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IMMUNOGENETICS AND RESISTANCE TO AVIAN MALARIA IN HAWAIIAN HONEYCREEPERS (DREPANIDINAE)

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Abstract. Although a number of factors have contributed to the decline and extinction of Hawai'i's endemic terrestrial avifauna, introduced avian malaria (*Plasmodium relictum*) is probably the single most important factor preventing recovery of these birds in low-elevation habitats. Continued decline in numbers, fragmentation of populations, and extinction of species that are still relatively common will likely continue without new, aggressive approaches to managing avian disease. Methods of intervention in the disease cycle such as chemotherapy and vaccine development are not feasible because of efficient immune-evasion strategies evolved by the parasite, technical difficulties associated with treating wild avian populations, and increased risk of selection for more virulent strains of the parasite. We are investigating the natural evolution of disease resistance in some low-elevation native bird populations, particularly Hawai'i 'Amakihi (*Hemignathus virens*), to perfect genetic methods for identifying individuals with a greater immunological capacity to survive malarial infection. We are focusing on genetic analyses of the major histocompatibility complex, due to its critical role in both humoral and cell-mediated immune responses. In the parasite, we are evaluating conserved ribosomal genes as well as variable genes encoding cell-surface molecules as a first step in developing a better understanding of the complex interactions between malarial parasites and the avian immune system. A goal is to provide population managers with new criteria for maintaining long-term population stability for threatened species through the development of methods for evaluating and maintaining genetic diversity in small populations at loci important in immunological responsiveness to pathogens.

Key Words: avian malaria; Drepanidinae; genetics; Hawai'i honeycreeper; *Mhc*; *Plasmodium relictum.*

The Hawaiian honeycreepers (Drepanidinae) are a morphologically and ecologically diverse subfamily of cardueline finches that probably evolved from a single finch species that colonized the Hawaiian Islands an estimated 4–4.5 million years ago (Tarr and Fleischer 1993, 1995; Fleischer et al. 1998). The honeycreepers radiated very rapidly to fill a variety of ecological niches. While the progenitor of the subfamily was presumably a finch-billed, granivorous form, the more than 50 species and subspecies of honeycreepers derived from this ancestor had great diversity of morphological types ranging from nectivores with long, decurved sickle bills to seedeaters with massive, powerful beaks (Perkins 1903, Amadon 1950, Raikow 1977, Freed et al. 1987a, James and Olson 1991).

While the Drepanidines have long been considered an exceptional example of adaptive radiation, they, along with many other Hawaiian birds, are also an unfortunate paradigm of extinction and endangerment (Scott et al. 1986, 1988; James and Olson 1991). Of a total of 54 described species, 14 became extinct after Polynesian colonization and are known only from subfossil remains. Another 14 became extinct following Western contact and are present in museum collections from the 1800s. Of the remaining 26 species, 18 are currently listed as endangered by the U.S. Fish and Wildlife Service and many of these are perched on the brink of extinction, and some may be extinct (Jacobi and Atkinson 1995, Reynolds and Snetsinger *this volume*). Although a large number of factors contributed to the extinction of Hawaiian honeycreepers (Ralph and van Riper 1985, Scott et al. 1988, James and Olson 1991), introduced disease and disease vectors are likely the greatest threat facing them today, particularly at elevations lower than 1,500 m (Warner 1968, van Riper et al. 1986, Atkinson et al. 1995).

PARASITES IN PARADISE

There are no native mosquitoes in Hawai'i, but a bird-biting species (*Culex quinquefasciatus*) was accidentally introduced to Maui in 1826 (Hardy 1960). The spread of this mosquito throughout low- and mid-elevation habitats and introduction and release of domestic fowl, game birds, and cage birds allowed two introduced diseases to escape into native populations, avian malaria (*Plasmodium relictum*), and avian pox (*Poxvirus avium*). Although there is little direct evidence that diseases caused by these organisms were responsible for the major declines and extinctions of honeycreepers during the past 100 years, considerable indirect evidence has accumulated in recent years that supports this hypothesis. Anecdotal reports by early naturalists of sick and dead birds with large pox-like tumors suggests that avian poxvirus was having major impacts on forest bird populations as early as the 1890s (Perkins 1903). It is less clear when malaria was first introduced to Hawai'i since the

TABLE 1. MORTALITY IN NONNATIVE FOREST BIRDS IN HAWAI'I EXPERIMENTALLY INFECTED WITH HAWAI'I ISOLATES OF *P. relictum*

Species	Sample size	Route of inoculation	% Mortality
Red-Billed Leiothrix[a] (*Leothrix lutea*)	5	Blood inoculation	0 (0/5)
Japanese White Eye[a] (*Zosterops japonicus*)	5	Blood inoculation	0 (0/5)
Nutmeg Mannikin[b] (*Lonchura punctulata*)	7	Mosquito bite	0 (0/7)

[a] van Riper et al. (1986).
[b] Atkinson et al. (1995).

TABLE 2. MORTALITY IN HONEYCREEPERS EXPERIMENTALLY INFECTED WITH HAWAI'I ISOLATES OF *P. relictum*. (SCIENTIFIC NAMES FOR ALL BIRD SPECIES LISTED BELOW CAN BE FOUND IN TABLE 2 FOLLOWING THE INTRODUCTION OF THIS VOLUME.)

