GENETIC DIVERSITY, POPULATION STRUCTURE, AND DEMOGRAPHIC HISTORY OF THE HAWAI'I AKEPA

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We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Master of Science in Zoology (Ecology, Evolution, and Conservation Biology).

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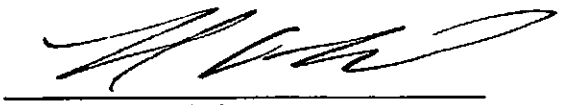

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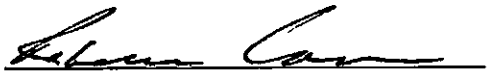

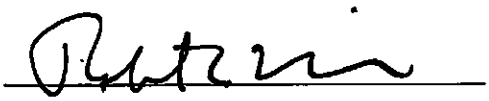

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ABSTRACT

As a result of disease, habitat destruction, and other anthropogenic factors, the Hawaii Akepa (*Loxops coccineus coccineus*) currently occupies less than 10% of its original range and exists in five widely separated populations, raising concerns about what effect such reduction and fragmentation has had on the connectivity and diversity of Akepa populations. In this study, both historical and contemporary samples were utilized to assess genetic diversity and structure in this endangered Hawaiian honeycreeper. Sequence data from ND2, control region, and two nuclear introns were obtained from three of the five current populations, and control region sequence data were obtained from museum specimens collected over 100 years ago throughout the historical range of the bird. Results indicate that despite recent declines and fragmentation, genetic diversity has not yet been lost. No clear phylogeographic breaks were observed across the historical range of Akepa, but rather genetic differentiation was modest and seemed to follow a pattern of isolation-by-distance. Low levels of differentiation between the contemporary populations observed with mtDNA but not nuclear sequences indicate that not much divergence, if any, has occurred post-fragmentation. Rather, the present structure seen likely reflects historical isolation-by-distance. Ironically, this declining species exhibits the genetic signal of an expanding population, demonstrating that earlier demographic events are outweighing the effects of recent changes in population size, and genetic estimates of N_e, though crude, suggest Hawaii Akepa were at least an order of magnitude more abundant prior to the decline.

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CHAPTER 1

INTRODUCTION

There is an undeniable link between human-related activities and the loss and endangerment of native species (Wilcove et al. 1998). Using a metaphor to characterize these threatening activities, Wilson (1992) describes the four "horsemen of the environmental apocalypse" as overexploitation, habitat degradation, introduced species, and the diseases spread by these exotic organisms. As a result of one or a combination of these factors, many species once abundant and widespread now have reduced and fragmented populations, and human intervention is often needed to stave off extinction (IUCN 2006).

An important aspect of conservation efforts today is the use of genetic markers to study endangered and threatened species (Moritz 1995; Haig 1998; Moran 2002; Piggott and Taylor 2003; DeYoung and Honeycutt 2005). Genetic data can contribute to conservation in a variety of ways. For example, molecular information can be useful in identifying and tracking animals non-invasively, determining sex ratios, identifying whether inbreeding and low genetic diversity threaten population persistence, describing connectivity between fragmented populations, documenting hybridization threats, delineating management and evolutionarily significant units, and estimating population sizes prior to declines (Frankham et al. 2002; Avise 2004). Given this utility, genetic assessments offer unique insight into the effects of declines on the viability of populations and can contribute information critical to the development of effective management plans.

Preserving genetic diversity is a major focus in endangered species conservation. Genetic theory predicts that as a population declines, molecular variation will increasingly be lost as a result of intensified genetic drift and inbreeding (Nei et al. 1975). Indeed, endangered species often demonstrate reduced genetic diversity when compared to non-endangered relatives (Frankham 1995; Spielman et al. 2004). The relative importance of genetic factors in species extinctions has been debated (Lande 1988; Amos and Balmford 2001; Spielman et al. 2004), but a growing body of literature provides compelling support that genetics do play a role in determining the fate of small populations (Frankham 2005). Empirical evidence from experimental, captive, and wild populations suggests that in the short-term, inbreeding and low genetic diversity can reduce reproductive fitness and the probability of persistence (Ralls and Ballou 1983; Crnokrak and Roff 1999; Westemeier et al. 1998; Dietz et al. 2000; Reed and Bryant 2000; Saccheri et al. 1998; Newman and Pilson 1997). In the long-term, low genetic diversity can reduce a species' evolutionary potential and impede its ability to adapt in the face of environment change (Frankel and Soulé 1981; Lande and Shannon 1996; Frankham et al. 1999). Because of these concerns, maintaining or restoring genetic diversity is a key component of recovery strategies for endangered species such as the Florida panther (Pimm et al. 2006), black-footed ferret (Wisely et al. 2003), and greater prairie chicken (Westemeier et al. 1998).

Not only are endangered species threatened with reduced population size, but fragmentation of the remaining individuals into isolated populations typically enhances genetic and demographic problems. In general, fragmented populations experience higher levels of inbreeding and genetic drift, and thus reduced genetic diversity,

compared to a single population of the same total size, and without gene flow, these fragmented populations experience genetic drift independently and diverge over time (Frankham et al. 2002). Empirical studies have demonstrated that small, isolated populations often do show lower levels of genetic diversity and increased differentiation compared to large continuous populations (e.g., Gaines et al. 1997; Tallmon et al. 2002; Stangel et al. 1992; Bouzat et al. 1998; Segelbacher et al. 2003). However, the effects of fragmentation can vary depending on how greatly gene flow is restricted. In some cases, fragments may be close enough and/or the dispersal ability of the species great enough that the fragmented populations behave like a single large population (e.g., Driscoll and Hardy 2005; Rossiter et al. 2000). In other cases, translocations and habitat corridors are recommended to re-establish gene flow and minimize genetic risks of extinction (e.g., Xu et al. 2006; Loew et al. 2005; Oyler-McCance et al. 2005).

While examining patterns of genetic diversity has become almost routine in studies of threatened and endangered species, interpreting the results remains challenging. Both contemporary and historical factors influence a population's genetic composition, and it can be difficult to disentangle the effects of the two. Because of this interaction, alternative scenarios can explain the observed patterns. For example, lower genetic diversity in an endangered species compared to a non-endangered relative can be interpreted to be the result of the recent population crash (Hoelzel et al. 1993; Schaeff et al. 1997), but different demographic histories between the two can also account for the pattern (Matocq and Villablanca 2001). Furthermore, genetic differentiation among current populations can result from historically restricted gene flow rather than recent fragmentation (Cunningham and Moritz 1998; Miller and Waits 2003), and likewise, a

lack of differentiation can result from historical connectivity or shared ancestry rather than ongoing gene flow (Bulgin et al. 2003). Accurate interpretations of the data are critical, particularly when management decisions are based upon them. More and more, researchers are turning to historical specimens such as museum skins and subfossils to evaluate competing hypotheses, and these studies have produced some interesting results.

In an effort to confirm that observed patterns of low genetic diversity in endangered species result from recent population declines, several studies have used archived sources of DNA to directly compare pre- and post-bottleneck populations. True to expectations, many of these studies did observe significantly higher levels of variation in the pre-bottleneck samples (e.g., Larson et al. 2002; Bouzat et al. 1998; Glenn et al. 1999; Leonard et al. 2005; Hoelzel et al. 2002; Groombridge et al. 2000; Hauser et al. 2002). But some studies found the focal species already had reduced genetic diversity prior to recent demographic crashes. While the Morro Bay kangaroo rat, Yellowstone grizzly bear, and Scandinavian arctic fox exhibit low genetic diversity compared to non-imperiled relatives, comparison with pre-bottleneck samples indicate that no or only modest amounts of diversity had been lost (Matocq and Villablanca 2001; Miller and Waits 2003; Nyström et al. 2006).

Clearly, some historical factor played a role in the observed paucity of molecular variation. In the case of the Hawaiian goose and North Atlantic right whales, earlier humans (i.e., native Hawaiians and 16th century Basque whalers, respectively) may be implicated in the initial declines (Paxinos et al. 2002; Rosenbaum et al. 2000). In other situations, the causes may be abiotic or ecological in nature. Through examination of fossil material dating back to 10,000 ybp, low genetic variation in a small Patagonian

rodent extends back for millennia and may have resulted from climate shifts, a volcanic eruption, or competition with a congener (Chan et al. 2005). These cases illustrate the significance of historical factors in shaping genetic patterns seen today and provide hopeful evidence that some species can persist for long periods of time despite low genetic diversity.

Historical data can improve our understanding of current genetic patterns also by offering information on the past distribution and population structure of a species. Use of archived DNA affords researchers the opportunity to examine populations now extinct and to determine pre-fragmentation levels of connectivity between geographic regions. Such knowledge can be useful in guiding efforts to establish corridors, increase genetic diversity through translocations, reintroduce individuals to restored areas, and develop captive breeding programs.

While translocations can be useful in reducing inbreeding and increasing genetic diversity in isolated populations, many issues, including how many individuals should be transplanted and which populations should serve as sources and recipients, need to be addressed to ensure the measures are appropriate. Such actions carried out among differentiated populations could result in the loss of critical adaptive features and the possibility of outbreeding depression (Frankham et al. 2002). Historical data can help justify translocations between isolated populations if they are found to have been connected historically. For example, differentiation between current populations of Scandinavian and North Russian arctic foxes may be too high to justify translocation of Russian foxes to bolster declining Scandinavian populations, but analysis of museum specimens from an earlier time period indicate there was historically a greater connection

between these two areas (Nyström et al. 2006). Thus, translocations at rates that mimic the historical level of gene flow might be warranted between these areas. Comparison of Yellowstone grizzly bears from three different time periods with bears from a northern population suggests gene flow was historically limited, and thus translocations, if any, should be done at a low level to maintain current levels of both diversity and divergence (Miller and Waits 2003).

Historical information can also be useful in selecting appropriate source populations for reintroduction efforts. To help endangered species rebound, efforts are often made to re-establish populations in areas where the species have gone extinct (e.g., Clark et al. 2002; Vandel et al. 2006). A survey of the structure across the historical range of the species provides insights on suitable sites and source populations for these reintroductions. For example, white-headed ducks were found to lack structure across the species' historical range, easing concerns over the merits of using birds from Spain and Pakistan as source populations for reintroductions in other countries (Muñoz-Fuentes et al. 2005). Analysis of museum specimens of grey wolves indicated the Mexican wolf had a broader geographic range than previously supposed, and hence restoration plans should be modified to include additional reintroduction sites (Leonard et al. 2005).

The examples above illustrate the utility of historical data in interpreting genetic patterns. An historical perspective is important when evaluating the influence of recent bottlenecks on genetic diversity and population structure, especially when management actions are based on the interpretations. With this in mind, the aim of this study is to utilize both historical and contemporary samples to assess the genetic diversity and structure of an endangered Hawaiian honeycreeper, the Hawaii Akepa (*Loxops coccineus*

coccineus). In this and other honeycreeper species, all four of Wilson's (1992) "horsemen" have played a role in recent declines and fragmentation (van Riper and Scott 2001). In the following chapter, the genetic consequences of demographic declines in the Hawaii Akepa are explored and put in light of historical influences on patterns of genetic diversity.

CHAPTER 2

GENETIC DIVERSITY, POPULATION STRUCTURE, AND DEMOGRAPHIC HISTORY OF THE HAWAI'I AKEPA

Introduction

Current genetic patterns can be shaped by both historical and contemporary factors, and interpretations of genetic data can greatly change when viewed in this light. Take, for example, estimates of genetic diversity. Endangered species often demonstrate reduced genetic diversity when compared to non-endangered relatives (Frankham 1995; Frankham et al. 2002), and such a pattern is usually interpreted to be the result of recent, known bottlenecks in population size (Avise 2004). But recent studies have shown that in at least some cases, patterns of low genetic diversity can be due to historical factors rather than modern-day anthropogenic disturbances (Matocq and Villablanca 2001; Chan et al. 2005; Paxinos et al. 2002). Many studies of endangered species also examine the level of genetic differentiation between fragmented, remnant populations to determine whether gene flow is being limited as a result of recent isolation. However, observed genetic differentiation among current populations can result from historically restricted gene flow rather than recent fragmentation (Miller and Waits 2003; Williams et al. 2003), and likewise, a lack of differentiation can result from historical connectivity or recent ancestry rather than ongoing gene flow (Bulgin et al. 2003).

