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Molecular evolution, genetic diversity, and avian malaria in the Hawaiian honeycreepers

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MOLECULAR EVOLUTION, GENETIC DIVERSITY, AND AVIAN MALARIA IN THE HAWAIIAN HONEYCREEPERS

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By

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Dedication

To my wife Dawn.

Her support,

guidance, and understanding

made this dissertation possible.

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Abstract

This dissertation is an interdisciplinary study linking molecular and population genetics to basic problems in island ecology, evolution, and extinction. The Hawaiian honeycreepers (Aves: Fringillidae: Drepanidinae) are extremely morphologically diverse and have radiated into nearly all of the passerine behavioral niches. The group is currently threatened with extinction by anthropogenic disturbances including introduced diseases. Basic biological problems in the honeycreepers include resolving systematic issues, documenting population structure and identifying the role of disease in limiting remaining populations.

A molecular systematic study tested representative honeycreeper species for monophyly. I sequenced 13 honeycreeper species and used 3 outgroup taxa, 2 cardueline finches and a titmouse, for a 790 bp fragment of the mitochondrial cytochrome b gene. Phylogenetic trees constructed using distance, parsimony, and maximum likelihood methods all grouped the honeycreepers monophyletically and placed *P. montana* and *O. bairdi* in a basal clade. Two *Oreomystis* species were polyphyletic, indicating that they have been misclassified. The basis for this misclassification was strong convergence of morphological and behavioral characters associated with insectivory.

A population study of mitochondrial cytochrome b DNA sequence variability was conducted at three scales; single locations, single islands, and multiple islands. Mitochondrial variation was found for four species living in a center of distribution that is surrounded by highly disturbed regions. In the multiple location study, diversity was found for the Common Amakihi (*Hemignathus virens*). The multiple island study showed that a population of Kauai Amakihi is phylogenetically distinct from those on Maui and Hawaii and that the Maui and Hawaii populations share mitochondrial haplotypes. No variation was detected in the highly mobile Iiwi (*Vestiaria coccinea*) sampled from the same islands.

A PCR-based test for avian malaria was developed that detects the *Plasmodium* 18S rDNA. Quantitative Competitive PCR experiments established the sensitivity limits of the test. The PCR test was used to estimate prevalence of avian malaria in Hawaiian birds living at high elevations above the normal range of mosquito vectors. Malaria was highest in the nomadic Apapane (*Himatione sanguinea*) followed by the sedentary Common Amakihi (*Hemignathus virens*). Malaria was not detected in the mobile Iiwi (*Vestiaria coccinea*).

Table of Contents

List of Tables

List of Figures

Chapter 1

Background and Introduction

In recent years the application of molecular techniques to problems in evolutionary biology has led to fundamental advances in understanding basic systematic relationships and processes. Such studies have offered glimpses into the RNA world (Cech 1986), illuminated ancient phylogenetic relationships of all extant life (Woese 1987), and provided a framework for meaningfully discussing human origins (Cann et al. 1987). The power of the application of molecular technologies to evolutionary and population genetic studies is one of increasing resolution which in turn can lead to an increased awareness of the patterns underlying biological change. The continued application of molecular methods to evolutionary problems promises to add to the growing collection of new insights into biological diversification and the factors affecting it.

The Hawaiian Islands offer some of the most intriguing systems in which to apply molecular tools to evolutionary problems. The islands are the most isolated major archipelago in the world, located in the middle of the North Pacific, 2400 miles south west of San Francisco and 3800 miles south east of Tokyo. The islands are volcanic, formed by a hot-spot in the ocean crust currently located beneath

the Island of Hawaii. As the Pacific plate moves to the north west, away from the stationary hot-spot, a succession of islands is created that differ in age, with the youngest in the south east and the oldest in the north west. As the islands move away from their volcanic source, they gradually erode until they are submerged beneath the sea and eventually subducted under the Kuril Trench. The youngest island, Hawaii, is 0.5 MY old. The oldest is Midway, 28 MY old. The hot-spot itself is at least 65 MY old (Clague et al. 1975).

Hawaii's unique geology and geography have contributed to making the archipelago the "showcase of evolution" (Carson 1982). Hawaii is home to an unparalled diversity of life forms exemplified by over 3000 endemic plant (Simon 1987), 10,000 endemic insect, and at least 700 endemic *Drosophila* species (Carson 1987). Hawaii is also home to the most diverse assemblage known of island birds. From fossils, over 100 endemic bird species are known including a sea eagle, hawks, owls, crows, and flightless forms of geese, ibises, and rails (Olson and James 1982). The known endemic species in Hawaii represent 10 of the 30 orders of birds (Gill 1980). The extent of bird evolution in Hawaii is impressive, but the undisputed stars of the Hawaiian avian evolution stage are members of the endemic subfamily of small bodied finches known as the Hawaiian honeycreepers.

