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Genetic structure and evolved malaria resistance in Hawaiian honeycreepers

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Abstract

Infectious diseases now threaten wildlife populations worldwide but population recovery following local extinction has rarely been observed. In such a case, do resistant individuals recolonize from a central remnant population, or do they spread from small, perhaps overlooked, populations of resistant individuals? Introduced avian malaria (*Plasmodium relictum*) has devastated low-elevation populations of native birds in Hawaii, but at least one species (Hawaii amakihi, *Hemignathus virens*) that was greatly reduced at elevations below about 1000 m tolerates malaria and has initiated a remarkable and rapid recovery. We assessed mitochondrial and nuclear DNA markers from amakihi and two other Hawaiian honeycreepers, apapane (*Himatione sanguinea*) and iiwi (*Vestiaria coccinea*), at nine primary study sites from 2001 to 2003 to determine the source of re-establishing birds. In addition, we obtained sequences from tissue from amakihi museum study skins (1898 and 1948–49) to assess temporal changes in allele distributions. We found that amakihi in lowland areas are, and have historically been, differentiated from birds at high elevations and had unique alleles retained through time; that is, their genetic signature was not a subset of the genetic variation at higher elevations. We suggest that high disease pressure rapidly selected for resistance to malaria at low elevation, leaving small pockets of resistant birds, and this resistance spread outward from the scattered remnant populations. Low-elevation amakihi are currently isolated from higher elevations (> 1000 m) where disease emergence and transmission rates appear to vary seasonally and annually. In contrast to results from amakihi, no genetic differentiation between elevations was found in apapane and iiwi, indicating that slight variation in genetic or life-history attributes can determine disease resistance and population recovery. Determining the conditions that allow for the development of resistance to disease is essential to understanding how species evolve resistance across a landscape of varying disease pressures.

Keywords: Avian malaria, introduced disease, *Plasmodium relictum*, population structure

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Introduction

Although pathogens have afflicted wildlife for millennia, anthropogenic change and the global introductions of species have dramatically altered the frequency, distribution and impact of disease (Daszak *et al*. 2000; Dobson & Foufopoulos 2001). Infectious diseases threaten the extinction of wildlife populations worldwide (e.g. LaDeau *et al*. 2007), but rarely have we had a chance to observe population recovery

(Cleaveland *et al*. 2002). Determining population-level effects of pathogens on genetic structure is essential for understanding disease impacts on host abundance and distribution (Sorci *et al*. 1997; Uller *et al*. 2003). Why do some populations persist while others go extinct? Furthermore, to determine the genesis of disease resistance in wildlife we must understand how disease impacts populations across the landscape (Real *et al*. 2005). Where are the sources of resistant individuals and how do they repopulate when disease has decimated local populations?

Avian blood parasites (Haemosporidia) infect bird populations globally (Beadell *et al*. 2006) but long-term population effects have rarely been documented. In contrast, avian malaria (*Plasmodium relictum*) appears to have been a major factor in the extinction and decline of native Hawaiian birds over at least the past 60 years, particularly at low elevations (below ca. 1200 m) (Warner 1968; van Riper *et al*. 1986). Endemic forest birds of the Hawaiian Islands, particularly honeycreepers (Drepanidinae), are broadly susceptible to malarial infection, with drepanid mortality rates of 65–90% depending upon species van Riper *et al*. 1986; Atkinson *et al*. 1995, 2001b; Yorinks & Atkinson 2000). Birds that do not immediately die sustain low-level chronic infections that can decrease long-term survival (Kilpatrick 2006), and these resistant birds serve as a reservoir to infect mosquitoes and continue the disease cycle (Atkinson *et al*. 2001a; Jarvi *et al*. 2001, 2002). We define malaria resistance as the ability of birds to contract and survive the disease, although some individuals may never completely clear the parasite from their systems. As a result of disease, populations of native forest birds are primarily restricted to mid and high elevations, where malaria is rare due to physiological (thermal) constraints on the primary vector (*Culex quinquefasciatus*) and the malaria parasite (van Riper *et al*. 1986; LaPointe 2000). Malaria is, in fact, the second wave of disease in Hawaiian birds; avian pox (*Poxvirus avium*) arrived in the late 1800s and appears to have devastated native bird populations (van Riper *et al*. 2002). The relationship between malaria and pox remains unclear, but pox may have been the first selective disease pressure on lowland native birds.

Despite the prevalence of malaria and mosquitoes in low-elevation habitats, small numbers of native birds have been observed at low elevations but their status has been little known (van Riper *et al*. 1986; Reynolds *et al*. 2003). In fact, one species of Hawaiian honeycreeper, the Hawaii amakihi (*Hemignathus virens*), now densely populates low-elevation habitats despite extremely high prevalence of malaria and evidence for local transmission (Woodworth *et al*. 2005). Woodworth *et al*. proposed that constant disease pressure could drive selection of heritable host resistance, and experimental evidence from birds in captivity indicated that low elevation amakihi are better able to cope with malarial infections than amakihi from higher elevations (C. Atkinson, unpublished). This is a clear example of a bird population rapidly evolving to the introduction of a novel pathogen. Yet, the origin of resistant birds has remained unknown.