Species	Sample size	Route of inoculation	% Mortality
Laysan Finch[a]	5	Blood inoculation	100 (5/5)
'I'iwi[a]	5	Blood inoculation	60 (3/5)
'I'iwi[b]	10	Mosquito bite	90 (9/10)
Maui 'Alauahio[c]	4	Mosquito bite	75 (3/4)
'Apapane[a]	5	Blood inoculation	40 (2/5)
'Apapane[e]	8	Mosquito bite	63 (5/8)
Hawai'i 'Amakihi[a] (high elevation)	6	Blood inoculation	66 (4/6)
Hawai'i 'Amakihi[a] (low elevation)	5	Blood inoculation	20 (1/5)
Hawai'i 'Amakihi[d] (high elevation)	20	Mosquito bite	65 (13/20)

Note: Mosquito bite method of inoculation duplicates natural conditions more closely and provides a more accurate estimate of expected mortality in the wild.
[a] van Riper et al. (1986).
[b] Atkinson et al. (1995).
[c] C. T. Atkinson (unpubl. data).
[d] Atkinson et al. (2000).
[e] Yorinks and Atkinson (2000).

earliest blood smears from native birds date only to the 1940s, and it is unlikely that early naturalists would have recognized signs and lesions of the disease. van Riper et al. (1986) has hypothesized that the introduction occurred in the 1920s, since this corresponds to a time when large numbers of exotic cage birds were imported from throughout the world and released into the wild. Limited collections of blood smears prepared from native species prior to 1950 also show little evidence of infection in native species, suggesting that spread of this disease in forest bird populations may be responsible for the major wave of extinctions in mid-elevation habitats that occurred in the second half of the 20th century (van Riper et al. 1986, van Riper 1991).

From historical collections and observations, as well as subfossil distributions, many honeycreeper taxa were known to have occurred at lower elevations prior to the introduction and spread of mosquitoes, pox, and malaria (Warner 1968, van Riper et al. 1986, James and Olson 1991). With the exception of several relatively common species, most honeycreeper taxa now occur only at elevations above 1,200 m. With few exceptions, they show little or no overlap with the current range of *C. quinquefasciatus*, even though otherwise suitable habitat is available at lower elevations (Goff and van Riper 1980, Scott et al. 1986). While nonnative passerines show little morbidity or mortality following infection with Hawaiian isolates of *P. relictum* (Table 1), most honeycreeper taxa tested thus far are severely debilitated and usually killed by acute anemia associated with fulminating erythrocytic infections (Warner 1968, van Riper et al. 1986; Atkinson et al. 1995, 2000; Yorinks and Atkinson 2000; Table 2).

Experimental studies (van Riper et al. 1986, Atkinson et al. 1995, Yorinks and Atkinson 2000) have provided evidence that elevational and geographical anomalies in honeycreeper distribution may largely be due to relative resistance or susceptibility to avian malaria (Fig. 1). Morbidity and mortality in 'I'iwi (*Vestiaria coccinea*), Hawai'i 'Amakihi (*Hemignathus virens*), 'Apapane (*Himatione sanguinea*), and Maui 'Alauahio (*Paroreomyza montana*) were extraordinarily high after a minimal dose of a single mosquito bite (Table 2). Both Maui 'Alauahio and 'I'iwi, two species that rarely occur below 1,500 m, were most susceptible with fatality rates of 75% and 90%, respectively. The range of both species appears to be contracting, particularly in mid-elevation habitats where mosquito populations have increased in recent years as a consequence of feral pig activity and human development (Goff and van Riper 1980). Mortality was lower for 'Apapane and 'Amakihi, although those individuals who recovered underwent a severe, acute illness that caused significant declines in food consumption, weight, and activity levels (Atkinson et al. 2000, Yorinks and Atkinson 2000). Laysan Finches (*Telespiza cantans*) are also highly susceptible to and suffer high mortality from malarial infection, based on experimental infection and exposure of caged captive birds to infected mosquitoes in lowland habitats (Warner 1968, van Riper et al. 1986). Much less is known about other threatened and

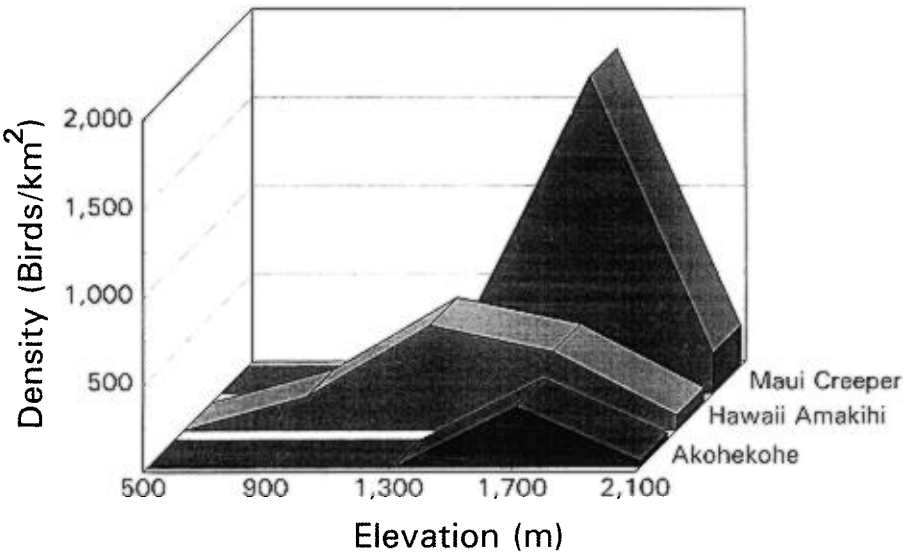

FIGURE 1. Differences in density of three species of honeycreepers on the northeastern slope of Haleakalā Volcano, island of Maui. Data are extracted from density maps published in Scott et al. (1986) to illustrate the lower elevational limits of species that are likely to be highly susceptible to avian malaria (i.e., Maui 'Alauahio and 'Ākohekohe, *Palmeria dolei*) and those more resistant to this disease (i.e., Hawai'i 'Amakihi).

endangered species, but it is likely that they share a similar high susceptibility to infection.