The Hawaiian Honeycreepers

The Hawaiian honeycreepers (Fringillidae: Drepanidinae) are a textbook example of extreme adaptive radiation in an island avifauna and have explored nearly the entire range of passerine behavioral, morphological, and evolutionary diversification (Freed et al. 1987). Evolutionary questions have surrounded the honeycreepers since they were first discovered by Western explorers and, although heavily studied over the years, the basic questions regarding their origin and relationships to one another remain (Amadon 1950, Raikow 1976, Olson and James 1982, Johnson et al. 1989, Pratt 1992a). The honeycreepers are currently classified into 26 species (AOU 1983, Pyle 1990) and are highly divergent morphologically, particularly at the generic level (Amadon 1950, Raikow 1976), although honeycreeper morphological and behavioral diversity seems to apply at various taxonomic scales. Some genera of honeycreepers are island endemics, such as the Maui Parrotbill (*Pseudonestor xanthophrys*). Among islands, some honeycreepers are morphologically divergent at the species level, such as the Kauai Akepa (*Loxops caeruleirostris*), and others at the subspecific level, such as the Kauai Amakihi (*Hemignathus virens stejnegeri*) (Pratt 1987, AOU 1983). A hierarchical approach to honeycreeper phylogeny can be used to address the general

problem of speciation in island birds as well as problems unique to the honeycreepers.

The Hawaiian honeycreepers are also an excellent group in which to pose basic population genetics questions. The honeycreepers vary strongly in population size (Scott et al. 1986), life history (Freed et al. 1987), movements (Baldwin 1953), and distribution patterns (Scott et al. 1986). Thus, they form a model system for investigating how these ecological differences might be reflected in the genetic structure of honeycreeper populations. The combination of a phylogenetic and a population genetic approach has the potential to reveal basic patterns of honeycreeper population structure that are applicable to speciation and island evolution in general. In this dissertation, I sought to make connections in phylogenetics and population genetics from a molecular evolutionary perspective with the hope of revealing basic processes associated with island speciation.

A Brief History of the Study of Honeycreeper Evolution and Extinction

The first reference to the Hawaiian honeycreepers by western explorers was made by Cook's officers aboard the Resolution (Cook 1778-9). The variability of the honeycreepers was so striking to the early naturalists that they classified them into several families of neotropical

origin (Bloxam 1826, Dole 1879). Toward the end of the nineteenth century, comprehensive studies of the honeycreepers were undertaken and a consensus emerged that the birds represented one extraordinary group (Wilson and Evans 1890 - 1900, Rothschild 1893-1900, Perkins 1903, 1913). In this century, studies have emphasized behavioral (Amadon 1950), anatomical (Raikow 1976), and genetic aspects of honeycreeper systematics (Sibley and Ahlquist 1982, Johnson et al.1986). Recently, the issue of honeycreeper monophyly (Pratt 1992a 1992b, James and Olson 1982) has been raised.

Disagreements regarding the phylogenetics of the honeycreepers have been fueled by attempts to understand the systematics of enigmatic forms (Amadon 1950, Raikow 1976). In 1973, a highly divergent living species, placed in a new genus, the Poouli (*Melamprosops phaeosoma*) was discovered (Casey and Jacobi 1974). The Poouli is so morphologically divergent that its classification in the honeycreepers has been questioned (Pratt 1992b). Other less divergent yet puzzling species are known from a handful of specimens collected around the turn of the century. These include the Greater Amakihi (*Hemignathus sagittirostris*) (Rothschild 1892, 1893-1900) and the Ula-ai-hawane (*Ciridops anna*). In most respects the Greater Amakihi resembles a huge Common Amakihi (*H. v. virens*) but with a straighter bill (Rothschild 1893-1900, Amadon 1950). The extinct Ula-ai-hawane had the most variable plumage and diversity of colors yet discovered

for the honeycreepers but is known from only 3 specimens collected from the island of Hawaii (Banko 1979) and 2 others possibly from Molokai (Olson 1992). Another unusual and rare form known from only a few sightings is *Oreomystis perkinsi*, which may have been a hybrid between the Common Amakihi (*H. v. virens*) and the Hawaii Creeper (*O. mana*) (Rothschild 1892, Amadon 1950), or merely an aberrant *H. virens* (Perkins 1913).

Unfortunately, the importance of the radiation of the honeycreepers to evolutionary biology is matched by the decline and extinction of these birds to conservation biology. Coincident with the landing of the Polynesians in Hawaii about 700 A.D. (Hunt and Holsen 1991), both rare and common species of native birds began declining (Olson and James 1982). The Polynesians introduced invasive species of rats, jungle fowl, pigs, and dogs which preyed on or competed with the native birds (Olson and James 1982). The agricultural methods of the early Hawaiians, which included clearing low elevation forests and altering water courses, were destructive to the native ecosystems (Olson and James 1982, Ralph and van Riper 1985). The ancient Hawaiians also hunted the birds for their feathers which were used in the construction of ceremonial capes and artwork (Kirch 1985). The Polynesian arrival heralded the first great wave of extinction of native Hawaiian birds (Olson and James 1982).

The Europeans landed in 1778 and accelerated the extinction of native Hawaiian species (Ralph and van Riper

1985). The Europeans brought goats, pigs, sheep, cattle, and horses that decimated mid-elevation forests (Atkinson 1977), drastically reducing the habitat available to native birds. In addition to habitat loss, an influx of introduced birds contributed to the decline of the honeycreepers possibly by direct competition or by introducing diseases such as avian malaria and avian poxvirus. The introduction of the mosquito vector combined with avian malaria and poxvirus probably took a heavy toll on honeycreeper species in the period between 1850 and 1930 (Warner 1968, van Riper et al. 1986).