In the presence of widespread malaria, where is the source of resistant individuals responsible for population recovery of amakihi? Were populations of low-elevation birds completely purged and then recolonization occurred from large populations that inhabit higher elevation forests < 15 km away? In this scenario, little genetic differentiation would be expected between elevations, and genetic variation at low elevations would be expected to be a subset of variation at higher elevations. Alternatively, did small remnant bird populations, subjected to extremely strong selection, rapidly evolve malaria resistance over several decades and re-establish? Evidence of genetic structure among elevations, unique local or private alleles, and a bottleneck at low elevation (i.e. lower genetic variation), would support the hypothesis of resistance developing among remnant low-elevation populations. Furthermore, comparison of genetic and life-history attributes will enable us to assess the source of variation in population-level effects among species. Although closely related to amakihi, apapane (*Himatione sanguinea*) and iiwi (*Vestiaria coccinea*) populations appear to respond quite differently to malaria and have not densely repopulated low-elevation areas.

In this paper, we provide evidence that the recovery of low-elevation amakihi occurred within multiple, small remnant populations at low elevation, where disease transmission occurs year round, rather than at the interface between the disease zone and large host populations, where transmission may vary seasonally and annually (van Riper *et al*. 1986; Woodworth *et al*. 2005). Determining source populations is essential for designing recovery efforts for threatened species impacted by disease and for developing models of how species evolve disease resistance across a landscape of varying disease pressures.

Materials and methods

We took blood samples from honeycreepers at nine primary study sites from 2001 to 2003, see Woodworth *et al*. (2005), as part of a broader study on the interactions of malaria hosts, parasites and vectors. For amakihi, we also used contemporary (1995–99) samples from additional sites throughout the island of Hawaii to place our results more broadly in the landscape. In addition, we utilized ancient DNA techniques (as described below) to obtain sequences from tissue from amakihi museum study skins collected in 1898 (low-elevation sites) and 1948–49 (mid-elevation sites). Historical samples allowed us to assess temporal changes in allele distributions. Nine samples from 1898 may antecede the arrival of avian

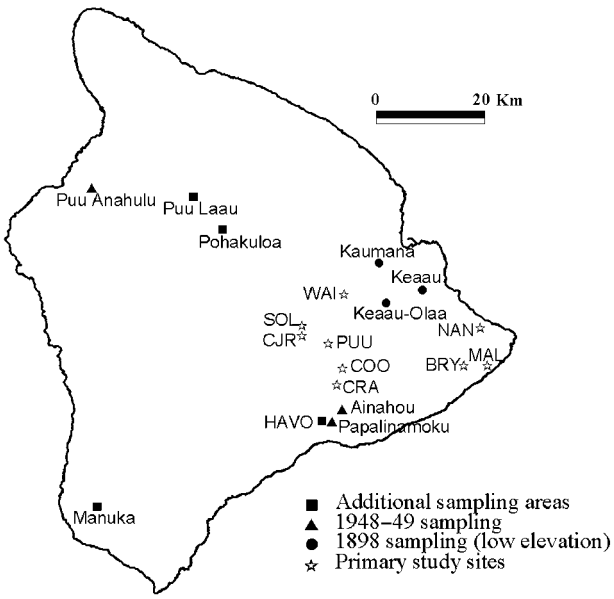

Fig. 1 Map of primary study sites on the island of Hawaii, with additional sampling areas from contemporary (1995–99) and historic sampling (1898 and 1948–49).

malaria in the Hawaiian Islands in ca. the 1940s (Fisher & Baldwin 1947; RCF *et al.* unpublished) and allow us to assess the past distribution of mitochondrial DNA (mtDNA) haplotypes.

Study sites and bird capture

We captured birds in mist-nets at nine sites on the eastern slopes of Mauna Loa and Kilauea on the island of Hawaii (Fig. 1). All 1-km^2 sites were within ohia-dominated (*Metrosideros polymorpha*) wet forest and selected along an elevational gradient, with three low-elevation sites (25–300 m), four mid-elevation sites (1000–1300 m) and two high-elevation sites (1650–1800 m). We took standard morphological measurements from birds and drew blood using jugular or brachial venipuncture. Separated red blood cells were deposited into lysis buffer (10 mM Tris-HCl, 10 mM NaCl, 10 mM EDTA, 1% SDS) in individual 1.5-mL plastic tubes and subsequently frozen at –20 °C. For additional information and methodology from the primary study sites see Woodworth *et al*. (2005). Bird handling adhered to animal care regulations of the University of Hawaii, IACUC # 00–035.

Sampling of birds from additional contemporary sites followed the same protocols as above. Samples (n) were grouped based on geography: Manuka (20); Puu Anahulu/Puu Laau/Pohakuloa (24); Kaumana/Keaau/Keaau-Olaa (9); and Ainahou/Papalinamoku (6). Hawaii Volcanoes National Park (3) was grouped with mid elevation sites COO and CRA. Samples used for ancient DNA came from birds collected in 1898 at Kaumana, Keeau and Keaau-Olaa by H.W. Henshaw (US National Museum, Washington, DC) and 1948–49 at Ainahou, Papalinamoku and Puu Anahulu by P.H. Baldwin (Museum of Vertebrate Zoology, Berkeley, CA). Tissue for extractions came from the toe pads of museum skins.