Such findings emphasize the importance of a historical perspective when evaluating the influence of recent bottlenecks on genetic diversity and population structure, especially when management actions are based on the interpretations. Indeed

many studies are now utilizing archived materials such as scales, bones, hair, feathers, and skins to conduct genetic analyses on extinct populations and/or populations from earlier time periods (e.g., Bouzat et al. 1998; Bouzat 2001, Paxinos et al. 2002; Leonard et al. 2005; Godoy et al. 2004; Hauser et al. 2002). Such samples have proven useful in providing a comprehensive picture of the historical range, diversity, and structure of declining species and in helping to sort out the role of historical and contemporary processes in shaping current genetic patterns.

In this study, contemporary and historical DNA samples are used to explore the genetic diversity and structure of an endangered Hawaiian honeycreeper, the Hawaii Akepa (*Loxops coccineus coccineus*). This bird was once widely distributed and abundant in ohia (*Metrosideros polymorpha*) forests on the island of Hawaii (Banko 1984; Freed 1999), being described as "common in all the forests" and "very generally distributed" by late 19th century collectors (Perkins 1903; Munro 1944). However, the Hawaii Akepa has since experienced a severe population decline and is now rare or absent from many former parts of its range (Scott et al. 1986). As a result of disease, habitat destruction, and other factors (van Riper and Scott 2001), the bird currently occupies less than 10% of its original range and occurs in five disjunct areas above 1300-m elevation (Figure 1) (Scott et al. 1986). Census estimates put the total population size at around 14,000 birds (Scott et al. 1986), but further declines in several of the populations (Camp et al. in preparation) and a potential upward bias of the census estimates due to male group displays at the time of the surveys (Lepson and Freed 1995) suggest this number could be somewhat lower. The population decline and fragmentation experienced by the Akepa raises both demographic and genetic concerns.

While several studies have been conducted on aspects of the species' ecology and breeding behavior (Lepson and Freed 1995, 1997; Freed 2001; Fretz 2002; Hart 2001; Ralph and Fancy 1994), little is known about levels and patterns of genetic variation (but see Feldman 1994).

Maintaining genetic diversity is a central concern in endangered species conservation because molecular variation provides the raw material that enables a species to adapt in the face of environmental change (Frankel and Soulé 1981), yet genetic theory predicts that it will increasingly be lost as a population declines (Nei et al. 1975). If the ultimate goal is to ensure long-term persistence of the Akepa, maintaining genetic diversity is a relevant concern, as these birds are facing the challenges of new diseases and parasites, introduced predators and competitors, and climate change (van Riper and Scott 2001; Benning et al. 2003). In this species, the recent population decline may have resulted in reduced genetic diversity due to increased genetic drift and inbreeding. Alternatively, since the total number of Akepa on the island is still relatively large, much of the molecular variation may still be preserved. Furthermore, given that the Akepa is an island endemic, it might historically have had low levels of genetic diversity (Frankham 1997). Historical samples can help to evaluate these competing hypotheses.

To further assess the potential impacts of the recent decline in the Hawaii Akepa, it is also important to know if and how Akepa populations were naturally subdivided on the island. In addition to the force of drift, loss of genetic diversity could result from the extinction of distinct populations with unique haplotypes. The Akepa is a philopatric bird that relies on old-growth forests for nesting sites and food resources (Lepson and Freed 1997; Freed 2001). The sedentary nature of Akepa, coupled with the natural

heterogeneous distribution of old-growth forests on the volcanically active island, suggests populations may have been structured even historically. Given the extirpation of the bird from most of its range, unique populations may have been permanently lost. In addition to assessing loss of diversity, knowing whether structure existed naturally in this species can be valuable in determining whether recent fragmentation has isolated populations that were historically connected.

Given the alternative scenarios set forth above, interpretation of the genetic composition of Akepa populations could be greatly enhanced by museum specimens, which offer information on regions from where Akepa have been extirpated and provide a baseline of pre-bottleneck levels of genetic diversity.

Thus, the objectives of this study were to utilize contemporary and pre-bottleneck samples to:

1. Estimate and compare current and historical genetic diversity to test the prediction that recent declines would be reflected by a lower level of genetic diversity in contemporary populations.
2. Assess population structure across the species' current and historical range to determine to what extent fragmentation has isolated historically connected populations.
3. Investigate the species' demographic history and estimate past population sizes to elucidate how the observed genetic data might be influenced by past events.

Materials and methods

Samples

Contemporary samples were obtained from the three largest remnant populations of Hawaii Akepa and consisted of 25 blood samples for the North Windward (NW) population, seven blood/tissue samples for the Central Windward (CW) population, and 15 blood samples from the Kau population (Figure 1). Samples from the Kau population were collected for this study, and samples from the NW and CW populations were donated from several sources (Appendix 2). Given the extreme rarity of birds recorded recently from the Hualalai and Kona populations (Camp et al. in preparation), collection of samples from these populations was not attempted. Historical samples were obtained from a total of 44 museum specimens collected during the years 1888-1903 and representing a broad geographic coverage of the island (Figure 1 and Appendix 1). Each sample consisted of a small piece of skin removed from the toe pad (Mundy et al. 1997).

DNA isolation, polymerase chain reaction (PCR), and sequencing

Museum samples were extracted with a phenol-chloroform method as outlined in Slikas et al. (2000). Blood samples from the NW and Kau populations were also extracted with a standard phenol-chloroform method (Quinn and White 1987), and blood and tissue samples from CW were extracted with DNeasy Tissue Kits (Qiagen) following manufacturer's protocols.

For the contemporary blood/tissue samples, both nuclear and mtDNA sequences were obtained. Intron XI from the glyceraldehyde-3-phosphate dehydrogenase (Gapd) gene and intron III from the Lamin (Lam) gene were amplified using primer pairs

GapdL890/GapdH950 and LamL724/LamH892, respectively (Friesen et al. 1997). These introns were amplified from all of the CW and Kau samples, but only from a subset of 18 of the 25 samples from the NW population. For mtDNA, a 550 bp piece of the ND2 region was amplified from all contemporary samples using primers L5758 (5'-GGMTGAATAGGMCTCAACCAAAC-3') and H6313 (5'-CTCTTRTTTAAGGCTTTG AAGGC-3'), which were modified from the universal primers described in Sorenson et al. (1999) to more efficiently amplify this region in passerines. In addition, a ~1.6 KB section consisting of the entire control region plus flanking tRNA-phe, tRNA-glu and portions of ND6 and 12S rRNA was amplified using four overlapping primer sets: L16581/H417, LCR3/HCR1045, LCR943/H1251rev, and LGL5/H1417 (see Figure 2). Products were amplified using the polymerase chain reaction (PCR) carried out in 25 µl reactions containing 1X buffer (ID Labs or Applied Biosystems), 2 mM Mg^{2+}, 200 µM each dNTP, 0.4 µM each primer, 0.7 U of ID Proof DNA polymerase (ID Labs) or Amplitaq Gold DNA polymerase (Applied Biosystems), and 25-100 ng DNA. Amplifications were carried out with 35 cycles and an annealing temperature of 55°C for mtDNA primer sets and 60°C for nuclear introns.

For the ancient samples, two of the most variable control region portions identified from the modern samples, a 725 bp piece of Domains 1 and 2 and a 231 bp piece of Domain 3, were targeted for amplification. Multiple primer sets were used to amplify small (<195 bp), overlapping regions (see Figure 2). In analyses, these two pieces were concatenated to form a single ~1 KB piece. All PCR amplifications consisted of 25 µl reactions containing 1X buffer (Applied Biosystems), 0.2 mM each dNTP, 1 µm each primer, 2 mM Mg^{2+}, 1.6 mg/ml BSA, 1U Amplitaq Gold (Applied

Biosystems), and 2 µl of DNA extract. Amplifications were carried out with 55 cycles and an annealing temperature of 50°C for all primer sets.

PCR products were cleaned using 96-well Qiaquick (Qiagen) kits following manufacturer's protocols or with the ExoSAP method (Werle et al. 1994) using the enzymes Exonuclease I and Antarctic Phosphatase (New England Biolabs), which degrades excess primer and removes free dNTPs, respectively. To 10 µl of PCR product, 2 µl of Exonuclease I (0.1 U/µl) and 2 µl of Antarctic Phophatase (0.02 U/µl) were added, and the mixture was incubated at 37°C for 15 minutes, followed by 80°C for another 15 minutes to inactivate the enzymes. Both directions were sequenced using the same primers used for PCR. Sequencing reactions were performed using BigDye terminator chemistry (Applied Biosystems) and were run on ABI 3730XL or ABI 3100 capillary-based DNA sequencers. Sequences were edited, assembled, and aligned using the program Sequencher 4.6 (Gene Codes Corporation). For both Gapd and Lam, different alleles were characterized by a single base pair substitution, so alleles could be defined unambiguously from direct sequences.

Ancient DNA precautions and verification of target sequences

Due to the low copy number and degraded nature of ancient DNA, measures were taken to avoid and detect contamination during all procedures (Slikas et al. 2000). Museum specimen extractions and PCR set-up took place in a Smithsonian Institution National Zoological Park laboratory that is dedicated solely to ancient DNA work and located in a building where no PCR amplifications are performed or stored. All laboratory surfaces and pipets were cleaned with a bleach solution prior to working with

the samples, only barrier pipet tips were used, all plasticware was exposed to UV light prior to use, and gloves were changed frequently. Each extraction run consisted of no more than five samples, and negative controls were simultaneously carried out in both extraction and PCR procedures. The negative controls were screened, and if an amplification product was present, it was sequenced. These cases were rare and all turned out to be non-specific human, insect, or bacterial amplification. For each individual, overlapping sequences were compared to check for possible contamination or misincorporated nucleotides. Mismatched base pairs were identified only 4 times in the entire dataset: 2 C→T and 1 T→C transitions, which are among the most common types of sequence artifacts seen in ancient DNA amplification products (Willerslev and Cooper 2005), and one involving an A→T transversion. These fragments were amplified and sequenced a second or third time, and the correct nucleotide was determined by majority rule.

There was no evidence of nuclear copies, or numts (Sorenson and Quinn 1998), in the mtDNA sequences. Double peaks in the electropherograms, which could suggest simultaneous PCR amplification of the mitochondrial gene and a nuclear paralog (Lopez et al. 1994), were not found, and protein coding regions (ND2 and ND6) did not contain any unusual placement of stop codons or deletions that might be indicative of a non-functional nuclear pseudogene (Smith et al. 1992). In addition, identical haplotypes were seen in samples from different tissue sources (toe pad, blood, and muscle tissue) which differ in the relative number of mtDNA and nuclear copies (Sorenson and Fleischer 1996).

Genetic diversity

Gene diversity, nucleotide diversity, mean number of pairwise differences among sequences, and their standard deviations were estimated using ARLEQUIN 3.1 (Excoffier et al. 2005). For the contemporary samples, these indices were estimated for the Lam and Gapd nuclear introns, ND2, and the 1.6 KB piece of control region plus flanking sequences. To allow for a comparison of genetic diversity before and after the population decline, the smaller 1 KB piece of control region was analyzed for both the historical and modern samples and the diversity indices compared. In addition, the degree of differentiation between the historical and modern samples was assessed by using Φst and testing significance with 1000 permutations as implemented in ARLEQUIN. Finally, to provide a direct comparison of genetic diversity estimates in Akepa versus other, non-endangered Hawaiian Honeycreepers, a 344 bp section of the control region used in other studies (using primers LGL2 and H417, Tarr 1995) was analyzed separately.

