. Laysan Ducks are restricted to the tiny 370 ha island of Laysan in the northwestern Hawaiian chain. They survived a severe bottleneck in the early part of the century, as their population was estimated to have plummeted to fewer than 10 individuals by 1911 (Moulton and Weller 1984). This precipitous population decline was caused by overhunting and by habitat destruction by introduced rabbits. A ban on hunting plus extermination of the rabbits allowed numbers of Laysan Duck to rebound to about 500 birds over the next few decades, but a severe drought in 1993 reduced the population to fewer than 150 individuals (Cooper et al. 1996), an indication of the extreme vulnerability of this species. Harsh environmental conditions on Laysan Island likely represent less than optimal habitat for the Laysan Duck.

Recent analysis of DNA isolated from late Holocene subfossils, found in lava tubes in forested habitats at elevations as high as 1,800 m on Hawai'i, indicates that they are Laysan Duck (Fig. 1), an indication that the species was once found elsewhere in the Hawaiian Islands (Cooper et al. 1996). Similar subfossil anatid specimens found on O'ahu, Kaua'i, and Moloka'i suggest that the range of Laysan Ducks was once more widespread. This situation is not unique: remains of over 30 other, now extinct, passerine and nonpasserine avian species of late Holocene age have also been found on Kaua'i, O'ahu, Moloka'i, Maui, and Hawai'i (James and Olson 1991, Olson and James 1991). These prehistoric avian extinctions are attributed primarily to predation by Polynesians and introduced predators and to habitat destruction (Olson and James 1991). It may be possible to reintroduce Laysan Ducks to other islands, provided predators such as rats, mongoose, feral cats, and dogs are controlled and wetland and upland nesting habitats are protected.

Koloa once occurred on all the major islands in the lower Hawaiian chain except Lāna'i and Kaho'olawe (Griffin et al. 1989). The only substantial population is now found on the island of Kaua'i, in montane areas and on the Hanalei National Wildlife Refuge. There are a few birds on O'ahu, but hybridization with introduced common Mallards (*A. platyrhynchos*) is a serious problem there (Browne et al. 1993). The total population of Koloa has been roughly estimated at 2,500 birds, (2,000 on Kaua'i-Ni'ihau, 300 on O'ahu, 25 on Maui and 200 on Hawai'i; Engilis and Pratt 1993), but in reality, there are few

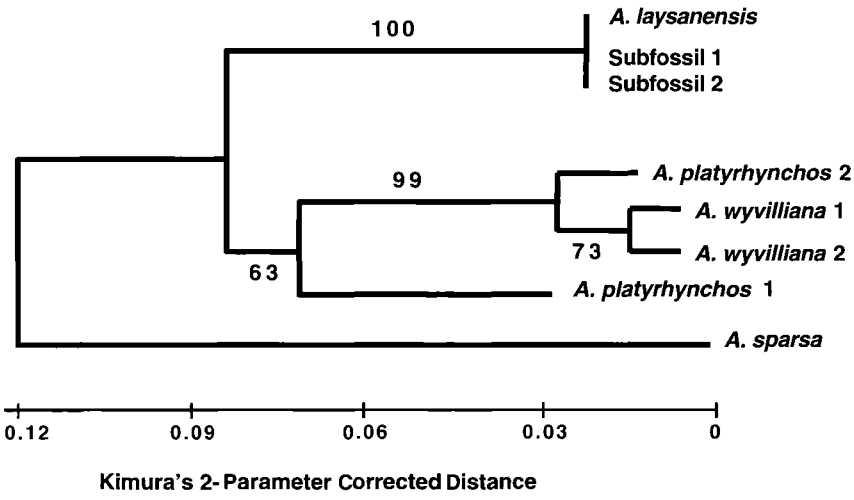

FIGURE 1. Neighbor-joining tree obtained with MEGA, based on Kimura's 2-parameter corrected distances using mtDNA control region sequences (after Cooper et al. 1996). Bootstrap values are shown. Sequences from Holocene subfossils are compared to those of extant Hawaiian anatids.

good data from which to estimate their current population size. Surveys do not cover montane streams and wetlands where most birds reside. In fact, little is known about their breeding ecology, reproductive success, movements, and annual habitat requirements. Specific conservation action has been limited, except for sporadic releases of captive-reared birds on O'ahu, Maui, and Hawai'i, which have had marginal success. Current recovery plans call for wetland protection and management and removal of the threat of hybridization (USFWS 1985). Management will include water level control, predator control, minimizing disturbance, improved census techniques, and monitoring of contaminants and avian disease.