The loss of habitat fragmented honeycreeper populations and drastically reduced their range and size (Scott et al. 1986, Scott et al. 1988). Fragmentation, range reduction, and small population size have enormous genetic consequences because of the increased potential for inbreeding (Frankel and Soule 1981). Scott et al. (1988) surveyed 53 surviving populations of drepanidines and found that 11 of them numbered less than 500 individuals and that 7 of these populations occupied less than 20% of their original range. The population sizes for many of the honeycreepers falls below the minimum viable population (MVP) size of 500 thought necessary for long term genetic survival (Gilpin and Soule 1986). An increased risk of extinction from stochastic forces is expected (Lande and Barrowclough 1986).

Conservation of remaining Hawaiian birds is currently an urgent priority because most of the remaining populations of

endemic birds are threatened with extinction. Of the 34 living endemic bird species in Hawaii, 24 are endangered (Pyle 1990), and of the 26 extant honeycreeper species and subspecies, 12 are listed as endangered (Pratt et al. 1987). This dissertation also provides basic information on honeycreeper mitochondrial DNA diversity which may be useful for conservation efforts.

Mitochondrial DNA for Evolutionary Studies and the PCR

The mitochondrial DNA molecule is useful for phylogenetic and population studies because it has a simple maternal transmission pattern, is haploid, and generally evolves faster than the nuclear genome (Wilson et al. 1985). It has a conserved gene order in vertebrates although slight rearrangements exist (Desjardins and Morais 1990, Paabo et al. 1991). The vertebrate mitochondrial genome contains 2 ribosomal genes, 22 transfer RNA genes, 13 protein coding genes, and a non-coding control region, or D-loop, that varies in size (Attardi and Schatz 1988). The length of the mitochondrial DNA molecule varies in vertebrates from 16,295 bp in *Mus* to 17,553 bp in *Xenopus* (Wolstenholme 1992). In the chicken, (*Gallus domesticus*) it is 16,775 bp long (Desjardins and Morais 1990).

Phylogenetic and population studies using mitochondrial DNA have been greatly assisted by the development of the

polymerase chain reaction (PCR) (Mullis et al. 1986). The PCR is based on the enzymatic amplification of DNA fragments using oligonucleotide primers from flanking sequence to characterize unknown regions. For studies involving mitochondrial DNA, primer sequence information is critical, and has proliferated with the development of "universal primers" that amplify the same gene regions from a variety of taxa (Kocher et al. 1989). The PCR proceeds by combining dNTPs, DNA primers, a Mg_2 containing buffer, and a small sample of the template DNA with a thermo-stable DNA polymerase, usually Taq DNA polymerase. The reaction is heated to denature the DNA, then cooled to allow the primers to bind. The reaction is heated again, and the DNA is copied by the polymerase. As the temperature rises to the DNA denaturation temperature, the strands separate again, the reaction is cooled, and the cycle is repeated. By repeating the procedure for many cycles, the original copy number of the DNA template is geometrically increased at the rate of 2^n, where n is the number of cycles (Saiki 1990). After 30 cycles, the DNA is amplified by 10^5 and purified for DNA sequencing.

PCR is also useful for the amplification of genes from pathogenic organisms and is becoming a basic tool for medical diagnostic laboratories (Kwok and Sninsky 1989). The procedures usually involve determining pathogen specific DNA regions for the design of specific DNA primers which are used

for the PCR. A positive PCR forms the basis for the diagnosis. In this dissertation, I report on the development of a PCR-based test for avian malaria and its use in studying the disease in natural populations of Hawaiian birds.

In this dissertation, DNA sequence from the mitochodrial cytochrome b (cyt b) gene is used for both the phylogenetic and population studies. The cyt b gene has become a popular choice in avian phylogenetics (Helm-Bychowski and Cracraft 1994, Edwards et al. 1991) largely because primers designed to conserved regions are successful for a wide variety of avian taxa (Kocher et al. 1989). The cyt b gene is generally useful for resolving relationships on the order of 1 to 100 MY. The growing use of this gene in avian systematics is creating a database from which to make comparisons and form molecular evolutionary hypotheses. This database will also allow for testing phylogenetic hypotheses generated by other molecular methods, such as the DNA hybridization studies by Sibley and Ahlquist (Sibley et al. 1988).

Questions Addressed in this Dissertation

Aspects of Honeycreeper Systematics

The primary systematic issue to resolve regarding the Hawaiian honeycreepers is whether or not they are monophyletic (Chapter 2). The discovery of the Poouli

(*Melamprosops phaseseoma*) (Casey and Jacobi 1974), a new genus and a highly divergent form, has brought this old problem (Wilson and Evans 1890-99) back to the forefront (Pratt 1992b). The systematic position of the Maui creeper (*Paroreomyza montana*) in relation to the Drepanidinae has also been questioned (Pratt 1992a). I performed a rigorous test of monophyly for the honeycreepers for representative taxa, including the *Paroreomyza*, using two Cardueline finches as outgroups. Also in chapter 2, the problem of species relationships in the genus *Oreomystis* (Pratt 1992a, James and Olson 1982) and the phylogenetic position of the Maui Parrotbill (*Pseudonestor xanthophrys*) are addressed. The interspecific relationships of the other honeycreepers were analyzed and compared to phylogenies produced by other studies. Unfortunately, the enigmatic Poouli, (*Melamprosops phaeseoma*) was not included in this study because no sample exists. It is exceedingly rare (Scott et al. 1988) and only 2 museum specimens exist (Banko 1979). Also no psittirostrinines were used so basal relationships within the honeycreepers could not be addressed.