Interestingly, several of the more abundant honeycreeper species, i.e. Hawai'i 'Amakihi, O'ahu 'Amakihi (*Hemignathus flavus*), and 'Apapane, appear to have fragmented but apparently stable populations in low- and mid-elevation habitats where pox and malaria transmission occurs. Based on differential mortality between mid- and high-elevation Hawai'i 'Amakihi that were infected experimentally with *P. relictum* (Table 2), van Riper et al. (1986) hypothesized that these low-elevation populations were evolving "immunogenetic" resistance to malaria from continual exposure to the parasite. van Riper (1991) also found some evidence that Hawaiian isolates of *P. relictum* are less virulent than those found on mainland North America, suggesting that both native hosts and introduced malaria are exerting strong selective pressures on each other and actively coevolving (Atkinson et al. 1995). There is some evidence that selection for less virulent strains of pox may also be occurring, since the massive, debilitating lesions that were described by workers in the early 1900s are found rarely today in naturally infected honeycreepers (C. T. Atkinson, unpubl. data). Evidence for this is still limited but consistent with current evolutionary theory that predicts selection for intermediate levels of virulence and host susceptibility after a pathogen is introduced into a highly susceptible host population (Anderson and May 1982, Ewald 1994).

This putative genetic basis for resistance to malaria in low-elevation honeycreeper populations could involve only a single gene of the immune system, such as a locus of the major histocompatibility complex. Or, like genetically based resistance to malaria in humans, there could be many loci and genetic systems involved to varying degrees, with epistasis also a possible factor (Nagel and Roth 1989, McGuire et al. 1994, Hill 1996, Riley 1996, Weatherall 1996, Hill and Weatherall 1998). If there is a genetic basis to resistance to malaria, then individuals who are resident at higher elevations represent a population that has not been tested significantly or subjected to natural selection by malarial infection. These individuals act as a control for the experimental or selected population that has lived with malaria at low elevations for many generations. Thus by comparing allele frequencies or heterozygosities of different genetic markers between these two populations, we may find evidence for the previous (and likely continuing) action of natural selection. This linkage disequilibrium caused by selection (Ghosh and Collins 1996) might also allow us ultimately to identify the specific target(s) of selection.

To understand why and how some species and individuals within species appear to be able to survive disease epidemics, while others succumb, requires knowledge of the complex interactions between malarial parasites and the avian immune system and how these interactions may

place selective pressures on both parasite and host. We have initiated research on genes of the immune system in Hawaiian honeycreepers and ribosomal genes and selected cell-surface molecules of Hawaiian isolates of *P. relictum* as a first step toward developing a better understanding of these complex relationships.

AVIAN IMMUNE SYSTEMS AND THE *MHC*

Many of the genes and molecules involved in immune system processes that are described in mammals are also known in birds and many were, in fact, initially discovered in birds. The domestic chicken (*Gallus domesticus*) has long served as the "laboratory mouse" of avian research, largely because of its agricultural importance and domestication. Thus, much of the current knowledge of avian immune systems comes from studies completed in chickens. Avian immune system cells appear to function in a way similar to those in mammals, but obvious distinctions in structure and distribution of lymphoid tissue exist in birds. Birds lack the lymph nodes that are so common in mammals, but they have unique avian lymphatic tissues such as the oculo-nasal Harderian gland and the bursa of Fabricious (Eerola et al. 1987). Early studies illuminating the role of the bursa of Fabricious in antibody production established the foundation for the T-cell, B-cell concept (Glick et al. 1956). The B-cell system involves production of specific antibody (humoral immunity) and the T-cell system involves cell-mediated immunity. This duality of the immune system is now known to be universal among all vertebrates. In mammals and birds, both humoral and cell-mediated responses are involved in the immune response against malaria. Among the many cells and molecules with important roles in immunity (e.g., antibodies, macrophages, neutrophils, natural killer cells, and a variety of cell communication molecules, such as interleukins and cytokines), molecules encoded by genes within the major histocompatibility complex (*Mhc*) are of special significance due to their critical role in both humoral and cell-mediated immunological responses.

Mhc class I and class II genes have been found in all well-characterized vertebrates and date as far back as cartilaginous fish. The *Mhc* was first identified as the genetic locus responsible for allograft (tissue) rejection, but it is now known to be responsible for determining what is viewed as "self" versus "non-self" by the immune system. Class I molecules are present on essentially all nucleated cells in the body (including erythrocytes in birds), whereas class II molecules are present on only certain cells of the immune system. *Mhc* molecules function to distinguish foreign invaders by presenting a peptide fragment of the invader (i.e., a parasite of any kind, or peptides from a foreign graft) in the antigen-binding region (ABR) of the molecule to T-cell receptors. This initiates a cascade of events that leads to production of lymphocytes, cytokines, and antibodies, and eventual elimination of the parasite.