The Laysan Duck and Koloa are thought by some to be derived from perhaps two waves of stray migratory Mallards that became isolated on the Hawaiian Islands and subsequently lost the Mallard's sexually dimorphic plumage (Weller 1980). They represent 2 of 14 closely related, nonmigratory, sexually nondimorphic species and subspecies in the worldwide mallard complex of waterfowl. The taxonomic status of many species in this complex has been controversial (e.g., Johnsgard 1961, Palmer 1976, Young and Rhymer 1998), and the specific status of Laysan Ducks and Koloa are no exception (Weller 1980). Detailed morphological analysis of the genus *Anas* by Livezey (1991) placed *wyvilliana* and *laysanensis* as sister species within a northern hemisphere mallard clade, not suprising given their close geographic distribution and small body size; they are about one-half to two-thirds the size of common Mallards. Livezey (1991) considered them to be full species, as did Berger (1972) and the American Ornithologists' Union (AOU 1983). In other studies, their status has variously been described as (1) both Laysan Duck and Koloa as subspecies of the Mallard (Delacour and Mayr 1945, Johnsgard 1978, Weller 1980); (2) Koloa as a subspecies of Mallard, but Laysan Duck as a full species (Ripley 1960); (3) Koloa as a full species with Laysan Duck as a subspecies of Koloa (Brock 1951b, Griffin et al. 1989); and (4) Laysan Duck as a full species that evolved from Koloa (Warner 1963).

Three issues have been raised that have important implications for conservation of the Laysan Duck and Koloa: (1) recognition of species limits—are the Laysan Duck and Koloa distinct species from one another and from the common Mallard and, therefore, more worthy of protection? (2) hybridization with introduced species—what is the extent of hybridization with introduced Mallards and is it a possible threat to the species' integrity of Koloa? (3) loss of genetic variation—have small population size and population bottlenecks led to a loss of genetic variation in Koloa and Laysan Duck? These issues are addressed using molecular genetic analyses of mitochondrial and nuclear DNA.

METHODS

Molecular Analysis

As part of a larger study of phylogenetic relationships in the mallard complex of species, blood and/or muscle or heart tissue samples were collected from common Mallard (North America, N = 28; Europe, N

= 20); Koloa (Kaua'i, N = 19), Laysan Duck (founders of captive flock, Smithsonian Conservation Research Center, N = 15), African Black Duck (*A. sparsa*), the nondimorphic sister species to the mallard complex (Cooper et al. 1996, Johnson and Sorenson 1998, J. Rhymer, unpubl. data; captive flock, Wildlife Preservation Trust, N = 1); and Green-winged Teal (*A. crecca*; N = 2) as an outgroup (Johnson and Sorenson 1998). DNA was isolated from each sample using standard procedures (Rhymer et al. 1994).

Mitochondrial DNA (mtDNA)

The two most variable domains of the mitochondrial control region (631 base pairs, bp, from the 5′ and 3′ regions) were amplified using primers developed for waterfowl (Cooper et al. 1996). DNA sequencing was done on an ABI automated sequencer (model 373A) and sequences were aligned using Geneworks® (IntelliGenetics, Inc.) and by eye.

Single-copy nuclear DNA (scnDNA)

Five μg of DNA from each individual were digested with 10 enzymes that recognize six-base sequences. Fragments in digested samples were separated on 0.7%-1.2% agarose gels and transfered to nylon membranes (MSI Magnagraph) via Southern (1975) blotting. One avian oncogene, v-*myc* (Alitalo et al. 1983) and five anonymous single-copy nuclear DNA (scnDNA) clones were used as probes, for a total of 30 probe/enzyme combinations. Anonymous scnDNA clones were obtained using standard procedures (Quinn and White 1987a, Parsons et al. 1993). Two hundred ng of probe were labeled with ^{32}P for each hybridization, and membranes were then exposed to Kodak XAR film for 24–72 hours.