Hierarchical Aspects of Honeycreeper Mitochondrial DNA Variability

Studies of mitochondrial diversity in populations of the Hawaiian honeycreepers were also undertaken (Chapter 3). The

Hawaiian honeycreepers are variable in their degree of morphological divergence with geographic distance (Pratt et al. 1987), which is probably linked to differences in the dispersal habits of the species and recency of divergence. The most nomadic species of honeycreepers, the Apapane (*Himatione sanguinea*), Iiwi (*Vestiaria coccinea*), and Ou (*Psittirostra psittacea*), were described historically as highly mobile and comprised the bulk of dead animals that would litter the beaches after a storm, presumably blown off course by the high winds (Wilson and Evans 1890-1900). Enormous mixed flocks of over 40,000 Apapane and Iiwi have been observed over Volcanoes National Park on the island of Hawaii (MacMillen and Carpenter 1980). The highly mobile habits of the Iiwi and Apapane are linked to their feeding behavior. They are primarily nectarivores that track ohia-lehua (*Metrosideros polymorpha*) blooms. Common Amakihi are more generalized in feeding habits, taking nectar and some insects and are more sedentary, tending to stay close to where they fledge (Baldwin 1953).

In the population study, a hierarchical perspective was taken in addressing mitochondrial DNA diversity in the honeycreepers. First, a single site analysis was done on birds living at the Pua Akala Tract of the Hakalau Forest National Wildlife Refuge on the Island of Hawaii. This study was intended to provide information on mitochondrial diversity of 6 species (3 endangered), varying in population

size and dispersal habits, while living in the center of distribution and density on Mauna Kea. Second, a multiple site, single island scale for one species was examined to provide information on how populations close to Pua Akala would compare. At a single island scale, mitochondrial variation between populations of Common Amakihi living on the two major volcanoes on the Island of Hawaii was analyzed. Finally, a multiple island scale was taken to glean insights into phylogenetic relationships between island populations and possibly discern patterns of island colonization.

Studies of mitochondrial DNA diversity in the honeycreepers are relevant to conservation efforts for these species. The disappearance of the honeycreepers, due in large part to habitat alteration from introduced infectious diseases, is one of the greatest tragedies in biology. This group offers unparalled opportunities for discovering basic evolutionary mechanisms and principles underlying gene flow in island species. My studies can help to identify unique populations for preservation and provide baseline information on genetic diversity of the drepanidinae. This type of work is underway for other endangered Hawaiian birds, such as the Hawaiian crow (NRC 1992), and should be a primary focus for work on the honeycreepers.

Molecular Studies of Avian Malaria in Hawaiian Birds

The two major studies on avian malaria in Hawaiian birds (Warner 1968 and van Riper et al. 1986) provide a framework for discussing the role of this disease in limiting honeycreeper distribution. Warner (1968) modeled an elevational incompatibility line above which malaria mosquito vectors did not exist and below which Hawaiian birds did not occur. This model forms the basis for much of the interpretation of honeycreeper demographics (Scott et al. 1986). The van Riper et al. (1986) challenge experiments suggested that the honeycreepers may be evolutionarily immunocompromised and more susceptible to malaria than other groups of birds.

One of the central questions regarding avian malaria in the Hawaiian honeycreepers involves defining the demographics of the disease and its vectors. Development of a standard molecular method of malaria screening that is more sensitive than the blood smear method would allow researchers to address questions regarding parasite load, tolerance and response. Over 100 species of birds from 5 continents were introduced to Hawaii (Moulton and Pimm 1983) and the ability to identify potential disease reservoirs is a necessity in controlling avian malaria. A PCR-based test which is quantitative, unambiguous and inexpensive, offers conservation biologists some hope in accurately estimating

disease prevalence, seasonal patterns of appearance, and tolerance or resistance. To this end, a PCR test for avian malaria was developed and used to screen high elevation populations for prevalence of the disease (Chapter 4). This test can be used in future studies to address questions regarding differences in experimental response to malaria challenge.

Chapter 2

Monophyly of and Polyphyly Within the Hawaiian Honeycreepers

Introduction

The Hawaiian honeycreepers (Aves: Drepanidinae) consist of a widely diverse group of over 60 species, known historically and from fossils (James and Olson 1991), that encompass nearly the entire spectrum of passerine morphological and behavioral adaptations (Amadon 1950, Raikow 1976, Freed et al. 1987). Feeding behavior in these birds ranges from insectivory in the tribe Hemignathini (e.g., *Paroreomyza*, *Loxops*, and *Hemignathus*) to granivory in the Psittirostrini (e.g., *Rhodocanthus*) and nectarivory in the Drepanidini (e.g., *Drepanis*). The differences in feeding ecology are manifest in highly variable bill morphology that ranges from the strong, broad, parrot-like bills of *Pseudonestor xanthophrys* to the long, slender, and decurved bills of *Hemignathus ellisianus* and *Vestiaria coccinea*.