The *Mhc* is polymorphic (multiallelic) and multigenic in most species investigated to date. The human *Mhc* is known to contain over 200 genes, many of which are directly involved in the adaptive immune response. The chicken *Mhc* (*B* system; Briles et al. 1948) is the smallest known *Mhc,* containing only 19 known genes coupled to a large family of B-G genes (Kaufman et al. 1999). Studies of the avian *Mhc* have recently been extended to galliformes other than chickens and include pheasants (Jarvi and Briles 1992, Jarvi et al. 1996; Wittzell et al. 1994, 1995), turkeys (Emara et al. 1993), and quail (Shiina et al. 1995) as well as passeriformes (Edwards et al. 1995, Vincek et al. 1995; S. I. Jarvi et al., unpubl. data) and cranes (Jarvi et al. 1995, 1999). B-G molecules are expressed on a variety of tissues in chickens (Miller et al. 1990, Salomonsen et al. 1991), but their function is still unknown. B-G genes have been shown to exist in pheasants (Jarvi and Briles 1992, Jarvi et al. 1996) and cranes (Jarvi et al. 1995, 1999). Recently a second cluster of at least two *Mhc* class I and two class II genes (called *Rfp-Y*) has been identified in chickens (Briles et al. 1993; Miller et al. 1994a,b) and pheasants (Wittzell et al. 1995, Jarvi et al. 1996). The function and expression of these genes is currently under investigation. The simplicity of *Mhc* structure and function in birds as compared to mammals has been thought to account for the more obvious associations of *Mhc* genotype and susceptibility to infectious disease (for review see Kaufman and Wallney 1996).

One of the earliest reported *Mhc* associations with infectious disease was in chickens with Marek's disease (Hansen et al. 1967, Briles and Oleson 1971). Marek's disease is a naturally occurring, herpes-virus induced lymphoma of chickens. The virus initially infects B-cells and macrophages, and eventually T-cells, which result in lethal lymphomas. Birds possessing the B21 *Mhc* haplotype show strong resistance (as much as 95% survival; Pazderka et al. 1975, Longenecker et al. 1976; Briles et al. 1977, 1980, 1983; Bacon and Whitter 1980). The B21 haplotype occurs frequently in many apparently unrelated populations of chickens, including Red Jungle Fowl (*Gallus gallus*; the hypothesized species progenitor), indicating that it may have

special survival value for the species (Longenecker and Mossman 1981).

Rous Sarcoma virus (RSV) is a retrovirus which causes fatal tumors in some chickens but not others. When studied in congenic strains of chickens, *Mhc* haplotype B12 is involved with tumor regression (more resistant to RSV) whereas B4 is a progressor (more susceptible to RSV; reviewed in Plachy et al. 1992). Further studies show the existence of 17 virally derived peptides that are capable of binding the major B12 class I molecule, whereas only two virally derived peptides were identified with the motif of the major B4 class I molecule (Kaufman and Wallney 1996). This may partially explain the resistance (or enhanced immune response) that occurs in B12 chickens. Other strong disease associations with different *Mhc* alleles in chickens include coccidiosis (Clare et al. 1989) and fowl cholera (Lamont et al. 1987). Association between specific *Mhc* alleles and resistance to disease in humans exists, but it is not as apparent. Substantial evidence exists for protective alleles against severe malaria in some human populations but not in others (Hill et al. 1991, 1994; Riley 1996, Hill and Weatherall 1998, Gilbert et al. 1998), and that particular class II haplotypes affect the probability that a hepatitis B infection will become persistent (Thursz et al. 1995).

It has been shown that *Mhc* heterozygotes have higher hatchability and viability than homozygotes (Schultz and Briles 1953), and that individual *Mhc* haplotypes are associated with level of hatchability and viability in chickens (Briles and Allen 1961). Evidence for overdominance of *Mhc* alleles (heterozygous advantage) was demonstrated very early in chickens for traits such as viability, hatchability, body weight, and survivor egg production (Briles 1954, Briles et al. 1957, Abplanalp et al. 1992, Sato et al. 1992) and in humans for susceptibility to hepatitis B infection (Thursz et al. 1997) and survivorship to HIV-1 infections (Carrington et al. 1999). Retention of polymorphism (multiple alleles) as well as heterozygosity for genes in the *Mhc* is likely important for long-term population stability. The *Mhc,* therefore, appears to be an excellent candidate gene region in which to search for disease relationships in Hawaiian honeycreepers.

GENETIC ANALYSES OF HOSTS

We know that susceptibility to malaria differs among and within honeycreeper species and that relative susceptibility or resistance can explain their current elevational distribution to this disease. To investigate the natural evolution of disease resistance in honeycreepers, we have initiated studies of the *Mhc* in Hawai'i 'Amakihi, a species with some evidence of natural resistance to malaria, and also the 'I'iwi, a highly susceptible species with declining numbers that is currently limited to high-elevation habitats. Because of the extreme variability and putative disease and fitness associations of the *Mhc,* a number of authors have suggested that selection for increased *Mhc* diversity occurs via interactions of the molecule with diverse antigen types, but the type of selection has been open to debate (e.g., Hughes and Nei 1988, Hedrick et al. 1991). Selection may be directional in situations where a particular *Mhc* variant provides immunity to a particular disease (Hedrick et al. 1991). If a number of different diseases infect a population over time, selection could balance the frequencies, resulting in a high level of variability (i.e., frequency dependent selection; Hedrick et al. 1991). If a population, such as low-elevation 'Amakihi, experiences a devastating epidemic of a single disease, we may see the "signature" of the selection as a greatly increased or modified frequency of a particular haplotype in the population of survivors. Alternatively, selection may favor heterozygous individuals (heterozygote advantage), because the *Mhc* products of two alleles can recognize more, different antigens (i.e., polypeptide products) of a particular disease than one allele.