Amplified fragment length polymorphisms (AFLP)

The amplified fragment length polymorphisms (AFLP) technique is based on the detection of genomic restriction fragments by polymerase chain reaction (PCR) amplification, which produces fingerprints without prior sequence knowledge (Vos et al. 1995). Protocols provided with the AFLP™ Analysis System I and AFLP Starter Primer Kit (GibcoBRL) were followed. Briefly, this includes an initial restriction digestion of 150 ng genomic DNA with *Eco*R I and *Mse* I, followed by ligation of *Eco*R I and *Mse* I adapters, amplification of the restriction fragments, labeling of an *Eco*R I primer with [$\gamma^{33}P$]ATP, reamplification with the labeled *Eco*R I primer and an *Mse* I primer, and separation of labeled, amplified fragments on a 6.0% denaturing polyacrylamide sequencing gel. Primers used were *Eco*R I (AAG) with *Mse* I (CAG), and *Eco*R I (AA) with *Mse* I (CAA).

DNA fingerprinting with minisatellites.

Five μg DNA were digested with *Hae* III, fragments were separated on agarose gels, and were then transferred to nylon membranes via Southern blotting using standard procedures (Loew and Fleischer 1996). Membranes were hybridized with ^{32}P labeled Jeffrey's 33.15 minisatellite probe and exposed to Kodak XRP-1 x-ray film for 24 hours.

STATISTICAL ANALYSES

Phylogenetic relationships using mitochondrial DNA control region sequences were estimated using maximum parsimony (PAUP 3.1.1; Swofford 1993) and the neighbor-joining algorithm (Saitou and Nei 1987) with Kimura's 2-parameter model (MEGA 1.01; Kumar et al. 1993). One thousand bootstrap replications were performed to estimate robustness of tree topologies and decay indices (the number of additional steps in the shortest tree(s) without a given node) were also calculated (Bremer 1988). For AFLPs, alleles at polymorphic loci were scored as 1 (present) or 0 (absent), and the resulting data matrix was also analyzed using maximum parsimony. A strict consensus of most parsimonious trees was calculated.

For scnDNA data, genetic distances were estimated for each pair of species according to Nei's (1987) method for unmapped fragment data, using the analysis package RESTSITE (v1.1; Nei and Miller 1990), which allows for the inclusion of multiple individuals of each taxon analyzed with several probe/enzyme combinations. Relationships among species were estimated using the neighbor-joining method. Data were not available for an outgroup for either AFLP or scnDNA analyses.

Two methods were used to estimate genetic diversity within species. First, band-sharing coefficients were calculated from Jeffrey's 33.15 minisatellite DNA data, comparing unrelated individuals of Mallards (N = 5), Koloa (N = 5), and Laysan Ducks (N = 5) on the same gel. Second, proportion of polymorphic loci (P) were calculated for each species using AFLPs, as the number of loci at which the most common allele had a frequency of less than 0.95 divided by the total number of individuals in the sample.

RESULTS

RECOGNITION OF SPECIES LIMITS

Phylogenetic analysis of mtDNA control region sequences indicate that there are two divergent lineages of common Mallards in the world (Figs. 1 and 2), one that has a Holarctic distribution (Mallard 1) and one that is apparently found only in North America (Mallard 2; Young and Rhymer 1998). The Koloa is very closely related to the Mallard, particularly lineage 2 (as are the other North American nondimorphic mallard species, the Mottled Duck, *A. fulvigula,* and American Black Duck, *A. rubripes*; J. Rhymer, unpubl. data). Divergence of Laysan Duck from the Koloa/Mallard clade is well supported (Fig. 2).

The occurrence of two divergent mtDNA Mallard lineages suggests either retention of an ancestral polymorphism or hybridization among taxa in North America. One of the problems that can arise from analysis of maternally inherited mtDNA is the possibility that the gene tree is not congruent with the species phylogeny (Avise et al. 1990). This possibility prompted analysis of biparentally inherited nuclear DNA molecular markers (scnDNA and AFLPs) to determine if