Laboratory procedures

We extracted DNA from blood samples using DNeasy® Tissue Kits (QIAGEN) and followed tissue extraction protocols. We amplified regions of mtDNA and nuclear DNA (nDNA) using polymerase chain reaction (PCR). For mtDNA, we focused on a sequence of ca. 345 bps of the non-coding control region using primer pair LGL2 and H417 (Tarr 1995). We amplified the DNA under the following conditions: 25 μL volume containing 10X PCR Buffer (50 mM KCl, 15 mM tris-HCl), 0.2 mM each dNTP, 0.4 μM each primer, 2 mM $MgCl_2$, 2 mg/mL BSA and 1 unit AmpliTaq® DNA polymerase. The cycling profile was as follows: 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min, and a final extension of 72 °C for 7 min.

Extraction and preparation of ancient samples were performed in an isolated lab dedicated to ancient DNA and followed strict protocols to prevent contamination (Fleischer *et al*. 2001). The control-region sequence was assembled in overlapping pieces through multiple PCR amplifications with the following primer sets developed for smaller DNA fragments: CR1: LGL2.5 to H417 (Tarr 1995); CR2: LGL2.5 to HCR-3 (5′ATGCAAAGGTGTACTGGGAC′3); CR3: LGL-3 (5′TTATCTCCAAAACGGACCTC′3) to H417; and CR4: LGL1 (5′-ATCGCACTCTCTGCCACATC′3) to HGL-1 (5′-GTAGGTAGGAGCACTTGGGC′3), both from Reding (2007). PCRs were set up in the ancient DNA lab and followed conditions provided in Fleischer *et al*. (2001), except that we used AmpliTaq® polymerase and total reaction volumes of 25 μL.

Nuclear DNA was amplified with primer pairs specific to two introns, lactate dehydrogenase (LDH), (605 bp) (Friesen *et al*. 1999) and α-enolase (ENOL), (310 bp) (Friesen *et al*. 1997). Cycling conditions for nDNA followed the above profile, with the exception that we ran 35 cycles at specific annealing temperatures: LDH 68 °C, ENOL 55 °C.

We assessed PCR amplification on EtBr-stained agarose gels and purified PCR product with QIAquick® PCR purification kits. PCR products were then cycle-sequenced in both directions using the same primers and we purified this product with G-50 fine Sephadex® gel filtration. Sequences were generated in an Applied Biosystems 3100 DNA sequencer. We assembled, aligned, and edited sequences in SEQUENCHER vs. 4.1. We separated the alleles of heterozygous individuals at nuclear introns in SSCP gels stained with GelStar, cut out unique bands, and sequenced to determine the sequence of each allele (Friesen *et al*. 1997).

Analyses

For mtDNA, we sequenced at least 40 individuals from each site in an attempt to equalize sample sizes. When fewer birds were captured, we sequenced samples from all possible individuals. Unique haplotypes were all verified for both strands and re-sequenced if necessary. For nDNA, we selected a subset of individuals for which we had a mtDNA sequence and generated sequence for roughly 15 birds per site at six sites.

We employed multiple population genetic methods to assess population structure and history. Comparisons were made based on the groups of birds at each of the main study sites, with pooling of individuals at different sites at the same elevation where sample sizes were limited (Table 3). Haplotype networks were generated in TCS version 1.21 (Clement *et al*. 2000). Pie charts of a given size were made to correspond to the indicated frequency of the haplotype in the population. We determined the most appropriate nucleotide substitution model in MODELTEST 3.7 (Posada & Crandall 1998). For amakihi, a modified General Time Reversible model (GTR + I) best fit the data based on Akaike's Information Criterion (AIC). The proportion of invariant sites (I) was 0.8814, with the following base frequencies: A = 0.3150, C = 0.3391, G = 0.1448, T = 0.2011. For apapane, the Hasegawa-Kishino-Yano model (HKY) best fit the data and had the following base frequencies: A = 0.2861, C = 0.3432, G = 0.1554, T = 0.2153.

We then analysed population structure with either pairwise comparisons of ϕ_{ST} and when possible, an Analysis of Molecular Variance (AMOVA) in ARLEQUIN using the most similar substitution model (Schneider *et al*. 2000). Comparisons were made as follows; between sites, nested within elevations, and among elevations. In the island-wide comparison in amakihi, sites were grouped into seven areas based on geography: NAN/BRY/MAL, Kaumana/Keeau/Keaau-Olaa, Manuka, COO/CRA/HAVO, Ainahou/Paplinamoku, SOL/CJR and Pohakuloa/Puu Laau/Puu Anahulu. For amakihi, we used the Tamura & Nei model as the closest model to the GTR + I available in ARLEQUIN. For apapane, Tamura & Nei best approximated the HKY model. In ARLEQUIN, we also generated standard genetic diversity indices, molecular diversity, and estimates of Tajima's D. We assessed the significance of test statistics with 1000 permutations of the data. We also assessed population structure using pairwise comparisons of ϕ_{ST} for haplotypes of nuclear introns. Results were similar when comparisons were made as genotypes. We used Kimura two-parameter and Tamura & Nei (Gamma = 0.54, estimated in PAUP*; Swofford 2002) models for distance calculations for ENOL and LDH, respectively.

We tested the nuclear introns for linkage disequilibrium in ARLEQUIN and found no apparent linkage between ENOL and LDH (Exact test, $P > 0.05$). Both introns, however, failed to meet expectations of Hardy–Weinberg equilibrium within each elevation and across all elevations (Exact test, all $P < 0.001$). Nonetheless, this failure did not appear to affect differentiation estimates.