Given the extreme level of morphological diversity, it is not surprising that the systematics of the Hawaiian honeycreepers has remained enigmatic since the discovery of these birds by Western explorers in 1778. Naturalists of the late eighteenth and early nineteenth centuries originally believed the birds represented several familes of neotropical

origin (Cook 1778, Latham 1781-5, Bloxam 1826, Sclater 1871, Dole 1879). But as comprehensive studies of the honeycreepers were undertaken, a consensus of opinion emerged, based mostly on the presence of the "drepanid specific odor" (Perkins 1903), limited anatomical diversity, and tongue morphology (loss of lingual wings), that these birds represented one extremely variable taxon (Amadon 1950). Identifying the group of passerines from which the honeycreepers were derived has remained problematic (Wilson and Evans 1890-99, Sushkin 1929, Amadon 1950, Sibley and Ahlquist 1982, Johnson et al. 1989). Questions have also arisen regarding the taxonomic rank of the Hawaiian honeycreepers (James and Olson 1991) and the species relationships within the group (Johnson et al. 1989, Pratt 1992a, Pratt 1992b, Olson and James 1982).

The Hawaiian creeper-like birds, which are the main focus of this study, have been especially problematic. They are straight-billed insectivores that forage by gleaning or probing for arthropods on the trunks or heavy branches of trees (Raikow 1974, Pratt 1992a). Extant creeper species, with AOU accepted classification (AOU 1983; Pratt 1979, 1992a), include the Kauai Creeper (*O. bairdi*), the Hawaii Creeper (*O. mana*), and the Maui Creeper (*P. montana newtoni*). The Lanai (*P. m. montana*), Oahu (*P. maculata*), and Molokai Creeper (*P. flammea*) are probably extinct (Pratt et al. 1987). Rothschild (1893-1900) classified all of the creeper-

like birds as *Oreomyza* and developed an identification key based on bill morphology and color. Stejneger first used the name *Oreomystis* making *bairdi* the type species (Pratt 1992a). He separated it from *Paroreomyza*, of which *maculata* was the type (Pratt 1992a). Amadon (1950), mostly on the basis of bill morphology, lumped the Common Amakihis (*H. virens* on different islands), the Anianiau (*H. parva*), the Greater Amakihi (*H. sagittirostris*), all of the creeper-like birds (*Paroreomyza* and *Oreomystis*), and the Akepa (*Loxops coccineus*) under the genus *Loxops*. Richards and Bock (1973) studied the feeding anatomy of the genus *Loxops*, using Amadon's lumped definition, and found that the tongues of the Hawaii and Maui Creepers were shorter, broader, straighter, and trough-like rather than tubular like the rest of the honeycreepers excluding the psittirostrinines. Raikow (1974, 1976), in a broader study on the evolution of the honeycreepers, re-elevated *Paroreomyza* (including the Hawaii *Oreomystis* and the Maui *Paroreomyza*) to full generic status, separating it from *Loxops*, on the basis of its nearly exclusive insectivory and non-tubular tongue.

The current accepted classification (AOU 1983) of the creepers is based on recommendations made by Pratt (1979, 1992a) and consists of the two genera *Paroreomyza* and *Oreomystis*. The characters that Pratt (1992a) used to unify *Oreomystis* to the exclusion of *Paroreomyza* include coloration (both *Oreomystis* are drabbly colored and slightly sexually

dichromatic; *Paroreomyza* are brightly colored and highly dichromatic), bill shape and color (*Oreomystis* have pale, slightly decurved bills; *Paroreomyza* bills are darker, more pigmented, and straighter), features of the nasal setae (present in *Oreomystis*, absent in *Paroreomyza*) (Perkins 1903), nasal operculum (partially developed in *Oreomystis*, fully developed in *Paroreomyza*) (Richards and Bock 1973), and body proportions (*Oreomystis* have short tails relative to wing lengths; *Paroreomyza* do not). The tongues of both *Paroreomyza* and *Oreomystis* are similar in general anatomy, and are nontubular with the distal end bifid, and with small terminal and lateral laciniae, but *Paroreomyza* has prominent lingual wings while *Oreomystis* has small ones (Wilson and Evans 1890-99, Richards and Bock 1973). Foraging behaviors are different between *Oreomystis* and *Paroreomyza*. *Oreomystis* creep in "nut-hatch" fashion on the main branches and trunks of trees. *Paroreomyza* gleans leaves and sometimes makes aerial sallies (Raikow 1974). Both *Oreomystis* and *Paroreomyza* are primarily insectivores, but *Paroreomyza* sometimes takes nectar. Pratt claims that the calls of the two *Oreomystis* are "more or less different" than those of *Paroreomyza* and that the song of *Paroreomyza montana* does not even remotely resemble those of the *Oreomystis*. Nests of the two *Oreomystis* are the typical honeycreeper open-cup types, and the *O. mana* nest is nearly identical to that of *H. virens* (Perkins 1903). From these investigations, Pratt concluded

that *Oreomystis* possesses all of the characters that unify the Drepanidinae, although in most respects the genus appears highly divergent.