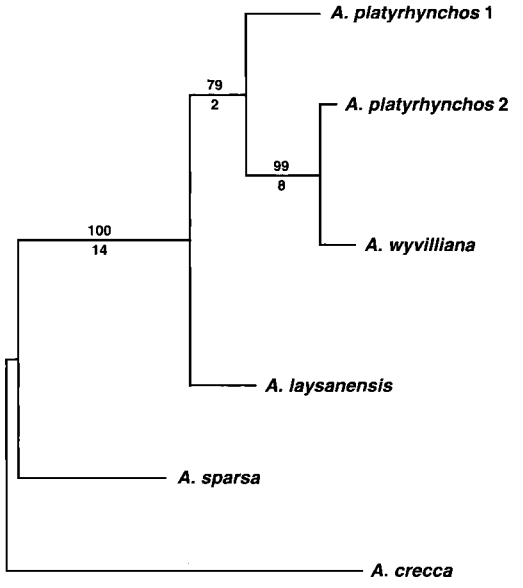

FIGURE 2. Single most parsimonious tree obtained with PAUP 3.1.1, relating mitochondrial control region sequences for *A. platyrhynchos, A. wyvilliana, A. laysanensis* and *A. sparsa* (length = 127, CI excluding uninformative characters = 0.73, RI = 0.70). Tree rooted with *A. crecca* as an outgroup. Branch lengths are proportional to the number of inferred changes along each branch. Decay indices are shown below each node; bootstrap values are shown above

relationships among species suggested by the mtDNA results would be upheld. Only one lineage of Mallards was found at the nuclear level and both nuclear DNA datasets support the very close relationship between the common Mallard and the Koloa (Figs. 3 and 4). The divergence between Laysan Duck and Koloa was also highly repeatable, regardless of the nuclear DNA method employed.

Hybridization with Introduced Species

Based on morphology, many of the Koloa-like birds on O'ahu appear to be hybrids. However, hybrid individuals are increasingly difficult to identify morphologically after more than one or two generations of backcrossing to one of the parental species (e.g., Rhymer et al. 1994). Molecular methods provide an unambiguous assessment of the extent of hybridization and introgression between species. One putative Koloa/Mallard hybrid has been analyzed with mtDNA and nuclear markers so far. This individual was phenotypically similar to Koloa but possessed a Mallard 2 mitochondrial haplotype. Analysis of nuclear DNA using AFLPs indicates that the hybrid is indistinguishable from Koloa (Fig. 4). These data suggest that the hybrid individual was not an F1 but a backcross into the Koloa. Further, because mtDNA is inherited only from the female parent, whereas AFLP loci are biparentally inherited, these data also indicate that the initial cross involved a female Mallard hybridizing with a male Koloa.

Loss of Genetic Variation

There is considerably less mtDNA haplotype diversity in both Hawaiian and Laysan Ducks than in the Mallard. Five to ten haplotypes (with minor changes) have been found in each of the two Mallard lineages (Avise et al. 1990; J. Rhymer, unpubl. data), whereas only two haplotypes are found in the Koloa and one in Laysan Duck (Fig. 1). Analyses of minisatellite DNA and AFLPs also indicate an apparent loss of variation in Laysan Ducks (Table 1). Average numbers of scorable bands for the Jeffrey's 33.15 probe were similar for Mallards and Koloa but much reduced in Laysan Ducks. Similarly, band-sharing coefficients for Mallards and Koloa are within the range (0.2−0.5) for unrelated individuals in outbred avian populations (Haig and Avise 1996), while those for unrelated Laysan

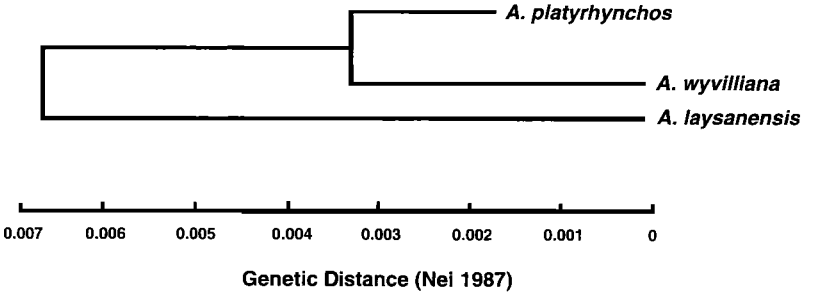

FIGURE 3. Neighbor-joining tree obtained with RESTSITE 1.1, based on scnDNA data using Nei's (1987) distances for unmapped restriction sites for *A. platyrhynchos, A. wyvilliana,* and *A. laysanensis*