Results

Amakihi populations

Amakihi populations at different elevations in the primary study area were highly differentiated genetically for both mtDNA and nuclear markers. We sampled 283 birds at seven sites and found 34 mtDNA haplotypes (Table 1). Two common mtDNA haplotypes (27% and 24% of samples) occurred throughout all elevations, with genetic structure driven by allele-frequency differences and private alleles — 18

Table 1 Summary of DNA sequences of three Hawaiian honeycreepers extracted from blood of birds captured at nine study sites on the island of Hawaii

		Elevation									
		Low			Mid				High		
Species	Data set	BRY	MAL	NAN	COO	CRA	PUU	WAI	CJR	SOL	Total
Amakihi	mtDNA	44	45	46	3	45	—	—	47	53	283
	ENOL	14	14	16	—	18	—	—	10	16	88
	LDH	14	10	13	—	16	—	—	16	10	79
Apapane	mtDNA	1	8	3	46	52	41	14	47	42	254
Iiwi	mtDNA	—	—	—	1	—	15	2	12	10	40

Genetic analyses of population structure were based on the mtDNA control region and the nuclear introns α-enolase (ENOL) and lactate dehydrogenase (LDH). Primary site codes for mist-netting were: BRY, Bryson's Cinder Cone; COO, Cooper's Center; CJR, CJ Ralph Site; CRA, Kilauea Iki Crater Rim; MAL, Malama Ki Forest Reserve; NAN, Nanawale Forest Reserve; PUU, Puu Unit Olaa Tract; SOL, Solomons Waterhole; WAI, Upper Waiakea Forest Reserve.

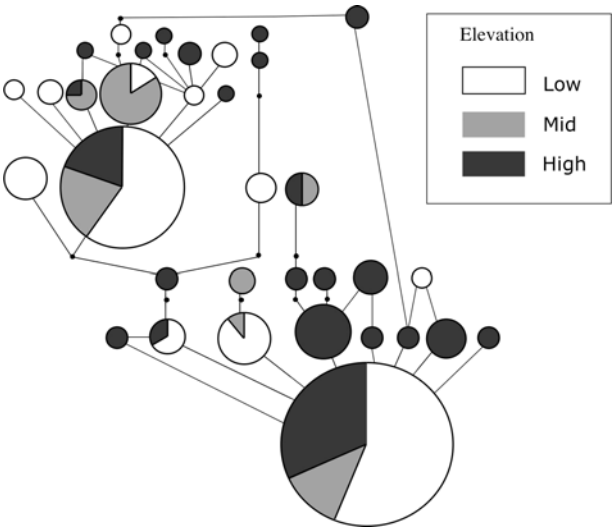

Fig. 2 Network diagram of all 34 mtDNA haplotypes in Hawaii amakihi from biocomplexity field sites at three elevations on the island of Hawaii. Sites are grouped based on elevation, and sample sizes varied by elevation (low = 135, mid = 50, high = 100). Size of the circle indicates frequency of the haplotype; smallest circles indicate only one individual with that haplotype. Haplotypes were typically separated by one bp mutation. Two haplotypes are common throughout all elevations; largest circles represent haplotypes of 67 and 75 individuals.

unique haplotypes at high-, one at mid- and eight at low elevation (Fig. 2). We found significant pairwise ϕ_{ST} values between mtDNA sequences at different elevations; low–mid = 0.085, low–high = 0.101, mid–high = 0.228, all $P < 0.001$. The highest ϕ_{ST} value occurred between mid- and high-elevation sites, despite only 17 km between the most distant locations, suggesting negligible effects of isolation by distance. Strong population structuring in amakihi by elevation also occurred when we assessed all sites separately, with minimal differentiation between sites at the same elevation but considerable differences between most sites at different elevations (Table 2). Of the total nucleotide variation, 0.36% occurred among sites at the same elevation, 11.33% among sites across elevations and 88.31% within sites (AMOVA, $F_{ST} = 0.117$, $P < 0.001$). All measures of mtDNA genetic diversity in amakihi indicated highest diversity at high elevation, despite greatest sampling at low elevation (Table 3). We found little evidence of a population bottleneck at low elevation, with Tajima's D not strongly negative. In comparing the number of haplotypes, there was no significant difference in diversity at high and low elevation (Mann–Whitney two-tailed test, $U = 6.0$, $P = 0.08$).

Table 2 Pairwise Φ_{ST} values for mtDNA sequences between sites at three elevations for Hawaii amakihi sampled on the island of Hawaii, 2001–03

	Low			Mid	High	
	BRY	MAL	NAN	COO-CRA	CJR	SOL
BRY	—					
MAL	0.033	—				
NAN	0.000	0.004	—			
COO-CRA	0.062***	0.076*	0.079**	—		
CJR	0.123***	0.064**	0.087***	0.197***	—	
SOL	0.140***	0.067**	0.104***	0.212***	0	—

Significance of population differences based on a 1000 permutations: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Strong population differentiation occurred with nDNA introns (ENOL, LDH), and the patterns resembled the population genetic structure found in mtDNA. For ENOL, significant population structuring occurred based on elevation; pairwise ϕ_{ST} values between elevations were as follows: low–mid = 0.051, low–high = 0.041, mid–high = 0.063, all $P < 0.001$. Structuring also occurred in LDH, although the mid–high comparison indicated no difference; pairwise ϕ_{ST} values were: low–mid = 0.028, low–high = 0.043, mid–high = 0.000, $P < 0.05$ for first two comparisons. Two common alleles (43% and 34% of total samples) occurred among the 16 haplotypes in the LDH intron, and only one common allele (27% of total samples) occurred in ENOL among the 25 haplotypes (Fig. 3). As with mtDNA, large