For all datasets, program MODELTEST 3.06 (Posada and Crandall 1998) was used to estimate the best-fit model of sequence evolution using the Akaike Information Criterion (AIC). In all cases, either the Hasegawa-Kishino-Yano (HKY) (Hasegawa et al. 1985) or Tamura and Nei (1993) were the best-fit models of nucleotide substitution. Because the HKY model is not available in ARLEQUIN, the Tamura and Nei model was selected as the distance method used to calculate genetic diversity indices and Φ-statistics. For the 1.6 KB control + flanking sequence, a gamma correction value of 1.0276 was also applied, since this was part of the model selected in MODELTEST.

Population structure

To visualize phylogenetic relationships among haplotypes, statistical parsimony networks (Templeton et al. 1992) were constructed using the program TCS 1.13 (Clement et al. 2000) considering gaps as a 5th state. In population-level studies, networks better represent relationships among DNA sequences than traditional methods since they take into account the unique characteristics of intraspecific data, such as persistence of ancestral haplotypes, lower levels of sequence variation, existence of multiple descendent haplotypes, and reticulations due to homoplasy and/or recombination (Posada and Crandall 2001). However, a neighbor-joining tree was also constructed for the combined modern and historical 1 KB control region dataset in program PAUP* 4.0b10 (Swofford 2002) using a HKY model of sequence evolution with a proportion of invariable sites I = 0.870 as estimated in MODELTEST. Statistical support for nodes was estimated by bootstrapping using 500 replicates.

To assess population genetic structure across the natural range of the species, a series of analyses of molecular variance (AMOVAs) were performed using Φ-statistics, analogues of Fst that take into consideration the genetic distance between haplotypes (Excoffier et al. 1992), and using the 1 KB control region dataset of combined historical and contemporary samples. Given that several of the historical sampling localities only consisted of one or a few samples, some were pooled with nearby localities to boost sample sizes and increase the power for detecting differentiation. "Populations" were defined as follows: (i) Kohala, Mana/Waimea, Horner's, Hanneberg's (KMHH; n=9); (ii) North Windward and Hilo (NWHi; n=25); (iii) Olaa and Kilauea (OlKi; n=9); (iv) Central Windward (CW; n=7); (v) Kau and Pahala (KauPah; n=17); (vi) Johnston's (Joh;

n=6); and (vii) Puulehua and Hualalai (PuHu; n=9). No genetic differentiation was found within these designated populations based on pairwise Φst (data not shown), though small sample sizes likely limited the power of these tests.

In the AMOVAs, molecular covariance components and Φ-statistics were calculated, and significance was assessed using a non-parametric permutation approach (Excoffier et al. 1992) as implemented in ARLEQUIN. Several AMOVA analyses were conducted using alternative groupings of populations based on geography (e.g., windward (east) vs. leeward (west) sides of the island, separate volcanoes, etc.). The groupings that maximized values of Φct were assumed to indicate the most parsimonious geographical subdivisions.

Genetic isolation by distance was assessed by plotting population pairwise Φst against distance (km), and the significance of the association was determined by a Mantel Test (Mantel 1967) as implemented in ARLEQUIN. Two different distance matrices were tested. The first disregarded potential geographic barriers to dispersal and used straight-line distance based on map coordinates between populations. The second matrix considered the summits of Mauna Kea and Mauna Loa, the saddle area between these two mountains, and North Kona as barriers to dispersal, since Akepa were not recorded from these areas historically (Banko 1984). In this matrix, distances were calculated as the shortest distance between populations with the restriction of remaining within the bounds of the historical distribution of the bird. Thus, distances between Johnston's and Puulehua/Hualalai and the populations on the windward side of the island were greater in this matrix. Distances were calculated using the "measure distance" function on the Pacific Basin Information Node's interactive map of the Hawaiian Islands (available

online at http://www.higap.org). Where sampling localities were combined into a single population, the approximate weighted center was used as the population's position.

Current genetic structure was examined separately at several loci. The null hypothesis that haplotypes are distributed randomly among populations was tested with exact permutation tests (Raymond and Rousset 1995) using 100,000 Markov chain steps and 10,000 dememorization steps. In addition Fst and Φst were calculated between pairs of populations. These statistics were estimated and evaluated for significance by random permutations using 1,000 replicates in ARLEQUIN.

Population sizes and demographic trends

Multiple approaches were used to examine population demographic history. First, distributions of the number of pairwise differences observed between all sequences, or mismatch distributions (Slatkin and Hudson 1991), were constructed to test the possibility of a population expansion. Mismatch distributions are usually ragged or multimodal for populations at demographic equilibrium but are typically unimodal for populations that have experienced a recent demographic expansion (Rogers and Harpending 1992). The parameters of a stepwise demographic expansion, $\tau = 2ut$, $\theta_0 = 2uN_0$, and $\theta_1 = 2uN_1$, were estimated from the mismatch distribution according to Schneider and Excoffier (1999). The parameters θ_0 and θ_1 describe the population sizes before and after the expansion, while τ represents the time to expansion. A parametric bootstrap method was used to obtain confidence intervals for the parameters, as well as to test the validity of the estimated stepwise expansion model using the sum of squared deviations (SSD) between the observed and expected mismatch distributions as the test

statistic (Schneider and Excoffier 1999). In addition, the raggedness index of the observed mismatch distribution was calculated (Harpending 1994). This index is expected to have a small value for smooth distributions characteristic of expanding populations and a larger value for multimodal distributions characteristic of stable populations. The above analyses were implemented in ARLEQUIN, and 1000 replicates were used in the bootstrap methods.

A variety of factors, such as population growth, selective sweeps, and background selection, can affect patterns of DNA polymorphism. A suite of neutrality tests have been developed to test whether the polymorphism observed fits with expectations of the neutral model, each test being more powerful at detecting certain forces than others (Fu 1997). For this reason, several neutrality statistics, including Fu's Fs statistic (Fu 1997), Fu and Li's D* and F* statistics (Fu and Li 1993), and Tajima's D statistic (Tajima 1989), were examined to test population equilibrium. Tajima's D and Fu's Fs were calculated in ARLEQUIN, and Fu and Li's F* and D* were calculated in DnaSP 4.10.8 (Rozas et al. 2003).

For mtDNA, the population parameter theta (θ) equals $N_{ef}\mu$, where N_{ef} is the female effective population size and μ is the mutation rate per site per generation. An estimate of the effective number of females in a population can be derived from this relationship if theta is estimated and a mutation rate is assumed. A number of methods have been developed to derive theta from genetic data (e.g., Watterson 1975; Tajima 1983; Ewens 1972; Felsenstein 1992). Here, a maximum likelihood method that utilizes genealogical data was used to simultaneously provide an estimate of present-day theta and the growth parameter (g) (Kuhner et al. 1998; Kuhner et al. 1995). These estimates

were made with the computer program LAMARC 2.0.2 (Kuhner et al. 2005). Starting parameters used empirical base frequencies and empirical transition/transversion ratios, Watterson's (1975) estimate of θ, and g=1. LAMARC was run with 10 initial chains of 1000 sampled genealogies each and two final chains with 20,000 sampled genealogies each. The first 1000 trees in each chain were excluded as the burn-in. A second run was performed to check the consistency of the results.

Results

Museum samples success

DNA sequence was obtained from 42 of the 44 museum samples extracted (95% success). However, for 6 of these, only a few of the shortest PCR fragments were successfully amplified and sequenced, and thus were excluded from the analyses (Appendix 1), bringing the number of historical samples down to 36 (82% success). All of the specimens that yielded little or no sequence were from the B.P. Bishop Museum (BPBM), Honolulu, HI. The loss of these eight samples and the preclusion of obtaining additional samples from this museum reduced sample sizes at several localities, and thus pooling was necessary.

Genetic diversity:

Number of haplotypes, haplotype diversity, nucleotide diversity, and mean number of pairwise differences were similar between the contemporary and historical samples (i.e., overlapping SDs) (Table 1). In addition, similar levels of genetic diversity were seen among the three current populations (Table 1). No significant differentiation

between the contemporary and historical samples was observed (Φst=0.001, P>0.05). Variation was high in the control region fragments, and these gene regions were characterized by many singleton (58% for 1 KB and 56% for 1.6 KB) and low frequency haplotypes, resulting in a high level of private haplotypes at the population level and between the two time periods (Table 2).

Based on the diversity indices for the 344 bp control region fragment (Table 1), Hawaii Akepa display similar levels of variation to that of the Hawaii Amakihi (*Hemignathus virens*) and higher levels than the Iiwi (*Vestiaria coccinea*) and Apapane (*Himatione sanguinea*) (Foster et al. in preparation), none of which are endangered. This result indicates Akepa did not have a history of small effective population size.

Population structure:

Haplotype networks do not provide evidence of geographic structure in the distribution of haplotypes (Figure 3). Haplotypes and alleles found in more than one individual were typically present in more than one population and often geographically widespread. Networks for most gene regions are star-like, but the control region haplotype networks are complex and show a high level of reticulation. The neighbor-joining tree did not reveal any clear phylogenetic groupings, with clades having little statistical support based on low (less than 50%) bootstrap values (data not shown). Again, no geographic pattern was seen in the relationships of the haplotypes.

Results of AMOVAs reveal modest but significant structure across the Akepa's natural range (Table 3). The population grouping that yielded the highest Φct value and was statistically significant was when populations were grouped into three regions: the

leeward (west) side of the island and north and south windward (east) parts of the island. However, this grouping explained only 6% of the variation observed. In all groupings tested, the majority of the observed variation exists within populations rather than among populations or groups, indicating weak structure. The Mantel test was not significant when straight-line distance between populations was used (r=0.161; P>0.05), but it was when potential barriers to dispersal were considered (r=0.561; P=0.029). The plot of this non-linear distance vs. pairwise Φst (Figure 4 and Table 4) suggests Akepa populations were naturally structured via a combination of isolation-by-distance and natural barriers to dispersal. Scatter in this plot may be due in part to uneven sample sizes or to increased differentiation in modern populations. If only the four purely historical populations (KMHH, OlKi, Joh, and PuHu) are considered, the trend holds. There is no significant correlation between distance and differentiation when straight-line distance is used (r=0.438, P=0.240) but there is when unoccupied areas are viewed as dispersal barriers (r=0.902, P=0.035) (Figure 4).

Among the contemporary samples, pairwise Φst between populations using the 1.6 KB and 1 KB control region sequences revealed modest but significant differentiation between Kau and NW and between NW and CW, while pairwise Fst and exact test comparisons indicated differentiation among all three populations (Table 5). Results of the exact test comparisons for the ND2 sequences also indicate differentiation between Kau and NW. However, if the risk of making a type I error is minimized by applying a sequential Bonferroni correction (Rice 1989) to each of the three test statistics, only the Kau and NW populations remain significantly differentiated based on Fst and exact test comparisons using the 1 KB or 1.6 KB control region sequences. No structure is seen

among the contemporary populations using the slower evolving nuclear introns. The overall pattern of these statistics suggests weak structure, with the two most distant populations (NW and Kau) showing the most support for differentiation. These results are consistent with the isolation-by-distance pattern that is inferred to have existed naturally in this species, and thus historical factors may account for the differentiation seen.