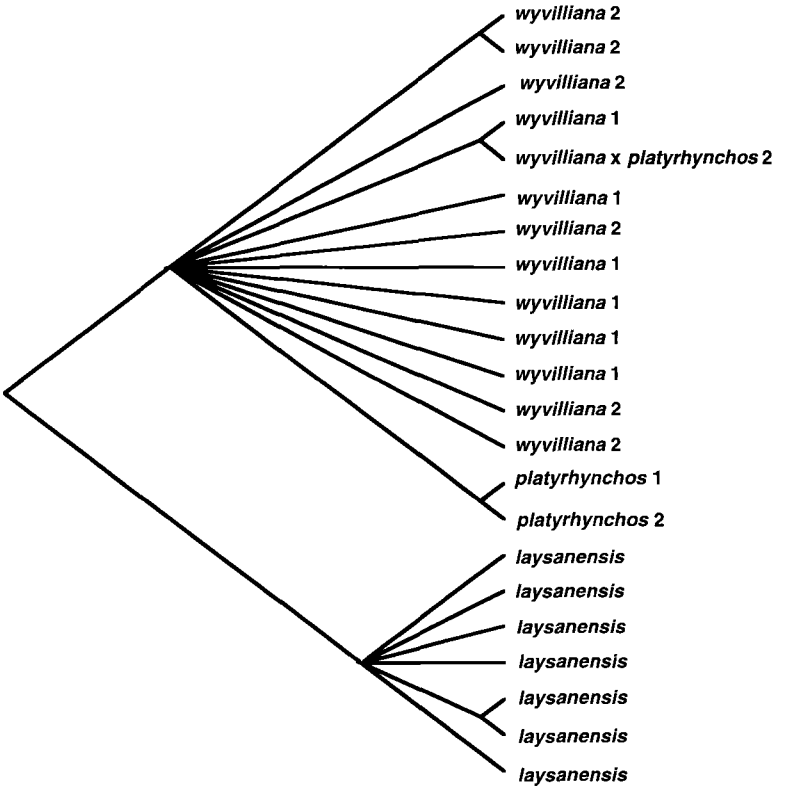

FIGURE 4. Strict consensus of 53 most parsimonious trees relating variation in AFLP loci of the two Mallard mtDNA lineages (*platyrhyncos* 1 and 2), Koloa (*wyvilliana* 1 and 2 refer to mtDNA haplotypes), and Laysan Duck (*laysanensis*). Several individuals are included to illustrate variation within and among taxa. One putative Koloa x Mallard hybrid with a Mallard 2 mtDNA haplotype clusters with Koloa, suggesting a backcross individual

Ducks were extremely high (> 0.8; Table 1). On two AFLP gels, the proportion of polymorphic loci (P; corrected for sample size) in Laysan Ducks was only about one-tenth that of Mallards with Koloa intermediate to the other two species (Table 1).

TABLE 1. COMPARISON AMONG SPECIES OF NUMBER OF SCORABLE BANDS (± SE) AND BAND-SHARING COEFFICIENTS (S), BASED ON JEFFREY'S 33.15 MINISATELLITE PROBE AND PROPORTION OF POLYMORPHIC LOCI (P), BASED ON AFLP DATA

	Minisatellite DNA			AFLP[a]	
	N	# scorable bands (± SE)	S	N	P
Mallard	5	42.8 ± 0.60	0.22–0.40	4	0.097
Koloa	5	43.4 ± 0.62	0.30–0.51	16	0.049
Laysan Duck	5	34.6 ± 0.26	0.82–0.90	10	0.014

[a] 104 of 401 scorable AFLP bands were variable.

DISCUSSION

Genetic analyses of both Laysan and Koloa provide insights into problems with systematics, hybridization, and loss of genetic variation of these endangered species that have important consequences for their conservation. Small body size of the two Hawaiian species places them together in recent systematic treatments based on detailed morphological analyses (Livezey 1991, 1993), but some plumage characters of the Koloa are more similar to Mallard than to Laysan Duck. Adding to the taxonomic confusion, however, are results of a recent allozyme study that showed a deep split between Mallard and Koloa (an order of magnitude greater than all anatid and most other avian congeneric genetic distances previously observed; Avise and Aquadro 1982), but virtually no differences between Laysan Duck and Koloa (Browne et al. 1993). In contrast, the analyses of mtDNA and nuclear DNA in this study show that the diver-

gence of Laysan Duck from a Koloa/Mallard clade is robust, whereas the Koloa and Mallard are very closely related (with only a few species-specific diagnostic markers). It is possible that the anomalous protein results stem from the analyses of different tissue types in different samples, which could artificially inflate estimates of divergence among taxa.