Table 3 Measures of genetic diversity in mtDNA control region for Hawaii amakihi, apapane, and iiwi collected in 2001–03 from study sites at three elevations on the island of Hawaii

	Elevation	N	Mean no. of haplotypes	Haplotype diversity (SD)	Nucleotide diversity (SD)	Tajima's
Amakihi	Low	135	8.3	0.795 (0.021)	0.007 (0.004)	– 0.001
	Mid	48	7	0.776 (0.033)	0.007 (0.004)	0.152
	High	97	17	0.882 (0.019)	0.008 (0.005)	– 0.614
Apapane	Low	12	2	0.530 (0.076)	0.002 (0.002)	1.381
	Mid	153	4	0.586 (0.027)	0.003 (0.002)	1.167
	High	89	3	0.535 (0.042)	0.002 (0.002)	0.567
Iiwi	Mid	17	1	0	0	—
	High	22	1	0	0	—

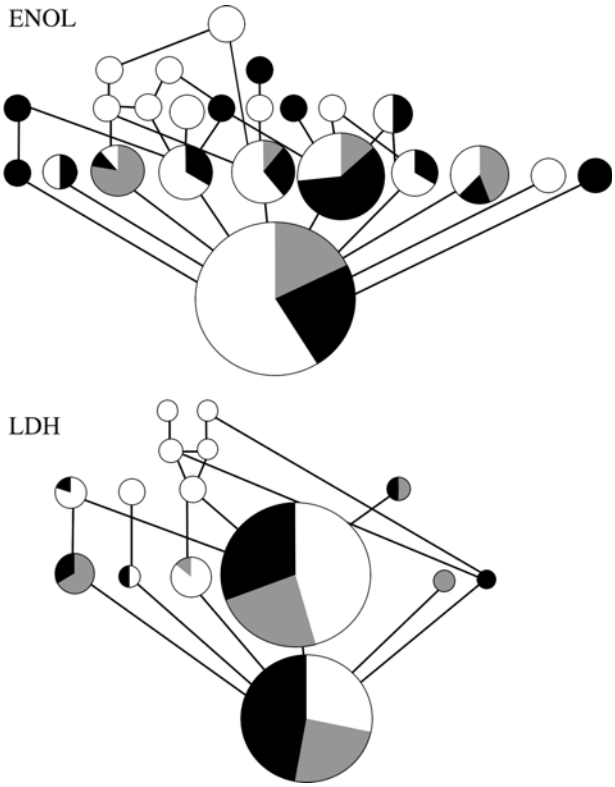

Fig. 3 Network diagrams of nuclear introns α-enolase (ENOL) and lactate dehydrogenase (LDH) in Hawaii amakihi from three elevations on the island of Hawaii. Methodology follows that used in Fig. 2.

numbers of private alleles differentiated the populations at different elevations in both introns; ENOL contained six private alleles at high elevation, nine at low; LDH had one at high, six at low.

Amakihi: whole island and ancient mtDNA

Amakihi collected in 1898 at low elevation near Kaumana and Keaau (Fig. 1) are differentiated from present high-elevation populations (CJR and SOL combined, $\phi_{ST} = 0.08$, $P = 0.04$), but not from populations at any other sites on the island of Hawaii. Of the nine samples from 1898, five shared unique haplotypes found only in current low- and mid- (COO and CRA) elevation populations in the main study area, and three haplotypes were not found in any other sampled birds. Amakihi samples collected in 1948 from mid elevations were not genetically differentiated from populations at any sites, but four of six haplotypes were shared only with populations from mid and high elevations in the main study area. Amakihi samples from other locations on the island of Hawaii, such as low-elevation Manuka and high-elevation sites of Puu Laau-Pohakuloa, were not different from current high-elevation and historic mid-elevation populations. However, these samples were significantly different from current low- ($\phi_{ST} = 0.10$, $P < 0.001$, $\phi_{ST} = 0.08$, $P = 0.007$, respectively) and mid-elevation birds ($\phi_{ST} = 0.15$, $P < 0.001$, $\phi_{ST} = 0.18$, $P < 0.001$, respectively) in our primary study area.

Apapane and iiwi populations

We found no evidence of population structure in apapane or iiwi throughout the main study sites. Genetic diversity was low in apapane with only four haplotypes present and there were no unique alleles based on elevation (Table 3). Iiwi exhibited no mtDNA variation in the control region. Few apapane were captured at low elevation (12 sampled), and no iiwi were captured at any of the low-elevation sites. In apapane, pairwise ϕ_{ST} values indicated no mtDNA divergence between elevations, all $P > 0.05$).

Discussion

As humans continue to alter habitats through the introductions of new species, we must be able to gauge the response of wildlife populations to these novel stressors. For instance, after decimation by disease, we need to document the recovery of populations and assess the factors responsible for their resurgence. Development of resistance to disease is expected to be highest in areas where selection is most intense, but some pathogens are so virulent that few or no survivors will persist. Knowing the source of recovering populations is an essential element for developing conservation strategies for species imperilled by disease, by targeting the most vital populations for long-term persistence. Populations may recover from small disease-resistant groups located within the region of direct disease impact or from large source populations where disease resistance has persisted on the periphery.