With this dataset, it is difficult to determine whether recent fragmentation has increased differentiation. Historical samples do not exist from the same localities as the current samples, which would allow for a direct comparison of Φst values between the two time periods. Based on geography, a reasonable historical proxy for the NW and CW population comparison might be the KMHH and OlKi populations. These populations do not show differentiation (Φst=-0.051, P>0.05), whereas NW and CW do (Φst=0.089, P<0.05).

Population sizes and demographic trends

Given that population structure was found to be modest, and since modern and ancient samples were not found to be differentiated, samples were pooled to investigate population history at each locus. To ensure this pooling of samples did not bias the results, analyses were also conducted separately for the ancient and modern samples, as well as separately for each of the three contemporary populations. Overall, similar results were seen whether samples were pooled or analyzed separately (Table 6).

Mismatch distributions were unimodal (Figure 5), consistent with expectations of population expansion (Rogers and Harpending 1992), and SSD and Raggedness tests

generally failed to reject the model of population expansion (P>0.05) (Table 6). In addition, a large difference between estimated values for θ_0 and θ_1 was seen (Table 6), indicating a rapid increase in population size, and similar values for τ (i.e. overlapping CIs) (Table 6) were obtained for each population/time period separately, supporting the idea of a single expansion event.

The overall pattern of results from neutrality tests was that of significantly negative values for Fu's Fs and negative but non-significant values for Tajima's D (Table 6). Fu and Li's F* and D* were not significant in any case (P>0.05). Both Fu's Fs and Tajima's D values are expected to have large negative values for demographic expansions, but Fu's Fs has been found to be considerably more sensitive than other tests at detecting population expansions and selective sweeps (Fu 1997). Fu and Li's F* and D* statistics are more powerful than Fs and Tajima's D in detecting background selection. Thus, an expansion (or selective sweep) is indicated when Fs and D are significant and F* and D* are not, while the reverse suggests selection. The pattern of significance among these neutrality statistics seen here supports the idea of population expansion rather than selection.

The maximum-likelihood estimates of exponential growth rate (Kuhner et al. 2005) are significantly greater than 0 (Table 6), consistent with expansion. Given the equation $\Theta = N_{ef}\mu$, estimates of Θ (Table 6) were used to derive female effective population size for the ND2 and 344 bp control region sequences, as estimates of mutation rate have been calibrated for these loci in Hawaiian honeycreepers (R. Fleischer personal communication). Generation time was assumed to be 3 years based on an annual female survival of 75% over a reproductive life of 7 years (Lepson and Freed

1997). Given a theta of 0.01 - 0.06 for ND2 and assuming a mutation rate of 7.7×10^{-8} per site per year, an estimate of N_{ef} is in the range of 50,000 to 300,000. Given a theta of 0.02 - 0.10 for the 344 bp control region sequence, and assuming a mutation rate of 6.1×10^{-8}, the estimate of N_{ef} based on this sequence is 100,000 – 600,000, which is similar to the one obtained from ND2. Given uncertainty in Θ, mutation rate, and generation time, these N_{ef} values represent very rough estimates. Lower theta values, faster mutation rates, and longer generation times than those used would work to decrease the estimates of N_{ef}. Nonetheless, the estimates of the number of effective breeding females are much larger than the total census estimate of 14,000 birds (Scott et al. 1986).

Discussion

Utility of museum specimens

Excluding the BPBM samples, the success rate for amplifying and sequencing DNA from 100 year-old museum specimens was 100%. Samples from the Bishop Museum seemed heavily degraded. Of the 10 BPBM samples used in this study, 2 did not yield any amplification products and 6 only yielded a few of the smallest fragments and had to be excluded from analyses. The degradation cannot be wholly explained by the age of the material since the birds were collected within a few years (+/-) of all of the other specimens successfully sequenced in the study. Other researchers have also had difficulty amplifying DNA from this museum's birds (R. Fleischer and S. Jarvi personal communication). It seems likely that the year-round warm, humid climate of tropical Hawaii, to which the museum skins would have been exposed for approximately 100 years prior to the installation of air-conditioning in the 1980s, played a role in speeding

DNA degradation in these specimens compared to their temperate-dwelling counterparts. Unfortunately, BPBM has one of the largest collections of Akepa, particulary of those containing specific locality information, so this limited sample size and locations for the historical analysis.

Despite the relatively young age of the material, misincorporated nucleotides, though rare, were found. DNA begins to break down after an organism dies. The molecule becomes increasingly fragmented, which limits the amount and size of template DNA available for PCR and escalates the risk of preferentially amplifying contaminant sequences, and miscoding lesions accumulate, which can lead to sequence artifacts via insertion of incorrect bases (Willerslev and Cooper 2005). Given that DNA degradation accrues over time, such issues are particularly of concern with specimens dating from earlier time periods such as the Pleistocene, but the findings of this study and Nyström et al. (2006) suggest measures should be taken to identify erroneous sequence even when using relatively young museum specimens.

Genetic diversity

Based on the comparisons between historical and contemporary Hawaii Akepa samples and between Hawaii Akepa and non-endangered Hawaiian honeycreepers, historical levels of genetic diversity seem to be maintained in the species. In addition, all three remnant populations examined appear to have similar levels of variation. Population declines and fragmentation have not yet caused a detectable reduction in mitochondrial genetic diversity in Akepa. This result is consistent with an earlier study

that found three cytochrome b haplotypes among seven Akepa samples from the NW population (Feldman 1994).

However, a number of factors, including severity and duration of the bottleneck and generation time of the organism, influence the rate of loss of genetic diversity (Frankham et al. 2002). In many of the studies that have documented reduced genetic diversity in contemporary vs. historical populations, the bottlenecks have been severe such that populations have been reduced to a few tens of individuals or less (e.g., northern elephant seal (Hoelzel et al. 2002), Mauritius kestrel (Groombridge et al. 2000), greater prairie chicken (Bouzat et al. 1998), whooping crane (Glenn et al. 1999), etc.). Furthermore, several empirical studies of long-lived organisms did not find low molecular variation even when declines have been severe, suggesting organisms may be buffered against loss of diversity by long generation times (Lippé et al. 2006; Goossens et al. 2005; Hailer et al. 2006; Kuo and Janzen 2004). The decline in Akepa populations has occurred gradually over the past century, and approximately 14,000 birds remain today. The bird is also relatively long-lived, with some individuals remaining in the breeding population for at least 7 years (Lepson and Freed 1997). These characteristics may explain why the decline is not yet reflected in the genetic data.

Population structure

No clear phylogeographic breaks were observed in the Akepa, but rather genetic differentiation seemed to follow a pattern of isolation-by-distance. This pattern was not seen when straight-line distance between populations was used but was when distances were calculated under the restriction of remaining within the bird's historical distribution.

If one assumes the isolation-by-distance pattern reflects the true structure of Akepa populations, then the difference in results between these two distance matrices suggests that high-elevation areas and the dry North Kona region may have acted as natural barriers to dispersal in this species.

While isolation-by-distance indicates restricted gene flow, and can be attributed to the strong natal and adult philopatry seen in this bird (Lepson and Freed 1997), the modest level of differentiation seen across the range suggests gene flow is not as restricted in the Akepa as in some other birds on the island of Hawaii. For example, three subspecies of the Hawaii Elepaio (*Chasiempsis sandwichensis*) have been described based on plumage variation across the island (Pratt 1980), and Burgess (2005) found large Fst values (0.1-0.4) between these subspecies using control region sequence. Ongoing work on the Hawaii Amakihi is revealing significant fine-scale differentiation in this species based on control region and nuclear intron sequence (Foster et al. in preparation), though larger sample sizes used in that study may affect the comparison. However, given the small geographic scale (Hawaii Island is only 95 miles long and 80 miles wide) and the type of organism (i.e., a vagile bird) involved, the fact that any geographical structure exists emphasizes the powerful role of philopatry in structuring populations (Brown et al. 2004; Woxvold et al. 2006; Burgess 2005).

Current populations of Akepa do exhibit modest genetic differentiation (Table 5). However, this differentiation is only seen in the mtDNA markers and not in the nuclear introns. This discrepancy could be attributed to male-mediated gene flow (e.g., Bowen et al. 2005). However, given the slower mutation rate of the nuclear introns and the relatively recent ancestry of Hawaii Akepa based on the young age of the island (Carson

and Clague 1995), these markers may evolve too slowly to see structure yet and thus recent coancestry rather than high rates of gene flow may explain the lack of genetic structure in these markers. Furthermore, since mtDNA markers have a fourfold lower effective population size compared to nuclear markers, they are more subject to the effects of genetic drift and thus respond more quickly to population subdivision (Avise 2004).

Unfortunately, historical samples from these same locations are not available, which would allow for a direct comparison between the two time periods to test if differentiation has increased. The lack of structure between historical "populations" KMHH and OlKi and the presence of structure between contemporary populations NW and CW might reflect an increase in differentiation resulting from fragmentation. However, smaller sample sizes and pooling of samples from a broader geographic range could have resulted in lower Φst values in the historical populations. Based on the low values of Φst between the contemporary populations, not much divergence, if any, has occurred post-fragmentation. Given that modest structure existed naturally in this species, it seems likely that the present structure seen is not completely due to recent fragmentation, but rather reflects historical isolation-by-distance.

Population history and effective population size

There is consistent evidence that Hawaii Akepa experienced an evolutionarily recent demographic expansion, as seen from the star-shaped haplotype networks, negative and significant values for Fu's Fs and non-significant values for Fu and Li's F* and D*, unimodal mismatch distributions, and large maximum-likelihood estimates for

the growth parameter *g*. Star-shaped networks are characteristic of expanding populations (Avise et al. 1984; Ball et al. 1988; Slatkin and Hudson 1991), and population expansion may also be responsible for the high number of reticulations seen in the haplotype networks of the highly variable control region (Malhi et al. 2002; Chen et al. 2006). Although both expansion and a selective sweep can lead to the pattern of results obtained from the various neutrality tests (Fu 1997), the consistent results achieved from a variety of methods using both nuclear and mitochondrial loci support population expansion as the most likely explanation.

The time since expansion can be estimated from the equation $t = \tau/2\mu$, where τ is estimated from the mismatch distribution, μ = mutation rate for the entire region of DNA under study, and time (t) is measured in units of generations. Based on this equation, the wave crest of the ND2 unimodal mismatch distribution obtained for the pooled dataset translates to an estimated expansion time of 60,000 - 110,000 years ago, given a τ estimate of 1.54 – 2.99 and assuming a per site mutation rate of 7.7×10^{-8} and a generation time of 3 years. The 344 bp control region sequence dates the expansion at 50,000 - 140,000 years, based on a τ of 0.74 – 1.90, a per site mutation rate of 6.1×10^{-8}, and a generation time of 3 years.

These results indicate that all Hawaii Akepa likely derive from a single, small ancestral population that expanded in size and distribution. This could stem from the initial colonization of the island by a small flock of birds that expanded in size and geographic range for the first time. The timing of the population expansion based on the ND2 and control region data is in line with the formation of the island, which began approximately 1 mya (Price and Clague 2002). Alternatively, the expansion could have

occurred following a catastrophic decline in a pre-existing Akepa population, perhaps the result of volcanic activity. Dynamic geological processes on the island have been implicated in influencing the population history of other species native to Hawaii Island (e.g., Carson et al. 1990, Burgess 2005, Vandergast et al. 2004).

This study has encountered the seemingly paradoxical situation where a declining, endangered species is actually exhibiting the genetic signal of an expanding population. Such a result has also been seen in coconut crabs (Lavery et al. 1996), white-headed ducks (Muñoz-Fuentez et al. 2005), and cerulean warblers (Veit et al. 2005). In these situations, it seems the historical pattern of growth is fairly robust to further changes in population size, and the population declines have occurred too recently for a new equilibrium to be reached (Lavery et al. 1996). Thus, earlier demographic events are outweighing the effects of recent changes in population size.