What sample sizes are needed to have sufficient power to detect this selection? We are currently comparing samples from low- and high-elevation Hawai'i 'Amakihi. Most of the low-elevation populations have been in contact with mosquitoes and malaria for 50–100 yrs. Given mortality rates of 50–70% in malaria-challenged high-elevation birds, there is potential for very strong selection with coefficients of 0.5 or higher. Using equations from Hartl and Clark (1989), we have modeled the potential impacts of directional selection by malaria on single-locus allele frequencies and on the power to detect significant differences (Cohen 1988) given sample sizes of 50 low- and 200 high-elevation birds (Fig. 2). Whether we use 50 or 100 generations of selection, or have an initial allele frequency of the positively selected allele as 5% (based on a "typical" *Mhc* haplotype frequency in chickens), or 30% (based on the survival rate in high-elevation challenges), we generally have sufficient power (>80%) to detect selection coefficients as low as 0.02 with our sample. Satta et al. (1995) recently estimated overdominant selection coefficients on mammalian *Mhc* loci to range from about 0.002 to 0.05; thus, this type of comparison should be able to detect at least the higher range of their values.

Our model does not include the ameliorating effects of migration. However, the 'Amakihi is

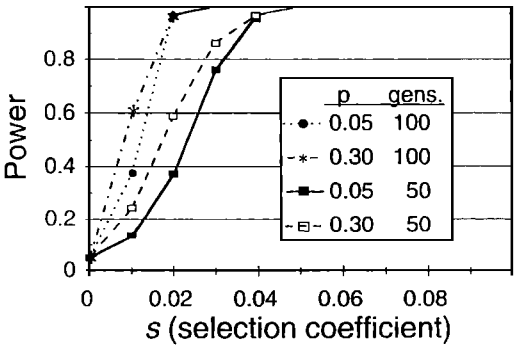

FIGURE 2. Power (Cohen 1988) to detect frequency differences between low- and high-elevation populations after 50 or 100 generations of malaria selection (Hartl and Clark 1989) in low-elevation populations.

known to have high breeding and natal philopatry (van Riper 1984; U.S. Geological Survey, unpubl. data), and it is our view that migration is not sufficient to counter the expected selection intensities. We also assessed the power to detect significant differences in allele frequencies between malaria challenge survivors, fatalities, and controls (Table 3). In this case, selection must be very strong (>0.25) in order for us to detect a significant difference given sample sizes from recent challenge experiments (Atkinson et al. 1995).

Our goals are to perfect genetic methods for evaluating *Mhc* diversity among and within species to look for elevation-dependent allele frequency distributions. Birds involved in experimental malaria challenges are also included in this ongoing study with the hopes of developing methods for identifying individuals with a greater ability to survive malarial infection. General methodology includes the use of the polymerase chain reaction (PCR) to amplify the *Mhc* antigen-binding region (ABR) of class II (beta) genes from the genomic DNA from several individuals (S. I. Jarvi et al., unpubl. data). This targets the gene region in which much of the variability of *Mhc* molecules is concentrated. Products from PCR were cloned using methods that allow specific cloning of homoduplex PCR products by either PCR+1 (Borriello and Krauter 1991) or single-stranded conformational polymorphism (SSCP) isolation (Oto et al. 1993), PCR reamplification, and direct cloning. Both strands of the cloned products were sequenced using a 373 ABI automated sequencing system, and sequences verified as originating from the class II ABR by comparison with numerous known class II sequences available through Genbank.™ Restriction fragment-length polymorphism (RFLP) analyses of 'Amakihi and

TABLE 3. POWER TO DETECT FREQUENCY DIFFERENCES BETWEEN SURVIVORS AND NONSURVIVORS AFTER DIRECTIONAL MALARIA SELECTION AT THE GIVEN SELECTION COEFFICIENT (s) WITH THE GIVEN SAMPLE SIZE (N)

	Selection coefficient			
N	0.05	0.10	0.25	0.50
25	0.061	0.077	0.153	0.440
100	0.077	0.120	0.354	0.915
250	0.097	0.184	0.655	0.999

'I'iwi class II genes were carried out (Figs. 3a and 3b, respectively). All blots were produced by digestion of approximately 15 μg of genomic DNA with either *PvuII, PstI,* or *HindIII.* Blots were then hybridized with a mixture of cloned, sequenced, ^{32}P-labeled *Mhc* class II antigen-binding region from Hawai'i 'Amakihi and 'I'iwi at 50°C in a rotating hybridization oven. Blots were washed in 1 × SSC for at least one hour and autoradiograms were produced. Band sharing coefficients (s) were calculated (using methods described in Lynch 1988) from banding patterns derived from individuals on four 'Amakihi blots and four 'I'iwi blots. Data are presented as composite histograms in Figures 3a and 3b. The *Mhc* class II banding patterns of 'Amakihi and 'I'iwi are markedly distinct, as is reflected by a mean band sharing coefficient of 0.617 over four 'Amakihi blots (Figure 3a) and 0.883 over four 'I'iwi blots (Figure 3b). The actual number of bands varies depending on restriction enzyme used (either *HindIII, PvuII,* or *PstI*). Digestion of genomic 'Amakihi DNA results in a range of four to nine bands/lane whereas 'I'iwi generally have from one to four bands/lane. One would expect these two species to possess a similar number of class II genes since they are thought to be monophyletic, and also since some 'I'iwi were found with six or more bands/lane. Therefore, the observed decrease in bands/lane among 'I'iwi as compared to 'Amakihi (particularly a decrease in polymorphic bands, i.e., an increase in band-sharing coefficients) likely represents a decrease in class II *Mhc* diversity. The limited *Mhc* diversity found in 'I'iwi could play a role in the high mortality observed in this species. That is, if *Mhc* class I and class II antigen-binding variability is low, it is less likely that malarial-encoded peptides will be presented to or be recognized by the host's immune system. Insufficient stimulus to the immune system could explain the prolonged parasitemias that are common in honeycreepers with acute infections, where the parasite reproduces unchecked in the bloodstream. This is in stark contrast to nonnative species where parasitemias peak sharply and immediately decline