Most recently, Pratt (1992a) suggested that the creepers in the genus *Paroreomyza* may not be closely related to the *Oreomystis* creepers and may not even be Hawaiian honeycreepers. The drepanidine specific musty odor (Perkins 1903) is absent in *Paroreomyza*. The presence of lingual wings in the tongue of *Paroreomyza* is unique in honeycreepers with the exception of the enigmatic Poouli (*Melamprosops phaeseoma*) (Pratt 1992b). *Paroreomyza* is the only honeycreeper that mobs predators. *Paroreomyza* nests are different from all the honeycreepers in that they are suspended from above. Pratt suggested that, based on these characters, *Paroreomyza* either is not a honeycreeper or must occupy a basal position in the phylogenetic tree, having branched off early from the main line of honeycreeper evolution. The question of the relationship of *Paroreomyza* to the Drepanidinae is one subject of this study.

Controversy also exists over the validity of grouping the two *Oreomystis* species together as congeners. The two *Oreomystis* species are known historically from the most distant islands of the range of the creepers, Kauai and Hawaii, and have not been found as fossils from the other islands (James and Olson 1991). Amadon agreed with Rothschild that the Kauai and Hawaii forms of the creepers

were distinct from one another, but did not think they were more closely related to each other than to the other creepers endemic to the islands between Kauai and Hawaii (Amadon 1950). Olson and James (1982) considered the Kauai Creeper, *O. bairdi*, to be a monotypic genus, although they offered no justification for this reasoning, and classified the Hawaii Creeper, *O. mana*, in the genus *Loxops*. Pratt (1992a) interpreted the skull drawings of Richards and Bock (1973) as indicating that *O. mana* was more similar to *L. coccineus* and *H. virens* than it was to *P. montana*. Unfortunately, Richards and Bock (1973) did not include the Kauai *Oreomystis* in their analysis, so a direct comparison of *O. bairdi* and *O. mana* could not be made from their data.

Existing biochemical systematic studies of Hawaiian honeycreepers include an allozyme (Johnson et al. 1989) and a mitochondrial RFLP study (Tarr and Fleischer [in press]). Neither study sampled both of the *Oreomystis* species and thus they cannot be used to address the validity of the *Oreomystis* taxonomy. Furthermore, the topological disagreement between Tarr and Fleischer's (in press) distance and parsimony trees suggest that too little data was generated to make meaningful phylogenetic trees. The relationship of both species of *Oreomystis* to each other, the *Paroreomyza*, and other drepanidines is the second subject of this study. In addition, I report on the first Maui Parrotbill (*Pseudonestor xanthophrys*) DNA sample, and its use to test Pratt's (1979)

assertion that this taxon is the most primitive of the Hemignathini.

I used DNA sequence, from the mitochondrial cytochrome b (cyt b) gene, to test Pratt's assertion that *Paroreomyza* may not be a drepanidine by examining representative honeycreeper taxa for monophyly. If *Paroreomyza* is not a honeycreeper, then a polyphyletic origin should be suggested in a phylogenetic study with closely related outgroups. The inability to split off the *Paroreomyza* (or any other honeycreeper taxa) with an outgroup would support a monophyletic origin for the honeycreepers. Thus, the test of whether or not *Paroreomyza* is a honeycreeper becomes one of whether or not it contributes to a monophyletic grouping with the honeycreepers, to the exclusion of closely related outgroups. I used the same gene to evaluate monophyly within the genus *Oreomystis*, and the position of *Pseudonestor* within the Drepanidinae.

Methods

Species Studied

Birds were sampled at Hakalau Forest National Wildlife Refuge on Hawaii, Hanawi Natural Area Reserve on Maui, and Na Pali Kona Forest Reserve on Kauai between May 1988 and July 1992. Honeycreepers examined in this study include a Maui and a Kauai Apapane (*Himatione sanguinea*), an Iiwi (*Vestiaria coccinea*), an Akohekohe (*Palmeria dolei*), two Hawaii Creepers (*Oreomystis mana*), one Hawaii Akepa (*Loxops coccineus*), two Hawaii Common Amakihis (*Hemignathus virens virens*), one Maui Parrotbill (*Pseudonestor xanthrophrys*), one Kauai Creeper (*Oreomystis bairdi*), and one Maui Creeper (*Paroreomyza montana*).

Non-honeycreeper outgroups included a White-Browed Rosefinch (*Carpodacus thura*), a Red Crossbill (*Loxia curvirostra*), and published sequence from a Plain Titmouse (*Parus inornatus*) (Edwards et al. 1991). The White-Browed Rosefinch (from Nepal) and the Red Crossbill (from Washington state) are in the subfamily Carduelinae within the Fringillidae. It is widely accepted on the basis of skull and tongue anatomy (Sushkin 1929), flocking behaviors (Richards and Bock 1973), limb musculature and tongue structure (Raikow 1976), and DNA hybridization studies (Sibley and Ahlquist 1982) that the fringillid subfamily

Carduelinae is the closest outgroup to the honeycreepers. The selection of cardueline specimens from the Old World and the New World reflects the cosmopolitan distribution of the subfamily, and the uncertainty as to which cardueline taxa are the closest relatives of the honeycreepers. The Plain Titmouse is in the family Paridae and represents a more distant outgroup.