Effective population sizes are typically much less than census sizes in wild populations because of the existence of non-breeding juveniles, variation in reproductive success among individuals, fluctuations in population size, unequal sex-ratios, and other factors (Frankham et al. 2002). But in the case of the Hawaii Akepa, N_e is actually much larger than the census size. In this study, the type of N_e measured is the inbreeding effective size, and it is not an uncommon result for this measurement of N_e to be orders of magnitude larger than census size in organisms experiencing recent declines (e.g., Florida grasshopper sparrows (Bulgin et al. 2003), grey wolves (Vilà et al. 1999), African wild dogs (Girman et al. 2001), marsh deer (Márquez et al. 2006), glade grasshoppers (Gerber and Templeton 1996), and Speke's gazelle (Templeton and Read 1994)). The inbreeding estimate is a backward looking statistic and reflects historical values of N_e

(Crandall et al. 1999). Thus, this measurement is useful in determining how many individuals existed prior to recent declines, which is relevant to setting restoration goals (e.g., Leonard et al. 2005; Roman and Palumbi 2003). Although the N_e estimates obtained in this study are crude, they suggest Hawaii Akepa were at least an order of magnitude more abundant prior to the decline.

Conservation Implications

Despite recent declines, genetic diversity is still high in Hawaii Akepa. Furthermore, diversity appears similar among the three remnant populations examined despite differences in population sizes. This is good news, as maintaining molecular variation is particularly important to native Hawaiian birds because it increases the chances they might evolve resistance to introduced diseases. However, these results do not mean that the population reduction has had no effect on Akepa populations. The three populations studied are the largest and may not reflect the level of genetic diversity remaining in the two remnant populations on the Kona side of the island. Akepa are extremely rare in these leeward populations, if not already extinct, and thus would be more likely to experience genetic consequences of low population size. The results also do not mean that genetic variation will remain unchanged in the future. Genetic diversity is lost at an increasing rate as effective population size decreases (Frankham et al. 2002). While the gradual nature of the decline has helped to slow the rate of loss of genetic diversity, variation will eventually decrease if the bottleneck persists or worsens. Thus, to preserve the evolutionary potential of this species, it is important that recovery begins sooner rather than later.

Since unsuitable habitat like mountain-tops may have served as natural barriers to dispersal in Akepa, it seems reasonable to expect that new man-made barriers will eventually lead to additional differentiation between current populations. Dispersal is likely restricted across barriers such as open pasture, roadways and developments, and disease-infested forest. Based on banding data, no movement has been detected between the fragmented populations (L. Freed personal communication), and the furthest distance an Akepa is known to have traveled within the North Windward population is 5 km (Lepson and Freed 1997). The lack of observed dispersal, coupled with modest genetic structure, indicate that from a demographic viewpoint, the populations should be considered as separate management units (Moritz 1994). Declining populations are unlikely to be sustained by immigration from stable or increasing populations in other areas. Since historical structure was found to be weak, translocation of birds between populations would be suitable in the future if it is deemed necessary to sustain populations and/or maintain levels of genetic diversity. However, given the high levels of genetic variation observed in Hawaii Akepa, the viability of this species is unlikely to be compromised by genetic factors in the near future.

CHAPTER 3

CONCLUSION AND FUTURE DIRECTION

This thesis represents the first study to combine historical and contemporary data to investigate patterns of genetic diversity in a Hawaiian honeycreeper. Many honeycreeper species have gone extinct, and a majority of those remaining are endangered (Pratt 2005). These birds have been extirpated from so much of their original habitat, that only through museum specimens and subfossils can we begin to comprehend the true genetic diversity and relationships of populations in these species and understand how anthropogenic disturbances have impacted natural processes. In this study, a comparison of contemporary and historical data sets has helped answer questions on whether recent demographic declines have resulted in reduced genetic diversity, whether fragmentation has isolated historically connected populations, whether unique populations have gone extinct, and whether natural barriers separated populations.

Despite recent population declines and fragmentation, Hawaii Akepa do not exhibit low genetic variation. Measures of genetic diversity were comparable in the pre- and post-bottleneck samples and among the three current populations examined. No significant differentiation between the contemporary and historical samples was observed, indicating remaining Akepa are not a depauperate subset of the historical population but rather capture the majority of the original genetic variation. While it appears genetic diversity has been maintained in Akepa, the two remnant populations on the leeward side of the island were not examined. Akepa are extremely rare in this area and would be more likely to experience genetic consequences of low population size,

thus it may be beneficial in the future to obtain genetic data from these populations to determine whether low genetic variation and inbreeding are issues. However, for the species as a whole, genetic factors are unlikely to threaten persistence in the near future.

Data from museum specimens suggest Akepa populations were naturally structured via isolation-by-distance. Population divergence was found to be modest, though, indicating gene flow was only mildly restricted across the range of this species. Mountaintops and unsuitable habitat seem to have served as natural barriers to dispersal, so it seems likely that expanses of open pasture and other disturbed areas will result in further restriction of gene flow among current populations. It is difficult to tell with this dataset whether recent fragmentation has already resulted in increased differentiation, but the observed divergence between populations is low and consistent with the isolation-by-distance pattern that is inferred to have existed naturally in this species. Thus historical factors may account for the differentiation seen. There is now a good baseline of differentiation between the three fragmented populations, so looking at genetic structure again in the future should reveal whether divergence is increasing.

The incorporation of microsatellite data would contribute to the findings of this study. First, these nuclear markers evolve much more quickly than introns, and therefore could help sort out whether the lack of structure seen in the introns is simply due to low mutation rate or if the discrepancy between mitochondrial and nuclear markers reflects male-biased dispersal. Use of high-resolution microsatellites could also allow for the identification of immigrants (e.g., Dalén et al. 2006; Seddon et al. 2006), which would provide evidence of ongoing gene flow between the fragmented populations. Microsatellite data could also be used to obtain genetic estimates of current N_e based on

linkage disequilibrium (Hill 1981; Bartely et al. 1992) and temporal changes in allele frequencies (Waples 1989; Anderson et al. 2000; Wang 2001), which would be critical in determining how fast Akepa populations will lose genetic variation. An attempt was made to obtain a moment estimator of contemporary N_e based on temporal changes in control region haplotype frequencies, but since data was available from only a single locus, the estimate had an extremely broad confidence interval (data not shown). Data from multiple microsatellite loci should result in a more precise estimate. Finally, it is always desirable to incorporate data from several loci to provide better representation of the entire genome and ensure inferences made on species and population history are not biased by unique characteristics of a particular locus.

Table 1. Akepa sequence data summary.
Sequence variation summary for North Windward (NW), Central Windward (CW), and Kau populations, all contemporary samples (Mod), all historical samples (Anc), and contemporary + historical samples (All). For mtDNA markers, *n* = number of sequences = number of samples. For nuclear markers, *n* = number of sequences = 2× number of samples.

Sequence	bp	Group	*n*	# haplotypes or alleles	Variable sites	Ti	Tv	Indels	Gene diversity	Nucleotide diversity	Mean # pairwise differences
Gapd	326	Mod	78	6	5	0	5	0	0.217 ± 0.062	0.0007 ± 0.0009	0.228 ± 0.271
Lam	209	Mod	80	4	3	2	1	0	0.351 ± 0.066	0.0018 ± 0.0019	0.381 ± 0.365
ND2	523	NW	25	9	9	8	1	0	0.893 ± 0.030	0.0041 ± 0.0026	2.128 ± 1.225
		Kau	15	8	10	9	1	0	0.876 ± 0.067	0.0043 ± 0.0028	2.228 ± 1.300
		CW	7	4	5	5	0	0	0.857 ± 0.102	0.0040 ± 0.0029	2.113 ± 1.333
		Mod	47	15	14	13	1	0	0.892 ± 0.027	0.0041 ± 0.0026	2.168 ± 1.224
Control + flanking	1631-1636	NW	24	17	28	22	1	5	0.971 ± 0.019	0.0028 ± 0.0016	4.568 ± 2.325
		Kau	15	9	24	21	2	1	0.933 ± 0.040	0.0041 ± 0.0023	6.679 ± 3.339
		CW	7	5	12	11	0	1	0.905 ± 0.103	0.0030 ± 0.0019	4.906 ± 2.718
		Mod	46	30	45	37	3	5	0.982 ± 0.008	0.0039 ± 0.0021	6.424 ± 3.098
Control	956-961	NW	24	16	20	15	0	5	0.964 ± 0.021	0.0036 ± 0.0021	3.505 ± 1.850
		Kau	15	9	19	17	1	1	0.933 ± 0.040	0.0058 ± 0.0033	5.522 ± 2.812
		CW	7	5	11	10	0	1	0.905 ± 0.103	0.0047 ± 0.0030	4.419 ± 2.478
		Mod	46	29	32	26	1	5	0.980 ± 0.008	0.0056 ± 0.0031	5.408 ± 2.654
		Anc	36	28	28	26	0	2	0.984 ± 0.011	0.0052 ± 0.0029	4.919 ± 2.452
		All	82	50	42	36	1	5	0.986 ± 0.004	0.0055 ± 0.0030	5.194 ± 2.540
Control (LGL2-H417)	344	Mod	46	7	5	4	1	0	0.747 ± 0.047	0.0034 ± 0.0025	1.165 ± 0.765
		Anc	35	9	6	6	0	0	0.766 ± 0.056	0.0037 ± 0.0026	1.265 ± 0.816
		All	81	12	9	8	1	0	0.749 ± 0.036	0.0034 ± 0.0025	1.180 ± 0.766

Table 2. Distribution of 1 KB control region haplotypes

Distribution of 1 KB control region haplotypes in contemporary and historical populations. The number of individuals with each haplotype at each locality are shown. Population abbreviations are as follows: NW=North Windward; CW=Central Windward; Koh=Kohala; Mana=Mana/Waimea; Horn=Horner's; Hann=Hanneberg's; Ola=Olaa; Kil=Kilauea; Pah=Pahala; Joh=Johnston's; Puu=Puulehua; Hua=Hualalai.

	Contemporary			Historical										
Haplotype	NW	CW	Kau	Koh	Mana	Horn	Hann	Hilo	Ola	Kil	Pah	Joh	Puu	Hual
Lcocc_1					1									
Lcocc_2	1													
Lcocc_3	3											1		
Lcocc_4	3													
Lcocc_5	1													
Lcocc_6		2												
Lcocc_7	2													
Lcocc_8	2													
Lcocc_9		1												
Lcocc_10		2					1				1			
Lcocc_11			1										1	
Lcocc_12												2		
Lcocc_13			2											
Lcocc_14														1
Lcocc_15	1													
Lcocc_16			2											
Lcocc_17	2													
Lcocc_18	1													
Lcocc_19			1											
Lcocc_20	1		2		1									
Lcocc_21	1													
Lcocc_22	1						1		1					
Lcocc_23	1													
Lcocc_24	1													
Lcocc_25			2											
Lcocc_26			3			1								
Lcocc_27	2													
Lcocc_28			1											
Lcocc_29	1													
Lcocc_30									2					
Lcocc_31									1					
Lcocc_32							1							
Lcocc_33												1	1	1
Lcocc_34			1											
Lcocc_35		1								1				
Lcocc_36		1					1							
Lcocc_37										1				
Lcocc_38												1		
Lcocc_39													1	
Lcocc_40									1					
Lcocc_41												1		1
Lcocc_42							1							
Lcocc_43									1					
Lcocc_44														1
Lcocc_45								1						
Lcocc_46				1										
Lcocc_47														1
Lcocc_48													1	
Lcocc_49										1				
Lcocc_50											1			

Table 3. Analyses of Molecular Variance (AMOVAs).
Hierarchical AMOVAs for different groupings of historical + contemporary populations. See text for population abbreviations.