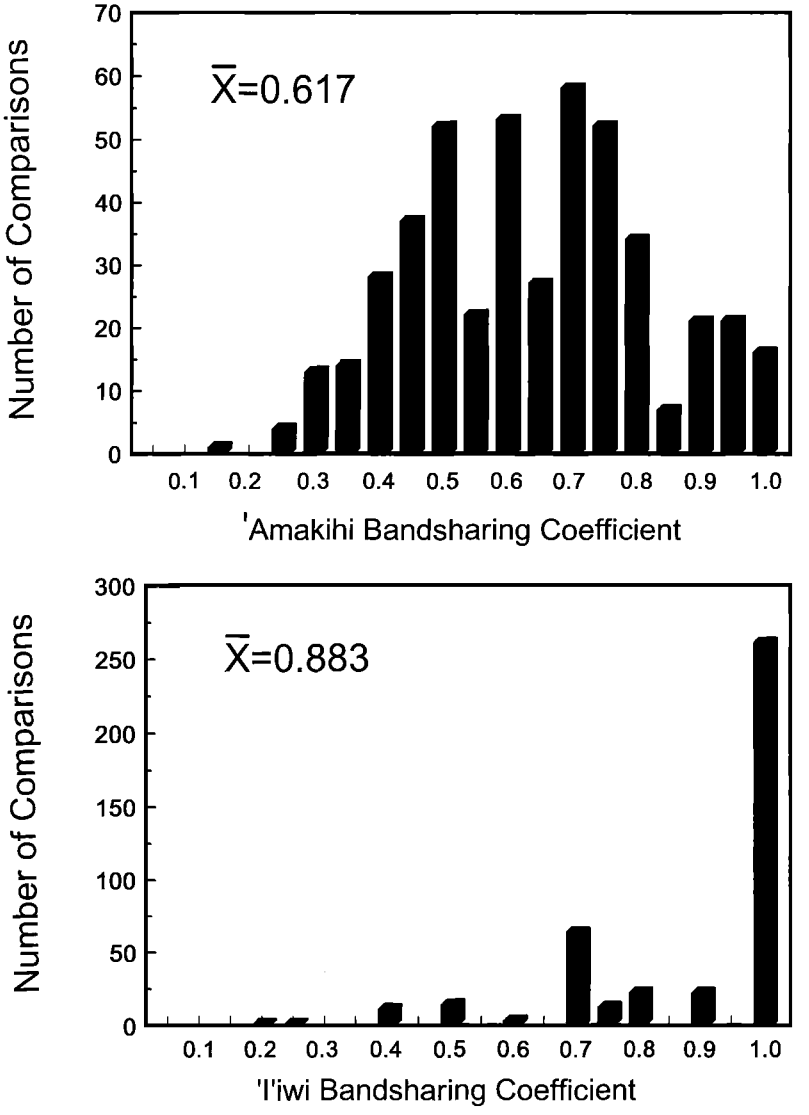

FIGURE 3. Summary of RFLP (Southern blot) analyses of Hawaiian honeycreeper *Mhc* class II genes. Data are compiled as the percentage of shared bands between individuals (i.e., the bandsharing coefficient, computed according to methods of Lynch 1988) versus the number of individual comparisons. A. Four Hawai'i 'Amakihi Southern blots include a total of 58 different individuals originating from several elevations on the island of Hawai'i. B. Four 'I'iwi blots include a total of 41 different individuals originating from high elevations on the island of Hawai'i.

(van Riper et al. 1986; C. T. Atkinson, unpubl. data).

Comparisons of mitochondrial DNA gene sequences derived from 'I'iwi and 'Amakihi reveal predictable levels of nucleotide diversity among 'Amakihi, but all sequences derived from 'I'iwi are invariant (Feldman 1994; C. Tarr, pers. comm.). Further studies are needed to clarify any potential selective role of the parasite.

In addition to systematically analyzing variation at the *Mhc* for its possible relationship to disease resistance, other genetic markers can be used to identify different genetic systems that may also confer resistance. These markers can include putatively random ones (such as microsatellites, minisatellites, Randomly Amplified Polymorphic DNA (RAPDs), or Amplified Fragment Length Polymorphisms (AFLPs)), or gene systems known to play a role in malaria resistance in humans (e.g., G6PDH, TNF-a, etc.;

Riley 1996, Weatherall 1996). Microsatellites have been developed for drepanidines (Tarr 1995, Tarr et al. 1998), and panels of chicken cDNA probes are available (Bumstead et al. 1995) for screening immune system and other genes. These markers may prove useful for three primary purposes: (1) An alternative measure of variability will be available for comparisons among elevations and susceptibility classes in 'Amakihi, and among different honeycreeper species. This tests whether a correlation between *Mhc* gene diversity (heterozygosity) and resistance is due to the variation within the *Mhc* itself (and/or its linked genes) or to genomic variability in general. (2) Markers can be assayed to determine if particular allelic variants or heterozygotes show strong associations with malaria resistance (i.e., differ between low and high elevation or challenge survivors and fatalities). This approach is standard in medical genetics and is outlined by Ghosh and Collins (1996) as the "linkage disequilibrium" approach, as it requires that the actual disease resistance mutations be in linkage disequilibrium with particular alleles. (3) Microsatellite and AFLP markers are excellent for the construction of linkage maps. Such a map would be important for locating the relative position of the *Mhc* and other immune system genes, or any other markers that show a relationship with disease resistance.