Native Hawaiian honeycreepers exhibited strikingly different population genetic structures in the face of disease. Amakihi had strong differentiation over a small spatial scale, but apapane and iiwi did not. MtDNA and nDNA of amakihi indicated significant population structure across elevations based on allele frequencies and on the abundance of private alleles at low- and high-elevation sites. Thus, the data suggest that currently abundant low-elevation amakihi populations of east Hawaii expanded out of low-elevation remnants and did not recolonize from higher elevation. Low-elevation populations of amakihi that may be able to tolerate malaria and pox are genetically unique compared with birds from the rest of the island. The relatively high level of genetic diversity that has been maintained in this low-elevation population is surprising. This suggests that contemporary low-elevation populations arose from multiple remnant populations or a sizeable and diverse population of undetected birds. Unique haplotypes

were present in low-elevation amakihi in 1898, before the arrival of avian malaria in Hawaii, giving additional support for *in situ* population growth of malaria-resistant birds rather than immigration of resistant birds from other areas. While it is feasible that amakihi may have been evenly distributed at low densities throughout low elevations, the historical lack of detection suggests that birds likely had clumped distributions. Historical and contemporary agricultural use of lowland areas could have contributed to this metapopulation structure with populations interbreeding only recently.

Haplotypes shared among sites represent ancestral haplotypes from an earlier spread, rather than movements by contemporary individuals, because only these haplotypes consistently occurred at all sites (and they show as ancestral to most of the haplotypes in Fig. 2 — i.e. a star radiation). Analysis of movement patterns suggests low levels of immigration from mid to low elevation, but not from high to low (data not shown). This result may be biased, however, due to the lack of private alleles at mid elevation and the ancestral haplotypes shared by birds at all elevations. Nonetheless, finer-scale assessment of population structure using microsatellites corroborates dispersal among elevations and indicates significant population structure in amakihi, but not in apapane and iiwi, which is consistent with this study (LSE, RCF unpublished). Thus, it is surprising that population structure has been maintained despite gene flow.

Why has the amakihi been able to re-establish in malaria-infested lowlands (Woodworth *et al*. 2005; Spiegel *et al*. 2006) while other native Hawaiian forest birds have not? High-elevation amakihi and apapane both survive malaria about 35% of the time (Jarvi *et al*. 2001), and nearly all honeycreepers appear to have some malaria resistance (Atkinson *et al*. 2000). Thus, differing levels of susceptibility among closely related honeycreepers suggest species-specific responses to disease. We suggest that dispersal behaviour, genetic diversity at disease-related loci and/or demographic effects are likely responsible for differences in levels of resistance and the subsequent effects on population genetic structure and distribution. Our analyses indicate that while amakihi have developed distinct genetic structure across the elevational gradient in windward Hawaii, two closely related honeycreepers, iiwi and apapane, have not. Amakihi are a relatively sedentary species (Lindsey *et al*. 1998), while iiwi and apapane make wide-ranging movements in search of their primary food source, ohia nectar, which varies seasonally across the landscape (Ralph & Fancy 1995; Fancy & Ralph 2000). While we do not think that they stay in these new areas to breed, higher rates of movement by iiwi and apapane suggest a higher rate of natal and breeding dispersal. Relatively sedentary species such as amakihi would be expected to face stronger selective pressures from disease. In contrast, the widespread movement of vagile species such as apapane and iiwi may prevent the development of malaria resistance among localized populations because of strong gene flow between resistant and susceptible populations. Native forest birds, particularly apapane, occur at low-elevation sites where malaria is prevalent but may represent individuals in search of nectar rather than local breeding populations.

Genetic diversity may affect the ability of species to evolve resistance in the face of new or emergent disease. The high degree of genetic diversity in amakihi and relatively low diversity in iiwi may indicate that past population bottlenecks have reduced their ability to resist disease. Iiwi have extremely low overall mtDNA diversity relative to other Hawaiian honeycreepers and are the most susceptible to malaria. However, iiwi have levels of diversity at loci in the Major Histocompatibility Complex (MHC), a region associated with immune response, that are similar to both amakihi and apapane, and this may indicate balancing selection (perhaps for disease resistance) in all three species (Jarvi *et al*. 2001, 2004). MHC alleles have been directly linked to malaria resistance in other passerines as well (Westerdahl *et al*. 2005; Bonneaud *et al*. 2006). Determining the relationships among disease susceptibility, exposure, genetic inheritance of resistance and fitness are new key areas of research.

Demography may also play a key role in resistance. Modelling and field studies suggests that amakihi may be the honeycreeper best adapted to survive malaria; chronic malaria infection had no negative effect on reproductive success in a mid-elevation population of amakihi, and populations infected with malaria can have positive population growth (Kilpatrick 2006; Kilpatrick *et al*. 2006). Persistence in amakihi, compared with other honeycreepers, may therefore depend on relatively high levels of reproduction and survival (Kilpatrick 2003). Low-elevation populations of amakihi from Manuka on the west coast of Hawaii were not genetically differentiated from high-elevation populations, despite being at a considerably greater distance from high-elevation populations than the distance between low- and high-elevation sites in the main study area. We speculate that amakihi persistence in this area is the result of drier conditions and consequently reduced disease pressure. Other amakihi species are present at low elevations on most of the other main Hawaiian islands (Lindsey *et al*. 1998), suggesting that development of resistance has occurred several times in this lineage. More research on nesting success and survival in amakihi across the archipelago is necessary.