Groupings	Source of variation	% Var	Fixation indices	P value
[KMHH/NWHi/OlKi/CW/	Among groups	8.34	$\Phi_{CT} = 0.083$	0.054
KaPa] [Joh/PuHu]	Among pops. within groups	0.45	$\Phi_{SC} = 0.005$	0.366
	Within populations	91.21	$\Phi_{ST} = 0.088$	0.035
[KMHH/NWHi]	Among groups	6.43	$\Phi_{CT} = 0.064$	0.053
[OlKi/CW] [KaPa]	Among pops. within groups	-1.95	$\Phi_{SC} = -0.021$	0.640
[Joh/PuHu]	Within populations	95.52	$\Phi_{ST} = 0.045$	0.031
[KMHH/NWHi]	Among groups	5.62	$\Phi_{CT} = 0.056$	0.036
[OlKi/CW/KaPa]	Among pops. within groups	-0.71	$\Phi_{SC} = -0.008$	0.606
[Joh/PuHu]	Within populations	95.08	$\Phi_{ST} = 0.049$	0.037
[KMHH/NWHi/OlKi/CW]	Among groups	1.52	$\Phi_{CT} = 0.015$	0.213
[KaPa/Joh/PuHu]	Among pops. within groups	2.79	$\Phi_{SC} = 0.028$	0.115
	Within populations	95.69	$\Phi_{ST} = 0.043$	0.043
[KMHH/NWHi]	Among groups	-0.54	$\Phi_{CT} = -0.005$	0.519
[OlKi/CW/ KaPa/Joh/PuHu]	Among pops. within groups	4.03	$\Phi_{SC} = 0.040$	0.070
	Within populations	96.51	$\Phi_{ST} = 0.035$	0.028

Table 4. Population pairwise Φst and geographical distance.
Pairwise Φst values between populations of Hawaii Akepa using the 1 KB control region historical + contemporary combined dataset are below the diagonal, and distances in kilometers between populations, calculated considering potential barriers to dispersal, are above the diagonal.

	KMHH	NWHi	OlKi	CW	KauPah	Joh	PuHu
KMHH	-	24.1	59.5	49.9	85.6	116.0	151.9
NWHi	0.000	-	35.1	26.7	66.5	91.9	127.8
OlKi	-0.051	-0.022	-	16.9	47.2	78.9	114.4
CW	-0.037	0.092*	-0.006	-	41.0	70.3	106.1
KauPah	-0.033	0.056*	-0.006	0.005	-	29.5	64.7
Joh	0.017	0.053	-0.003	0.045	0.017	-	35.2
PuHu	0.113*	0.097*	0.113*	0.173*	0.117*	-0.061	-

*P<0.05. None of the comparisons are significant at α=0.05 if a sequential Bonferroni correction (Rice 1989) is applied.

Table 5. Contemporary population differentiation.
Differentiation between the three current populations, given as pairwise Φst values (left), pairwise Fst values (middle), and pairwise p-values for the exact test (right).

	Pairwise Φst			Pairwise Fst			Pairwise exact test p-values		
	NW - Kau	Kau - CW	NW - CW	NW - Kau	Kau - CW	NW - CW	NW - Kau	Kau - CW	NW - CW
ND2	0.022	-0.011	0.036	0.024	-0.023	0.022	0.035*	0.611	0.155
Control [1.6KB]	0.073**	0.021	0.079*	0.042**†	0.079*	0.058**	0.002**†	0.005**	0.012*
Control [1 KB]	0.083**	0.014	0.089*	0.046**†	0.079*	0.062*	0.001**†	0.007**	0.006**
Gapd	-0.001	0.029	0.074	-0.015	-0.004	0.020	0.584	0.593	0.168
Lam	-0.014	0.000	0.066	-0.020	-0.013	0.016	0.538	0.594	0.064

*P<0.05, **P<0.01. †Significant at α=0.05 if a sequential Bonferroni correction (Rice 1989) is applied to each of the three tests.

Table 6. Demographic statistics summary.

Summary of demographic statistics for NW, Kau, and CW populations, all contemporary samples (Mod), all historical samples (Anc), and contemporary + historical samples (All). Parameters τ, θ_0, and θ_1 were estimated from the stepwise expansion models. Raggedness index (r) and sum of squared deviations (SSD) were calculated from mismatch distributions. Theta (Θ) and growth parameter (g) were estimated using a maximum-likelihood method. Given the computation time required, Θ and g were only estimated for the combined datasets.

Sequence	Group	τ (95% CI)	θ_0 (95% CI)	θ_1 (95% CI)	SSD	r	Taj D	Fu'sFs	Θ (95% CI)	g (95% CI)
ND2	NW	2.15 (1.09-3.23)	0.00 (0.00-0.72)	∞ (6.50-∞)	0.001	0.046	-0.368	-2.417	-	-
	Kau	2.42 (0.56-4.17)	0.02 (0.00-1.56)	18.8 (3.64-∞)	0.003	0.036	-1.072	-2.718*	-	-
	CW	2.32 (0.34-4.67)	0.00 (0.00-1.05)	35.4 (3.14-∞)	0.026	0.111	0.132	-0.042	-	-
	Mod	2.23 (1.54-2.99)	0.00 (0.00-0.56)	∞ (7.29 - ∞)	0.001	0.039	-0.969	-6.744**	0.024 (0.010-0.061)	1159 (288-3012)
Control +	NW	6.86 (3.46-9.70)	0.05 (0.00-2.53)	31.9 (16.5-∞)	0.008	0.023	-0.986	-6.345**	-	-
flanking	Kau	4.43 (1.64-12.6)	3.51 (0.00-12.0)	79.1 (20.2-∞)	0.018	0.028	-0.283	-0.146	-	-
1.6 KB	CW	8.97 (0.00-80.6)	0.00 (0.00-12.6)	13.8 (5.02-∞)	0.093	0.191	0.443	0.349	-	-
	Mod	5.24 (4.05-6.20)	0.00 (0.00-0.87)	∞ (38.2-∞)	0.015**	0.011	-1.378	-16.19**†	0.018 (0.009-0.045)	865 (225-2070)
Control	NW	5.48 (2.62-7.55)	0.01 (0.00-2.46)	57.8 (19.1-∞)	0.006	0.028	-0.483	-6.301**†	-	-
1 KB	Kau	6.58 (3.33-10.7)	0.50 (0.00-3.28)	25.9 (13.6-∞)	0.022	0.064	-0.051	-0.658	-	-
	CW	7.20 (1.00-11.8)	0.00 (0.00-8.26)	15.1 (5.96-∞)	0.080	0.191	0.394	0.161	-	-
	Mod	5.75 (3.85-6.97)	0.01 (0.00-1.97)	191 (39.8-∞)	0.005	0.022	-0.923	-17.24**†	-	-
	Anc	5.35 (3.20-6.60)	0.01 (0.00-1.93)	559 (45.4-∞)	0.002	0.015	-1.169	-22.41**†	-	-
	All	5.51 (3.77-6.31)	0.02 (0.00-1.67)	384 (54.5-∞)	0.002	0.014	-1.330	-25.46**†	0.038 (0.023-0.072)	1058 (534-2133)
Control	NW	1.72 (0.00-3.50)	0.00 (0.00-0.54)	5.01 (1.60-∞)	0.072	0.257*	1.103	-0.512	-	-
344 bp	Kau	1.40 (0.00-2.62)	0.00 (0.00-0.71)	∞ (3.89-∞)	0.004	0.082	0.319	-0.771	-	-
	CW	0.72 (0.00-22.8)	0.00 (0.00-0.02)	∞ (1.10-∞)	0.012	0.229	0.551	0.589	-	-
	Mod	1.29 (0.66-2.07)	0.00 (0.00-0.27)	∞ (3.29-∞)	0.007	0.090	0.041	-1.592	-	-
	Anc	1.40 (0.77-2.29)	0.00 (0.00-0.39)	∞ (3.41-∞)	0.010	0.092	-0.428	-3.917**	-	-
	All	1.32 (0.74-1.90)	0.00 (0.00-0.28)	∞ (4.09-∞)	0.007	0.083	-0.899	-5.714**	0.052 (0.016-0.097)	2876 (1265-4458)
Gapd	Mod	3.00 (0.00-3.50)	0.00 (0.00-0.01)	0.29 (0.00-∞)	0.002	0.371	-1.725**	-5.773**†	0.048 (0.018-0.161)	10,937 (6611-17011)
Lam	Mod	0.45 (0.20-0.94)	0.00 (0.00-0.11)	∞ (8.73-∞)	0.003	0.195	-0.706	-1.189	0.006 (0.002-0.010)	1313 (-326-7619)

*P<0.05. **P<0.01. †Significant at α=0.05 if a sequential Bonferroni correction (Rice 1989) is applied to each of the four tests.

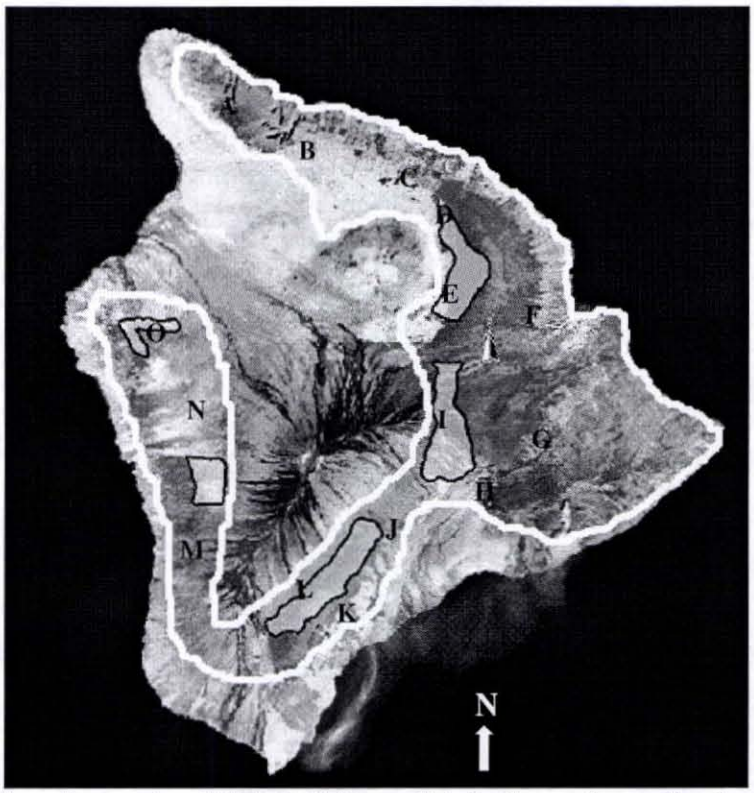
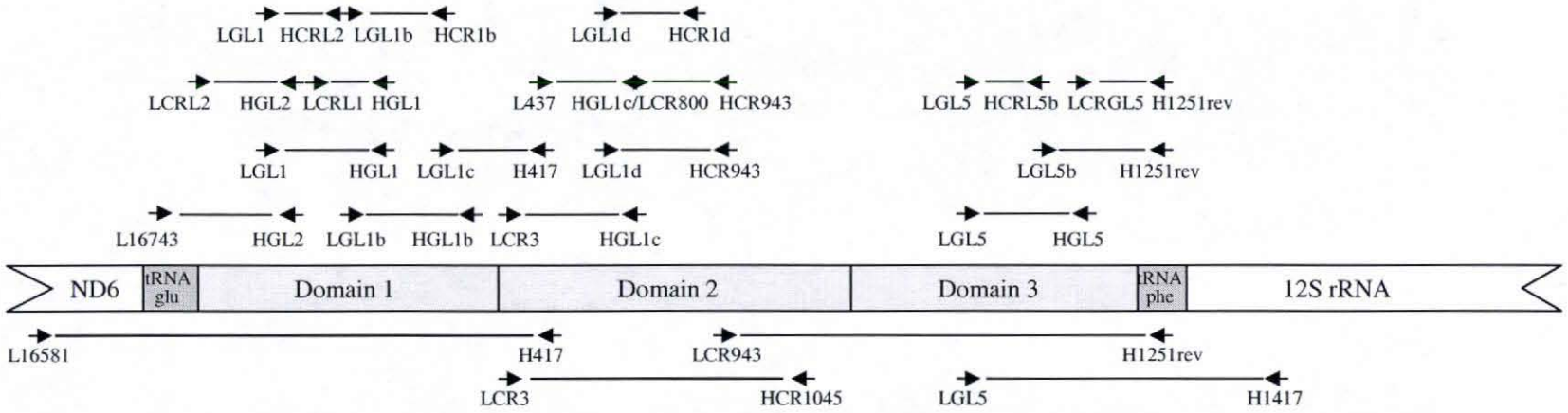