Birds were captured in aerial mist-nets, weighed, and banded. A blood sample, 50 - 100 µl, was taken using sterile techniques before releasing the bird. The blood was stored frozen in 0.5 ml 10 mM Tris, 10 mM NaCl, and 2 mM EDTA pH 8.0. Total genomic DNA was extracted after the method of Quinn and White (1987).

Cytochrome b PCR and DNA Sequencing

The mitochondrial cyt b gene was amplified by PCR using a set of four primers, #006 (L-strand, chicken #14991): 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3', #005 (H-strand, chicken #15297): 5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3', #073 (L-strand, chicken #15262): 5'-ATC GGA GTA ATC CTC CTC CTA ACC C-3', and #074 (H-strand, chicken #15823): 5'-GTG GTC GGA AGG TTA TTG TTC GTT G-3' (Desjardins and Morais 1990). Primers #006 and #005 are conserved primers (Kocher et al. 1989) that amplify a 370 bp (including the primer sites) fragment of cyt b. Primer #074 was

designed from the chicken sequence (Desjardins and Morais 1990) and #073 was designed from drepanidine sequence. Primer #006 and #074 amplify a fragment of 893 bp including the primer sites. A contiguous alignment of 790 bp was used for analysis.

For double strand PCR, 1 µl of a 1:10 dilution of the DNA extracted from bird blood was used with 2.5 µl of 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin, and 2 mg/ml BSA), 2.5 µl each primer (10 mM), 4 µl dNTPs (1.25 mM each), and 0.25 µl (1.25 units) of Taq DNA polymerase (Perkin-Elmer Cetus/ABI) in a total volume of 25 µl. The reactants were overlain with mineral oil and run for 30 cycles at 92°C, 30 seconds, 48°C, 2 minutes, 72°C, 1 minute followed by an extension cycle of 10 minutes at 72°C in a Perkin-Elmer Cetus DNA Thermal Cycler. Approximately one half the reaction was electrophoresed on a 2% low melting point (LMP) Nusieve (Seakem) gel in Tris-Acetate (TAE) buffer, stained in Ethidium bromide (ETBr), and visualized under UV light. Bands of the correct size (usually single fragments per reaction) were excised from the gel and stored frozen.

Single-stranded templates for sequencing were generated by the unbalanced or asymmetric priming method (Gyllensten and Erlich 1988). To the bands isolated in 2% LMP agarose, 0.5 ml sterile ddH_2O was added, and the mixture was heated at 90°C for 10 minutes to melt the agarose and free the DNA.

The tube of melted agarose and ddH_2O was centrifuged for 5 seconds and 10-15 µl was used as template for the second PCR. The ratios of the PCR reagents were the same as for the double stranded reaction but the primers were offset 50:1 in concentration and the total volume of the reaction was raised to 50 µl. The PCR profile for the second amplification was the same as the first except that it was extended to 40 cycles to allow time for the single strands to accumulate over the double strands (Gyllensten 1989).

Five microliters of the reaction were removed from beneath the oil and electrophoresed on a 2% agarose gel. The remaining 45 µl of the reaction products were added to 0.3 ml ddH_2O in Millipore ultra free-MC 30,000 NMWL filter units and centrifuged three times to concentrate the single strands and remove the unincorporated dNTPs and primers. The single strand DNA was collected from the filters, the volume was adjusted to 30 µl with ddH_2O, and 7 µl was used for DNA sequencing with T7 DNA polymerase (Sequenase, United States Biochemical Corp.) using ^{35}S-dATP and the manufacturer's protocol. Priming for the sequencing reaction was done with either the primer that was limiting in the asymmetric PCR or with an internal primer complementary to the single strands. The sequencing reaction products were electrophoresed on 6% poly-acrylamide, 7M Urea gels, and were fixed, dryed, and exposed to Fuji RX X-ray film for 18 hours.

Sequences were read, aligned by eye, and entered into the computer program MacClade (Maddison and Maddison 1992) for sorting and ordering. A sequence file was saved using the PHYLIP exporting option in MacClade. Phylogenetic trees were generated by distance using the Neighbor Joining (Saitou and Nei 1987) program in the PHYLIP package (Felsenstein 1989). Bootstrapping for the PHYLIP analysis was done with the Seqboot program for 1000 replications. Distances were calculated using the DNAdist program set to calculate the Kimura 2-parameter distances at a transition to transversion ratio of 2.6 to 1 (calculated from the total observed ratio of 208 to 80). The titmouse was designated as the outgroup. The consensus tree was found with the program Consense sampling 1000 data sets.

Parsimony trees were generated using the Phylogenetic Analysis Using Parsimony (PAUP 3.1.1) program (Swofford 1991). A heuristic search was done using the tree bisection-reconnection (TBR) swapping algorthim, weighting transversions over transitions by 3 to 1. A consensus tree was found from the seven shortest trees using the strict, majority rule (50%) option, again, defining the titmouse as the outgroup taxon. The consensus tree was bootstrapped 1000 times using the heuristic method.

Trees generated from PHYLIP and PAUP were exported to MacClade. Trees were rearranged in the MacClade program

using the "move branch" tool. This method allows one to selectively restructure the tree topology and test the resultant tree for statistical properties. The manipulated trees were compared to the original trees for investigations targeted at finding shorter topologies.