Resistance to malaria occurs in many birds throughout the world (Valkiunas 2005). Differences in susceptibility occur among amakihi at all elevations, even populations well above the elevational limit of the vector and parasite have some resistance to malaria (Atkinson *et al*. 2000). Yet,

it is apparent that immigration to low elevations from dense source populations in montane sites is not occurring at high rates. In fact, such an effect of migration would swamp out localized resistance to disease (Lenormand 2002), removing any population-genetic patterns in bird distributions. Determining direct disease effects of selection on bird populations will require identification of the gene(s) for resistance to avian malaria and other diseases such as pox. Furthermore, we present a relatively simple three-elevation scenario, when the actual system is likely more complicated. Knowledge of pathogen evolution in response to selective pressures in hosts and vectors is essential for a full understanding of this complex system.

Conservation of native forest birds in Hawaii will likely depend on the presence of disease-resistant individuals in each bird species. Global warming will reduce disease-free refugia for forest birds as the parasites and mosquitoes are able to survive at increasingly higher elevations (Benning *et al*. 2002). In the case of amakihi, this would suggest that the future of its populations lies in the resurgence of low-elevation populations. These areas are not just wastelands of introduced species but contain unique pockets of genetic diversity of native species. By documenting genetic isolation in amakihi populations, this study further supports the theory that the disease-driven evolution of tolerance to malaria led to the recovery of low-elevation amakihi (Jarvi *et al*. 2001; Woodworth *et al*. 2005). This scenario offers hope for population persistence if existing sources of malaria-resistant honeycreepers are able to withstand the continued spread of malaria into new areas.

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References

Atkinson CT, Woods KL, Dusek RJ, Sileo LS, Iko WM (1995) Wildlife disease and conservation in Hawaii: Pathogenicity of avian malaria (*Plasmodium relictum*) in experimentally infected Iiwi (*Vestiaria coccinea*). *Parasitology*, **111**, S59–S69.

Atkinson CT, Dusek RJ, Woods KL, Iko WM (2000) Pathogenicity of avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases*, **36**, 197–204.

Atkinson CT, Dusek RJ, Lease JK (2001a) Serological responses and immunity to superinfection with avian malaria in experimentally-infected Hawaii Amakihi. *Journal of Wildlife Diseases*, **37**, 20–27.

Atkinson CT, Lease JK, Drake BM, Shema NP (2001b) Pathogenicity, serological responses, and diagnosis of experimental and natural malarial infections in native Hawaiian thrushes. *Condor*, **103**, 209–218.

Beadell JS, Ishtiaq F, Covas R *et al*. (2006) Global phylogeographic limits of Hawaii's avian malaria. *Proceedings of the Royal Society, Series B: Biological Sciences*, **273**, 2935–2944.

Benning TL, LaPointe D, Atkinson CT, Vitousek PM (2002) Interactions of climate change with biological invasions and land use in the Hawaiian Islands: modeling the fate of endemic birds using a geographic information system. *Proceedings of the National Academy of Sciences, USA*, **99**, 14246–14249.

Bonneaud C, Perez-Tris J, Federici P, Chastel O, Sorci G (2006) Major histocompatibility alleles associated with local resistance to malaria in a passerine. *Evolution*, **60**, 383–389.

Cleaveland S, Hess GR, Dobson AP *et al*. (2002) The role of pathogens in biological conservation. In: *The Ecology of Wildlife Diseases* (eds Hudson PJ, Rizzoli A, Grenfell BT, Heesterbeek H, Dobson AP), pp. 139–150. Oxford University Press, New York.

Clement M, Posada D, Crandall K (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657–1660.

Daszak P, Cunningham AA, Hyatt AD (2000) Wildlife ecology – Emerging infectious diseases of wildlife — Threats to biodiversity and human health. *Science*, **287**, 443–449.

Dobson A, Foufopoulos J (2001) Emerging infectious pathogens of wildlife. *Philosophical Transactions of the Royal Society of London, Series B: Biological Sciences*, **356**, 1001–1012.

Fancy SG, Ralph CJ (2000) 'Apapane (*Himatione sanguinea*), No. 296. In: *The Birds of North America* (eds Poole A, Gill F). Birds of North America Inc., Philadelphia, PA.

Fisher HI, Baldwin PH (1947) Notes on the Red-billed Leiothrix in Hawaii. *Pacific Science*, **1**, 45–51.

Fleischer RC, Tarr CL, James HF, Slikas B, McIntosh CE (2001) Phylogenetic placement of the po'o-uli *Melamprosops phaeosoma* based on mitochondrial DNA sequence and osteological characters. *Studies in Avian Biology*, **22**, 98–103.

Friesen VL, Congdon BC, Walsh HE, Birt TP (1997) Intron variation in marbled murrelets detected using analyses of single-stranded conformational polymorphisms. *Molecular Ecology*, **6**, 1047–1058.

Friesen VL, Congdon BC, Kidd MG, Birt TP (1999) Polymerase chain reaction (PCR) primers for the amplification of five nuclear introns in vertebrates. *Molecular Ecology*, **8**, 2147–2149.