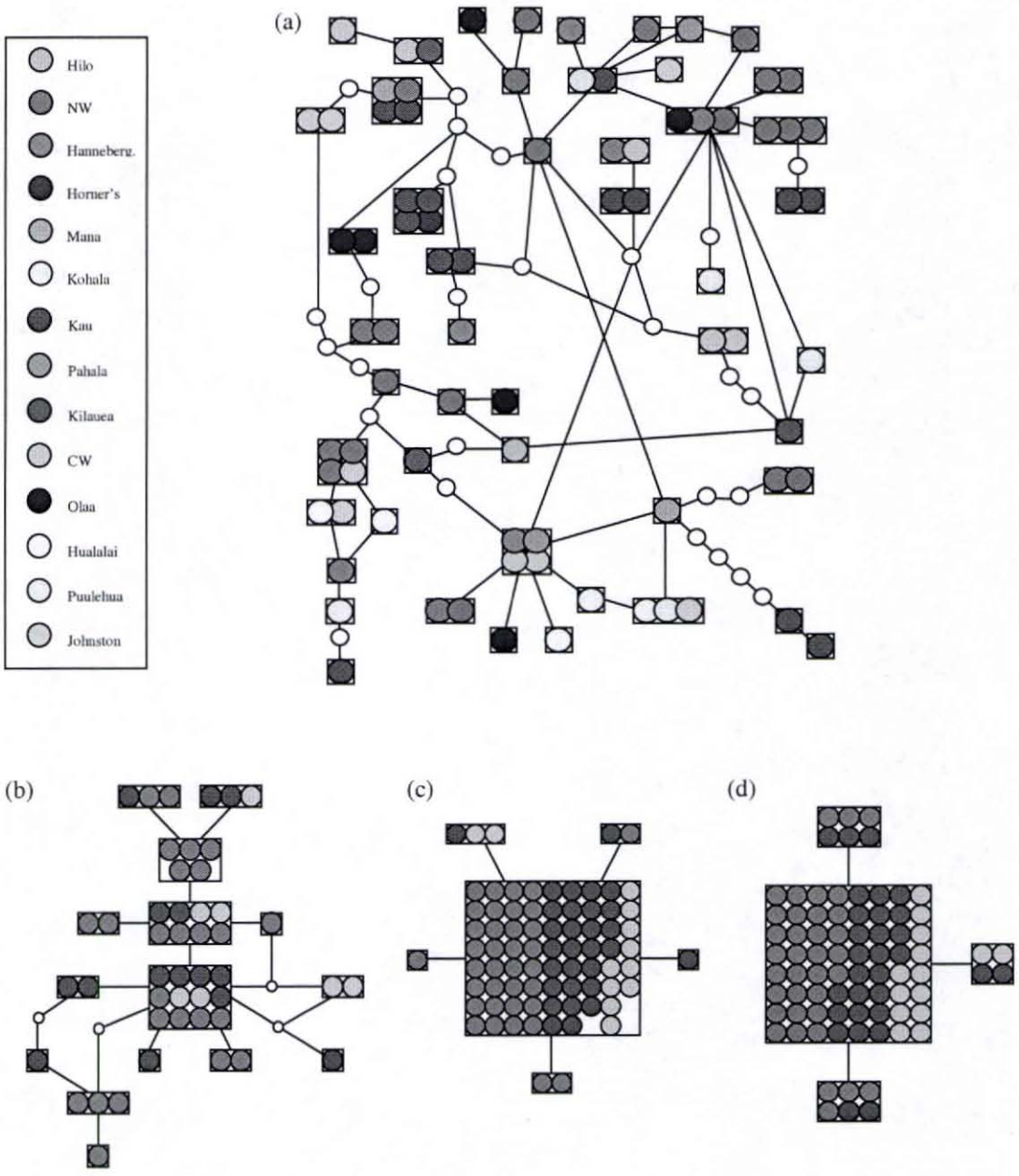
Figure 1. Map of current and historical Akepa distribution and sampling localities. Map of Hawaii Island showing current (outlined in black) and historical (in between the white lines) distribution of the Hawaii Akepa, as well as approximate location of sample sites. Contemporary and historical sampling localities, followed by number of samples obtained and number of samples successfully sequenced, are as follows: A) Kohala (1,1); B) Mana/Waimea (2,2); C) Horner's Ranch (1,1); D) Hanneberg's (5,5); E) North Windward (25,25); F) Hilo/Kaiwiki (4,1); G) Olaa (6,6); H) Kilauea (4,3); I) Central Windward (7,7); J) Ainapo (2,0); K) Pahala (4,2); L) Kau (15,15); M) Johnston's (6,6); N) Puulehua (4,4); O) Hualalai (5,5). Map used with permission of the Pacific Basin Information Node and the Hawaii Biodiversity and Mapping Program.



L16581	AAGAAACCCCCAAACTCACC	HCRL2	GCCGTATGAGGTCCGTTT
LCRL2	CTACAAGAACCCCTAGACCCA	HGL1	GTAGGTAGGAGCACTTGGGC
LGL1	ATCGCACTCTCTGCCACATC	HCR1b	GACATCCCTCGTTCTCTTGGTT
LCRL1	CGCTATGACCCTCCACGAA	HGL1b	TCAGTGGTCTAGGGGAATGC
LGL1b	CCAGAGACATTCTTGTTTCAGG	HGL1c	AGAAGGAAGGATGGGTGGAG
LGL1c	CAAGAGAACGAGGGATGTC	HCR1d	AAGTGTAGGAGGTCGGTATGC
LGL1d	GCTCTTTTGCGCTATTGGTT	HCR943	GCACTTGAGGGGCTTATTGA
LCR800	CTCCACCCATCCTTCCTTCT	HCR1045	GAGACGACCTTATCCGCAAA
LCR943	TCAATAAGCCCCTCAAGTGC	HCRL5b	GTTTTTGGGTGGGATGTGAT
LGL5	ATCCCTACATTTTACAAACTATCC	HGL5	TTTTCGATGGCGTAAAAAGG
LGL5b	ATCACATCCCACCCAAAAAC	H1251rev	TCTTGACATCTTCAGTGTCATGC
LCRGL5	CTTACCTTTTTACGCCATCGAA	H1417	CTTGGGCTACGGTTATGGTG

Figure 2. Location and sequence of mtDNA control region primers.
Horizontal position of primers and fragments indicate their location on the mtDNA molecule, which is represented by the illustration. Immediately below the mtDNA illustration are the four primer sets used to amplify the contemporary samples. Above the DNA segment are the primers used to amplify the museum specimens, with the bottom two rows showing the eight primer sets mainly used to amplify small PCR products (159-193 bp) and the top two rows showing the nine primer sets used to amplify even smaller PCR products (113-137 bp) from heavily degraded samples. Primers L16743, LCR3, L437, HGL2, and H417 are from Tarr (1995). Other primers were designed from Akepa sequence generated from two blood samples and using overlapping primer sets L16525rev/HGL2, L16525rev/H417, L16743/HGL2, L16743/H1858, L16743/Hphe1, LGL2/Hphe1, L437/Hphe1, and L437/H1858. Primer L16525rev (ACAAACACCACCARC-ATTCCMCC) was modified from Sorenson et al. (1999), primer H1858 is from Sorenson et al. (1999), and primers LGL2 and Hphe1 are from Tarr (1995). Sequences (5' to 3') of the Akepa-specific primers designed in this study are provided in the box.

Figure 3. Haplotype networks.
Haplotype networks for (a) 1 KB control region; (b) ND2; (c) Gapd; and (d) Lam. Each circle corresponds to one sequence, each box to one haplotype/allele, and each line to one mutation. White circles represent inferred haplotypes.



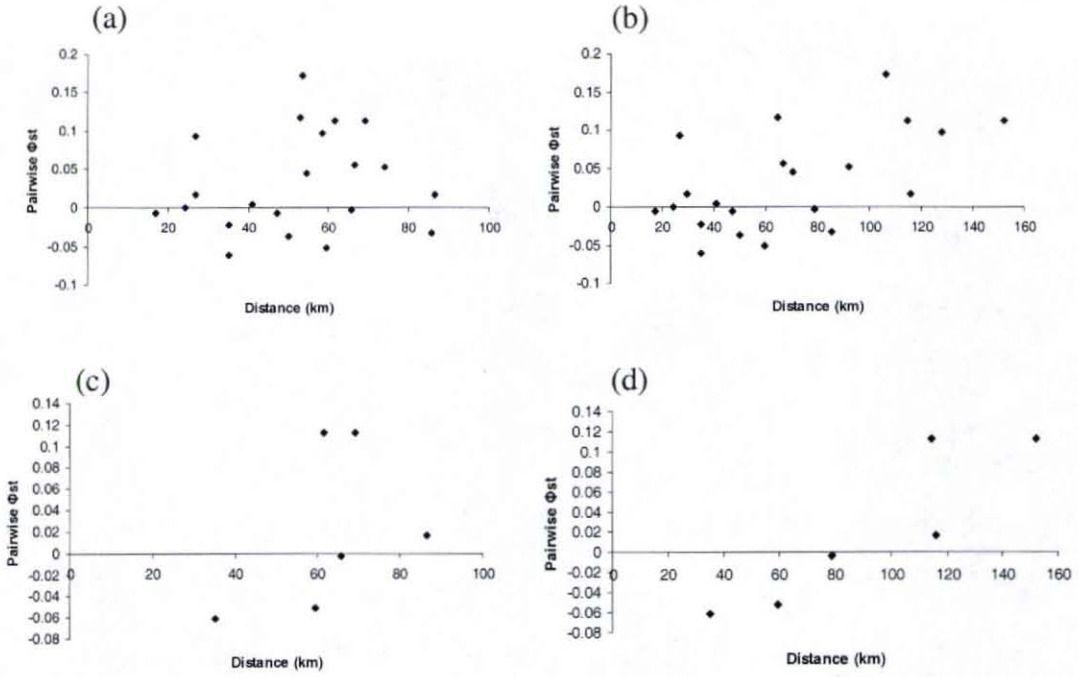
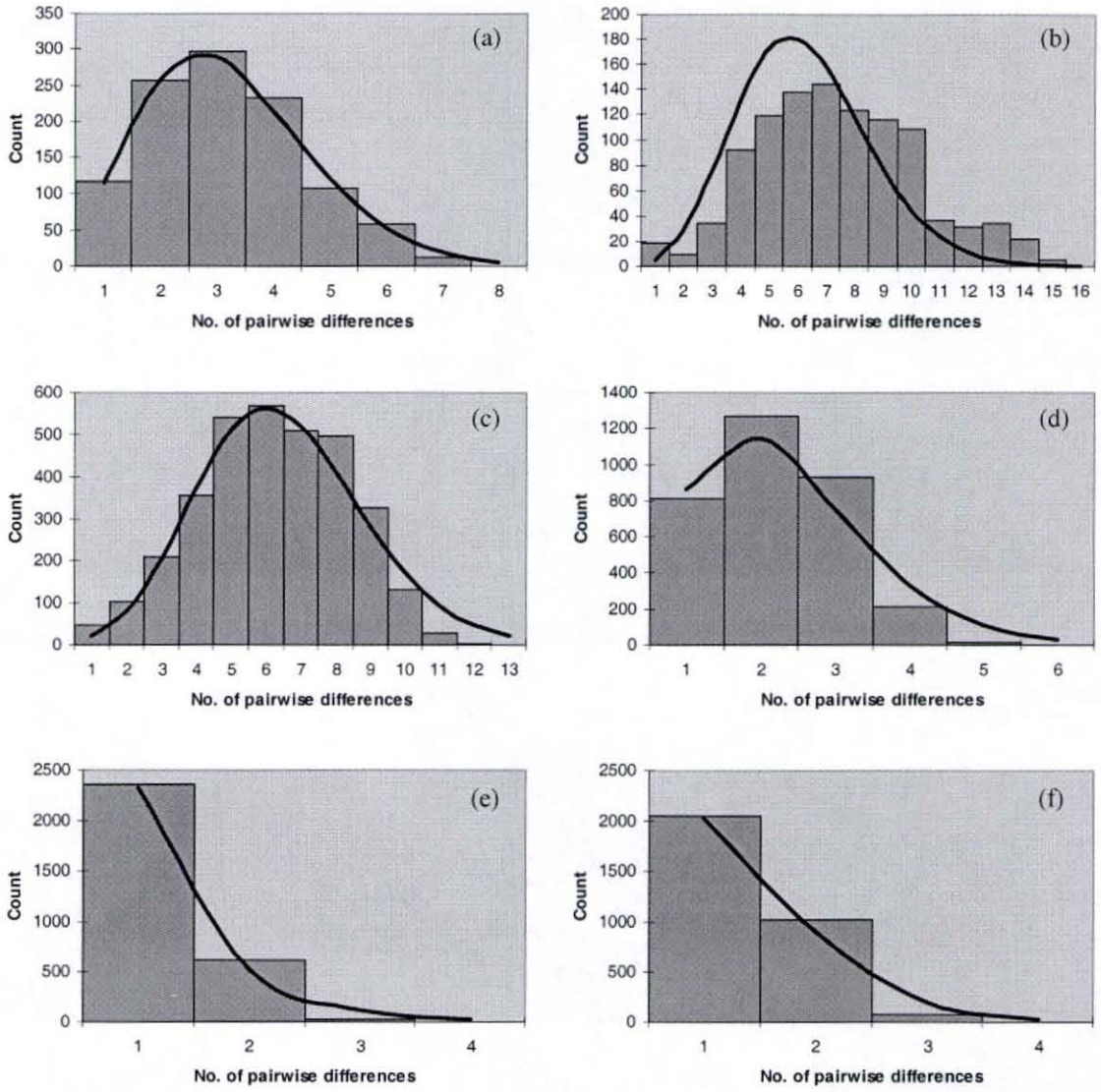