Using a clock calibration of about 8% sequence evolution per million years (calculated for the more variable 5′ end of the mitochondrial control region by Sorenson and Fleischer 1996), it is estimated that the Koloa may have diverged from the North American lineage of Mallards (Mallard 2) as recently as 130,000 years ago, but from the Holarctic lineage (Mallard 1) as long ago as 0.8 million years ago (Ma). Divergence of the Laysan Duck from both common Mallard lineages, as well as from the Koloa, also appears to be on the order of 0.8 Ma. The evolution of these species from *A. sparsa* (about 1.7 Ma) is well supported even when all 14 species and subspecies in the mallard complex are included in the analysis (J. Rhymer, unpubl. data). In addition, some species in the mallard complex, such as the Laysan Duck, may well have evolved from the nondimorphic ancestor rather than the common Mallard (Fig. 2; see also Johnson and Sorenson 1998, Young and Rhymer 1998).

Confusion over the taxonomy and evolutionary history of these species has been compounded by the propensity of introduced Mallards to hybridize whenever possible with some of the nondimorphic species in the mallard complex, e.g., Grey Duck (*A. superciliosa*) in New Zealand (Rhymer et al. 1994), Black Duck and Mottled Duck in North America (Johnsgard 1967, Mazourek and Gray 1994), and the former Mexican Duck (*A. diazi*; Hubbard 1977). Extensive hybridization with introduced species can lead to a kind of genetic extinction of rare native flora and fauna (Rhymer and Simberloff 1996). As a result, the specific status of the taxa involved can be called into question, with important consequences for the protection of some endangered species (Meffe and Carroll 1994, Avise and Hamrick 1996). Nevertheless, current thinking does not consider the retained ability to interbreed as sufficient evidence to preclude specific status and protection (O'Brien and Mayr 1991, Rhymer and Simberloff 1996).

Hybridization between Koloa and introduced Mallards on O'ahu has been so extensive that this population is no longer considered to have pure Koloa. Removal of the threat of hybridization is an essential component for the species recovery (USFWS 1985). As an aside, the contention that hybridization between Mallards and closely related nondimorphic species occurs primarily because females of the nondimorphic species are more attracted to the colorful Mallard male was not upheld in a detailed study of New Zealand Grey Ducks and introduced Mallards (Rhymer et al. 1994), and the same appears true for Koloa. Only one known Koloa x Mallard hybrid has been analyzed so far and this individual resulted from a Mallard female x Koloa cross. More importantly, there is a population of Koloa on Kaua'i that is largely unaffected by hybridization, so far. Knowledge of the potential threat and the availability of diagnostic molecular markers can now help to monitor incursion of hybridization on this island. Apart from guarding against hybridization, detailed studies of Koloa ecology are of the utmost importance in understanding its population dynamics. It is the Kaua'i population that will provide a stronghold for the Koloa, so it is suprising that little is known about the ecology of this endangered species. Captive breeding programs and/or translocations are a final resort. It is better to understand the species' ecology in planning the prevention of further declines.

The Laysan Duck is in an even more precarious situation with fewer than 150 individuals surviving the drought of 1993 (Cooper et al. 1996). In this case, a captive breeding program seems warranted. Results of mitochondrial and nuclear DNA analyses indicate that repeated bottlenecks, inbreeding, and/or low population numbers have probably contributed to a loss of genetic variation in this species. Only one mitochondrial haplotype remains and the number of minisatellite DNA bands and polymorphic loci (using AFLPs) is reduced compared to that found for either the Koloa or the Mallard. High levels of band sharing among apparently unrelated individuals suggest a history of inbreeding, similar to those observed in another species of endangered Hawaiian waterfowl, the Nēnē (*Branta sandvicensis*; Rave et al. 1994). Although few empirical data are available showing a direct link between loss of genetic variation (as indicated by molecular markers) and fitness (Lynch 1996), it is generally understood that adaptive evolutionary change is the primary means of responding to selective challenges (i.e., genetic variation is important for isolated species to adapt to environmental perturbations). All indications are that the beleaguered Laysan Duck does not adapt well to the harsh environmental conditions on Laysan Island. A captive program should be undertaken to reintroduce the Laysan Duck to other islands, provided predators are controlled and the habitat protected. It is now possible to plan a captive breeding program to maximize maintenance of the remaining

genetic variation in this species (e.g., Haig et al. 1990).

We now know what the conservation issues are for the endangered Koloa and Laysan Duck and genetic considerations provide one starting point for developing comprehensive strategies to ensure their protection.

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