Jarvi SI, Atkinson CT, Fleischer RC (2001) Immunogenetics and resistance to avian malaria in Hawaiian honeycreepers (Drepanidinae). *Studies in Avian Biology*, **22**, 254–263.

Jarvi SI, Schultz JJ, Atkinson CT (2002) PCR diagnostics underestimate the prevalence of avian malaria (*Plasmodium relictum*) in experimentally-infected passerines. *Journal of Parasitology*, **88**, 153–158.

Jarvi SI, Tarr CL, McIntosh CE, Atkinson CT, Fleischer RC (2004) Natural selection of the major histocompatibility complex (Mhc) in Hawaiian honeycreepers (Drepanidinae). *Molecular Ecology*, **13**, 2157–2168.

Kilpatrick AM (2003) *The Evolution of Resistance to Avian Malaria (Plasmodium relictum) in Hawaiian Birds*. University of Wisconsin, Madison, WI.

Kilpatrick AM (2006) Facilitating the evolution of resistance to avian malaria in Hawaiian birds. *Biological Conservation*, **128**, 475–485.

Kilpatrick AM, LaPointe DA, Atkinson CT *et al*. (2006) Effects of chronic avian malaria (*Plasmodium relictum*) infection on reproductive success of Hawaii Amakihi (*Hemignathus virens*). *Auk*, **123**, 764–774.

LaDeau SL, Kilpatrick AM, Marra PP (2007) West Nile virus emergence and large-scale declines of North American bird populations. *Nature*, **447**, 710–713.

LaPointe D (2000) *Avian Malaria in Hawai'i: the Distribution, Ecology and Vector Potential of Forest-dwelling Mosquitoes*. PhD Dissertation, University of Hawaii, Manoa, HI.

Lenormand T (2002) Gene flow and the limits to natural selection. *Trends in Ecology and Evolution*, **17**, 183–189.

Lindsey GD, VanderWerf EA, Baker H, Baker PE (1998) Hawai'i (*Hemignathus virens*), Kaua'i (*Hemignathus kauaiensis*), O'ahu (*Hemignathus chloris*), and Greater 'Amakihi (*Hemignathus sagittirostris*), No. 360. In: *The Birds of North America* (eds Poole A, Gill F). Birds of North America Inc., Philadelphia, PA.

Posada D, Crandall KA (1998) Modeltest: testing the model of DNA substitution. *Bioinformatics*, **14**, 817–818.

Ralph CJ, Fancy SG (1995) Demography and movements of Apapane and Iiwi in Hawaii. *Condor*, **97** (3), 729–742.

Real LA, Russell C, Waller L, Smith D, Childs J (2005) Spatial dynamics and molecular ecology of North American rabies. *Journal of Heredity*, **96**, 253–260.

Reding DM (2007) *Genetic Diversity, Population Structure, and Demographic History of the Hawaii Akepa*. University of Hawaii, Manoa, HI.

Reynolds MH, Camp RJ, Nielson BMB, Jacobi JD (2003) Evidence of change in a low-elevation forest bird community of Hawaii since 1979. *Bird Conservation International*, **13**, 175–187.

van Riper C III, van Riper SG, Goff ML, Laird M (1986) The epizootiology and ecological significance of malaria in Hawaiian (USA) land birds. *Ecological Monographs*, **56**, 327–344.

van Riper C III, van Riper SG, Hansen WR (2002) Epizootiology and effect of avian pox on Hawaiian forest birds. *Auk*, **119**, 929–942.

Schneider S, Roessli D, Excoffier L (2000) Arlequin: A software for population genetics data analysis. Version 2.001. Genetics and Biometry Laboratory, Department of Anthropology, University of Geneva, Geneva.

Sorci G, Moller AP, Boulinier T (1997) Genetics of host–parasite interactions. *Trends in Ecology and Evolution*, **12**, 196–200.

Spiegel CS, Hart PJ, Woodworth BL, Tweed EJ, LeBrun JJ (2006) Distribution and abundance of forest birds in low-altitude habitat on Hawai'i Island: evidence for range expansion of native species. *Bird Conservation International*, **16**, 175–185.

Swofford DL (2002) *PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods), Version 4.0*. Sinauer Associates, Sunderland, MA.

Tarr CL (1995) Primers for amplification and determination of mitochondrial control-region sequences in oscine passerines. *Molecular Ecology*, **4**, 527–529.

Uller T, Olsson M, Madsen T (2003) Family and population effects on disease resistance in a reptile. *Heredity*, **91**, 112–116.

Valkiunas G (2005) *Avian Malaria Parasites and Other Haemosporidia*. CRC Press, Boca Raton, FL.

Warner RE (1968) The role of introduced diseases in the extinction of the endemic Hawaiian avifauna. *Condor*, **70**, 101–120.

Westerdahl H, Waldenstrom J, Hansson B *et al*. (2005) Associations between malaria and MHC genes in a migratory songbird. *Proceedings of the Royal Society, Series B: Biological Sciences*, **272**, 1511–1518.

Woodworth BL, Atkinson CT, LaPointe DA *et al*. (2005) Host population persistence in the face of introduced vector-borne diseases: Hawaii amakihi and avian malaria. *Proceedings of the National Academy of Sciences, USA*, **102**, 1531–1536.

Yorinks N, Atkinson CT (2000) Effects of malaria on activity budgets of experimentally infected juvenile Apapane (*Himatione sanguinea*). *Auk*, **117**, 731–738.

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