Figure 4. Plots of pairwise Φst vs. distance.
Plot of population pairwise Φst vs. distance for the 1 KB control region using (a) historical + contemporary populations and straight-line distances; (b) historical + contemporary populations and non-Euclidean distances; (c) historical populations and straight-line distances; (d) historical populations and non-Euclidean distances. Straight-line distances were calculated based on map coordinates and non-Euclidean distances were calculated considering Mauna Loa, Mauna Kea, the saddle area between the two mountains, and the North Kona region as geographical barriers to dispersal (see text).

Figure 5. Mismatch distributions.
Observed (bars) and expected (line) mismatch distributions for Hawaii Akepa under the sudden expansion model (Rogers 1995): (a) ND2, all modern samples; (b) 1.6 KB control region, all modern samples; (c) 1 KB control region, modern + ancient samples; (d) LGL2-H417 control region, modern + ancient samples; (e) Gapd, all modern samples; (f) Lam, all modern samples.



Appendix 1. Summary list of historical samples used in this study

Sample ID	Collection Date	Locality[1]	Sex	Contributor[2]	Data[3]
AMNH 453393	17-Oct-1891	Hualalai*	m	AMNH	+
AMNH 453394	16-Oct-1891	Hualalai*	m	AMNH	+
AMNH 453395	16-Oct-1891	Hualalai*	m	AMNH	+
AMNH 453401	25-Sep-1891	Puulehua*	m	AMNH	+
AMNH 453402	29-Sep-1891	Puulehua*	m	AMNH	+
AMNH 453403	29-Sep-1891	Puulehua*	m	AMNH	+
AMNH 453404	29-Sep-1891	Puulehua*	m	AMNH	+
AMNH 453406	14-Mar-1892	Hanneberg sheep station*	m	AMNH	+
AMNH 453409	15-Mar-1892	Hanneberg sheep station*	m	AMNH	+
AMNH 453410	15-Mar-1892	Hanneberg sheep station*	m	AMNH	+
AMNH 453411	15-Mar-1892	Hanneberg sheep station*	m	AMNH	+
AMNH 453413	15-Mar-1892	Hanneberg sheep station*	f	AMNH	+
AMNH 453415	07-Dec-1891	Hualalai*	f	AMNH	+
AMNH 453416	18-Nov-1891	Johnston's Dairy*	f	AMNH	+
AMNH 453418	17-Nov-1891	Johnston's Dairy*	f	AMNH	+
AMNH 453419	17-Nov-1891	Johnston's Dairy*	f	AMNH	+
AMNH 453420	17-Nov-1891	Johnston's Dairy*	f	AMNH	+
AMNH 453421	17-Nov-1891	Johnston's Dairy*	f	AMNH	+
BPBM 3236	09-Feb-1900	Kaiwiki	m	BPBM	~
BPBM 3253	13-Feb-1900	Kaiwiki	m	BPBM	~
BPBM 3255	11-Feb-1900	Kaiwiki	m	BPBM	~
BPBM 3277	6-Jan-1902	Pahala, Kau	f	BPBM	-
BPBM 3278	30-Nov-1901	Pahala, Kau	f	BPBM	-
BPBM 3279	01-Mar-1902	Ainapo, Kau	m	BPBM	~
BPBM 3283	1-Mar-1902	Ainapo, Kau	m	BPBM	~
BPBM 6027	25-Feb-1892	Kohala Mts	f	BPBM	+
BPBM 7229-A	00-Sep-1896	Kilauea	m	BPBM	~
BPBM 7236-A	00-Sep-1896	Kilauea	m	BPBM	+
CAS 33103	22-Dec-1899	Olaa	m	CAS	+
LACM 27714	14-May-1902	Pahala, Kau,	m	LACM	+
LACM 27715	14-May-1902	Pahala, Kau,	m	LACM	+
MVZ 21533	23-Aug-1903	Horner's Ranch	m	MVZ	+
ROM 62777	16-Nov-1891	Johnston's Dairy*	f	ROM	+
ROM 62778	7-Dec-1891	Hualalai*	f	ROM	+
UMZC 27/Dre/6/a/1	28-Feb-1888	Puleokapa, Waimea	m	UMZC	+
UMZC 27/Dre/6/a/15	00-Aug-1894	Kilauea	f	UMZC	+
UMZC 27/Dre/6/a/16	00-Mar-1888	Mana Dairy	f	UMZC	+
UMZC 27/Dre/6/a/9	00-Aug-1894	Kilauea	m	UMZC	+
USNM 169325	6-Jun-1898	Olaa	m	USNM	+
USNM 169326	29-Jun-1898	Olaa	m	USNM	+
USNM 169327	5-Jun-1898	Olaa	m	USNM	+
USNM 172457	7-May-1898	Olaa	f	USNM	+
USNM 172458	8-May-1898	Olaa	f	USNM	+
ZMK 573	00-Dec-1895	Hilo 2000'	?	ZMK	+

[1]Museums contributing the samples are as follows: AMNH: American Museum of Natural History; BPBM: B. P. Bishop Museum; CAS: California Academy of Sciences; LACM: Los Angeles County Museum; MVZ: Museum of Vertebrate Zoology; ROM: Royal Ontario Museum; UMZC: University Museum of Zoology, Cambridge; USNM: National Museum of Natural History; and ZMK: Zoological Museum, Kopenhagen.

[2]An asterisk indicates that location was determined from the *Résumé of Palmer's Diary* (Rothschild 1893-1900) by corroborating the date of collection listed on the specimen tag with collector H.C. Palmer's whereabouts during that time.

[3]The following signs describe the level of sequence data obtained: - denotes samples that did not yield any sequence data, ~ denotes samples that yielded some sequence data but were too incomplete to be included in the analyses (<70% complete), and + denotes samples that yielded all or most of the targeted control region sequences and were included in the analyses.

Appendix 2. Summary list of contemporary samples used in this study.

Sample ID	Collection Date	Locality	Population	Type[1]	Sex	Contributor[2]
Kau17	03-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau20	04-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau25	04-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau29	05-May-2006	Kau Forest Reserve	Kau	B	F	-
Kau34	14-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau36	15-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau42	16-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau43	16-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau62	25-May-2006	Kau Forest Reserve	Kau	B	M	-
Kau69	01-Jun-2006	Kau Forest Reserve	Kau	B	M	-
Kau82	14-Jun-2006	Kau Forest Reserve	Kau	B	M	-
Kau83	14-Jun-2006	Kau Forest Reserve	Kau	B	F	-
Kau96	25-Jun-2006	Kau Forest Reserve	Kau	B	F	-
Kau98	26-Jun-2006	Kau Forest Reserve	Kau	B	F	-
Kau99	26-Jun-2006	Kau Forest Reserve	Kau	B	F	-
Sol242	24-Jan-2002	Solomon's Waterhole	CW	B	M	Biocompl.
Sol245	24-Jan-2002	Solomon's Waterhole	CW	B	M	Biocompl.
Sol472	19-Apr-2002	Solomon's Waterhole	CW	B	M	Biocompl.
Sol769	03-Oct-2002	Solomon's Waterhole	CW	B	M	Biocompl.
Rcf3025	13-Feb-1988	Powerline Road	CW	M	M	RCF
Rcf3026	13-Feb-1988	Powerline Road	CW	M	M	RCF
Rcf3027	13-Feb-1988	Powerline Road	CW	M	M	RCF
SRO84	22-May-1995	Hakalau Forest NWR	NW	B	M	LAF
UPA1502	22-Feb-2000	Hakalau Forest NWR	NW	B	F	LAF
UPA1504	22-Feb-2000	Hakalau Forest NWR	NW	B	F	LAF
UPA1505	22-Feb-2000	Hakalau Forest NWR	NW	B	M	LAF
UPA1548	26-Mar-2000	Hakalau Forest NWR	NW	B	F	LAF
UPA1992	28-Feb-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2035	26-Mar-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2045	27-Mar-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2047	27-Mar-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2060	30-Mar-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2068	03-Apr-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2069	03-Apr-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2094	14-Apr-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2099	15-Apr-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2104	16-Apr-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2127	24-Apr-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2144	03-May-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2211	20-Jun-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2250	29-Jun-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2287	15-Jul-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2288	15-Jul-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2289	15-Jul-2002	Hakalau Forest NWR	NW	B	M	LAF
UPA2310	03-Aug-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2313	03-Aug-2002	Hakalau Forest NWR	NW	B	F	LAF
UPA2340	25-Aug-2002	Hakalau Forest NWR	NW	B	F	LAF

[1] B=blood; M=muscle

[2] Samples were contributed by the Hawaii Biocomplexity Project (Biocompl.), Robert C. Fleischer (RCF), and Leonard A. Freed (LAF).

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