While mapping disease resistance to the *Mhc* is strong evidence favoring that *Mhc* itself is responsible, it does not rule out other genes linked within the region. In fact a number of other genes coding for immune system molecules have been localized to the *Mhc* region in mammals and birds (Bumstead et al. 1995), including tumor necrosis factors (Hedrick et al. 1991), complement proteins (Hedrick et al. 1991), proteasomes for antigen degradation (Fehling et al. 1994), and transporter-associated antigen processing proteins (de la Salle et al. 1994, Suh et al. 1994, Bumstead et al. 1995). Some of these may be involved in disease resistance and serve as the targets of selection.

GENETIC ANALYSIS OF PARASITES

A second key to understanding disease resistance in Hawaiian honeycreepers concerns genetic diversity of malarial parasites themselves and how they may exert selective pressure on the host. The malarial parasites of vertebrates (*Plasmodium* spp.) are a closely related group of Apicomplexan parasites that share common morphological and developmental characteristics in all of the reptilian, avian, and mammalian hosts in which they occur (Garnham 1966). Species of *Plasmodium* are thought to have diverged from other members of the Apicomplexa approximately 129 million years ago (Escalante and Ayala 1994), possibly explaining why more species are found in reptiles and birds (110+) than in mammals (40+; Levine 1988). Most of what we know about the life cycles, pathogenicity, and immunology of the avian parasites was established during the first half of the 20th century when several species that readily infect domestic birds (e.g., *P. gallinaceum* and *P. lophurae*) were used as primary laboratory models for studies of human malaria. With the development of rodent, primate, and in vitro models, research shifted away from avian parasites in the 1950s and we consequently know relatively little about how unique avian immune system molecules and processes might influence parasite interactions with the avian host.

Having successfully persisted in a variety of vertebrate species over such a long period of time, *Plasmodium* spp. have necessarily evolved effective mechanisms for survival. Immune-evasion strategies and the processes involved in natural immunity to malaria are complex and poorly understood, even in mammalian hosts where most research has focused in recent years. Much of this complexity is due to multiple stages of the parasite life cycle that alternate between the vertebrate host and the mosquito vector. In mammals, transmission occurs to a new host when an infected anopheline mosquito inoculates sporozoites into the bloodstream during a blood meal. These invade hepatocytes, undergo one generation of asexual reproduction, and release merozoites into the bloodstream, which invade circulating erythrocytes. Multiple cycles of asexual reproduction occur in the circulating blood cells, during which some merozoites are produced that invade erythrocytes and develop into gametocytes. These circulating gametocytes complete the vertebrate phase of the life cycle and are capable of infecting new mosquito hosts. The complex interactions that occur between developing parasites, host cells, and the host immune system in mammals results in production of antibodies, activation of a variety of different effector cells, production of lymphokines, and a cascade of events that control parasite numbers without completely eliminating the infection. Production of nonsterilizing immunity is characteristic of *Plasmodium* in its various vertebrate hosts, including birds, and has been termed concomitant immunity.

A number of key differences exist between the life cycles of avian and mammalian malarial parasites that may be important in how parasites interact with the immune system. The pre-erythrocytic stages of avian parasites (i.e., those that develop from sporozoites) invade and develop in blood forming cell types, such as hemocytob-

lasts, and cells of the lymphoid-macrophage system rather than hepatocytes (Huff 1969). These cell types include macrophages, stem cells, and endothelial cells that line blood capillaries. Avian parasites undergo several cycles of reproduction in these cell types before invading erythrocytes and, unlike most mammalian parasites that have a self-limiting cycle in the host liver, persist in cells of the lymphoid-macrophage system for the duration of the infection and most likely for the life of the host. These persistent tissue stages provide a source of parasites for relapsing erythrocytic infections and, more importantly, stimulate concomitant immunity in the host, providing protection from reinfection with homologous strains of the parasite.

We have initiated genetic studies of Hawaiian isolates of *P. relictum* to determine if multiple strains that differ in pathogenicity are present in Hawai'i and whether they are responsible for periodic epidemic outbreaks that occur in mid-elevation habitats (C. T. Atkinson, unpubl. data). We are also interested in developing reliable PCR-based methods for diagnostic purposes. To accomplish this, we are evaluating diversity of regions of several genes including the more conserved 18S ribosomal genes (Waters and McCutchan 1989, Feldman et al. 1995), and several variable genes encoding cell-surface proteins including thrombospondin-related analogous protein (TRAP), circumsporozoite protein (CSP), and merozoite surface antigen 2 (MSA-2). The genes encoding cell-surface proteins were initially characterized in *P. falciparum,* and we are currently developing PCR primers specific for the homologous gene regions in *P. relictum* (Felger et al. 1993, 1994; McCutchan et al. 1996, Templeton and Kaslow 1997). The 18S ribosomal genes have a low mutation rate of approximately 2% per 110 million years (Ochman and Wilson 1987, Wilson et al. 1987) and are especially useful for phylogenetic analyses (e.g., Escalante and Ayala 1994). Sporozoite and merozoite cell-surface proteins are all quite variable and are thought to be under positive Darwinian selection by the host immune system, i.e., amino acid variability is higher, especially within certain gene regions of the molecules, than would be expected under circumstances of neutrality (Hughes and Hughes 1995). Balanced host-parasite interactions may likely involve selective pressure by the parasite on molecules of the immune system (e.g., *Mhc* molecules) as well as selection on variable parasite molecules (e.g., TRAP, CSP, MSA-2) by the host's immune system. These highly variable parasite molecules are important in fundamental primary mechanisms of immune evasion, antigenic diversity and antigenic variation. Antigenic diversity refers to the expression of different alleles of a gene in different populations, whereas antigenic variation is the process by which a clonal parasite population can switch its antigenic phenotype (reviewed in Reeder and Brown 1996). In fact, polymorphic regions of the CSP have been shown to serve as T-cell epitopes (Good et al. 1988).

We are using a variety of molecular techniques to evaluate these genes or portions of these genes in *P. relictum* as a means of identifying variation sufficient to warrant strain divergence. We are also using these techniques to develop a PCR-based diagnostic test for *P. relictum* that will supplement both the PCR test described by Feldman et al. (1995) and serological tests for antibodies to the parasite that we are currently using (Atkinson et al. 2001).

We began analyses of the 18S ribosomal genes using highly conserved PCR primers specific for an approximately 580 base pairs (bp) segment of 18S ribosomal genes (Feldman et al. 1995, Shehata et al. *this volume*). We selected individuals that had been previously screened for the presence or absence of *Plasmodium* by blood smear and immunoblot methods (C. T. Atkinson, unpubl. data) to provide a basis for comparison. PCR-based techniques for studies of human *Plasmodium* spp. are generally used in combination with serological or other immunological methods due to the high percentage of false negatives (0.05) and false positives (0.16) in PCR-based diagnostic tests (reviewed in Weiss 1995). These ribosomal primers are also highly conserved. This means that they would likely anneal to ribosomal regions of DNA of any number of organisms under the appropriate conditions, as has been demonstrated in other species (Perkins and Martin 1999). In our hands we have found that these primers amplify multiple fragments from individuals which makes it difficult to distinguish the (theoretically) *Plasmodium*-specific 580 bp band from other similar-sized bands that may also be amplified from whole blood. Upon cloning and sequencing amplified DNA from 20 individuals, we have found that the length of this region varies, ranging from approximately 570 to 600 bp in length. From initial nucleic acid comparisons (MEGA and PC Gene), no distinct groupings were seen based on geographic origin of the samples.

The actual number of ribosomal genes in *P. relictum* is unknown but ranges from 4–10 rDNA units in other *Plasmodium* species (McCutchan 1986). For understanding more completely the diversity levels in this gene region, we are using an SSCP-based approach. The patterns produced by SSCP reveal the presence of as many as 10 bands, suggesting that *P.*

relictum, if haploid, contains multiple rDNA gene units with a minimum copy number of five. We have cloned and sequenced a nearly full length TRAP gene from *P. relictum* in order to obtain DNA sequence for designing strain-specific primers (S. I. Jarvi, unpubl. data); these are for use in diagnostic tests to supplement the currently available PCR test that is based on ribosomal genes (Feldman et al. 1995, Shehata et al. *this volume*), as well as for use in evaluating diversity at a variable and likely selected gene.

Because the bionomics of the mosquito vectors of avian malaria can affect evolution of the bird-parasite interactions, research on dispersal, host preferences, behavior, susceptibility, and genetics of *C. quinquefasciatus* is needed to help interpret findings from research being conducted on both honeycreepers and malarial parasites. It is beyond the scope of this paper to report in detail on projects in progress, but field and laboratory studies currently underway are using mitochondrial DNA and a number of microsatellite markers as well as other techniques for examining geographic diversity, patterns of introduction, dispersal rates, and vectorial capacity of *Culex* populations in Hawai'i (Fonseca et al. 1998; D. A. Fonseca, D. A. LaPointe, C. T. Atkinson, and R. C. Fleischer, unpubl. data).

CONCLUSIONS

Genetic studies that help to clarify the complex interactions between host and parasite can provide information critical for the survival and management of native forest birds. Immunogenetic studies of honeycreepers will provide natural resource managers new criteria for maintaining and increasing genetic diversity in fragmented populations of threatened or endangered species. Because of dynamic coevolutionary interactions among hosts, parasites, and vectors, the best overall strategy may be to aggressively use translocations and captive propagation to maximize heterozygosity to prevent loss of rare alleles, especially at loci important in immunological responsiveness to pathogens. At the same time, detailed information about genetic diversity in parasite populations can have important applications in monitoring epidemics and developing quarantine protocols for preventing introductions of new strains of the parasite. Recent data indicates that the dispersal and flight range of *Culex quinquefasciatus* in densely forested habitats may be much greater than initially anticipated, making it less likely that vector-control techniques based on elimination of breeding sites or application of environmentally compatible larvicides will be effective unless applied over large geographic areas (D. A. LaPointe, C. T. Atkinson, unpubl. data). Other approaches for breaking the disease cycle, such as chemotherapy or vaccine development, are even less feasible because of efficient immune-evasion strategies evolved by the parasite, technical difficulties associated with treating wild avian populations, and the increased risk of selecting for more virulent strains of the parasite. Until we know more about genetic diversity and its relationship to disease susceptibility in remaining threatened and endangered forest bird populations, protection of high-elevation habitats, prevention of new introductions of pathogens, and intensive management of adjacent mid-elevation forests to reduce oviposition sites for *Culex* mosquitoes may be the best short-term approach for preventing further extinctions.

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