Testing for Monophyly

I used several methods to test for monophyly. First, I applied the bootstrap, which allows one to place confidence limits on specific topologies (Felsenstein 1985a,b). The bootstrap procedure involves resampling from a given dataset, with replacement, until a new dataset of the same size as the original is obtained (Efron and Tibshirani 1991). When applied to DNA sequence data, the taxa are held constant and the characters (nucleotides) are resampled with replacement (Hedges 1992). The bootstrap has been used to test monophyly of certain groups within a given phylogeny (Felsenstein 1985b, Archie 1989). In the present study, the bootstrap value for a monophyletic grouping of the honeycreepers including *Paroreomyza* would give an indication of statistical support of this node and a direct measure of whether or not this genus should be included with the rest. By choosing in advance the particular group being tested for monophyly, I have avoided the necessity of correcting for a multiple-tests problem (Felsenstein 1988).

Second, I applied the permutation tail probability (PTP) test to compare the tree lengths of the observed data with that of the data after randomization (Archie 1989, Faith 1991, Faith and Cranston 1991). The PTP test can be useful to investigate parts of a topology for monophyly versus nonmonophyly (Faith 1991). The observed data are constrained to fit two trees, 1) the monophyly tree and 2) a nonmonophyly tree, and a difference value of minimum tree length is calculated. The character state assignments are then randomly permuted and the same two trees, showing monophyly or nonmonophyly, are generated from the randomized data. The process is repeated for the randomized data a large number of times and a frequency distribution of tree length differences is generated. The test statistic compares the difference values for minimum tree length of the two topologies under consideration for the observed and the randomized data. The monophyly constraint statement includes *Paroreomyza* with the honeycreepers, while under nonmonophyly, *Paroreomyza* is excluded. Support for including *Paroreomyza* in the honeycreepers would be indicated if the shortest tree found was one that included *Paroreomyza*, and if the minimum tree length difference for this tree versus permuted trees (manipulated to exclude *Paroreomyza*) was significant.

The permutation tail probabilites were calculated after the methods of Faith (1991) using a program developed by George Roderick at the University of Hawaii (Roderick, pers.

comm.). A NEXUS data block was set up with two constraint statements representing the two trees to be tested. Roderick's program Permute was run 500 times using a Monte Carlo method to randomize the data set. Output trees were imported to PAUP using the heuristic TBR branch swapping algorithm, designating the titmouse as the outgroup. The differences of tree lengths between the two trees for each round of tree construction were calculated. The values for the differences of each round were collected and sorted from the PAUP files using the program Extract.

The third method used to assess honeycreeper monophyly was the Delta Q test proposed by Templeton (Templeton 1983). The Delta Q method performs a statistical test of the permuted trees against the best tree using the mean and variance of step differences between trees across sites and is analogous to the test using log likelihood differences (Felsenstein 1989, Kishino and Hasegawa 1989). The Delta Q test has an advantage over the PTP test because, unlike the PTP test, it uses all the DNA sequence data and not just the informative sites. The test is included in the DNApars program of the PHYLIP package and is run by selecting the "user trees" option (Felsenstein 1989). The same 5 tree topologies were examined in the PTP and Delta Q tests.

The fourth method used was the maximum likelihood analysis (Felsenstein 1981) in the DNAML program of the PHYLIP package (Felsenstein 1989). The maximum likelihood

method assumes that 1) each nucleotide site evolves independently, 2) different lineages evolve independently, 3) each site undergoes substitution at an expected rate, 4) all sites of the sequence are included for analysis, and 5) two types of substitutions can occur: a) replacement of a purine by a purine or a pyrimidine by a pyrimidine which can lead to either no change or a transition or b) replacement of an existing base drawn at random from a pool of bases at known frequencies independent of the identity of the base being replaced, which can lead to no change, a transition, or a transversion (Felsenstein 1989). The maximum likelihood method has the advantages of using all the sequence data, and taking base compositions into account, in contrast to the PTP test and the Delta Q method. Maximum likelihood tests were performed using a transition to transversion ratio of 2 to 1 and empirical base frequencies of A=0.27063, C=0.34329, G=0.14574 and T=0.24034.

Monophyly in *Oreomystis* was tested by evaluating the bootstrap values of the overall trees from the parsimony, maximum likelihood, and distance analyses, and by forcing the congeners together and evaluating the length of the tree in comparison to the original tree by the Delta Q method.

The phylogenetic position of *Pseudonestor* was evaluated by the bootstrap values of the trees, and by forcing it to assume the phylogenetic position of an ancestral

hemignathinine and comparing the length of the resultant tree by the PTP method.

For all tests, statistical significance was assigned to p values < 0.05. Exceptional values are noted.

Results

DNA Sequence Results

The alignment of the 790 bp cyt b fragment for the 15 taxa in this study (Figure 2.1) shows 211 variable positions, of which 122 are phylogenetically informative. A phylogenetically informative site is one at which a particular base is shared by at least two or more of the taxa while at least two or more share another base at that site (Nei 1987). Of the 211 variable sites, 175 (82.9%) are at third positions, 26 (12.3%) are at first positions, and 10 (4.7%) are at second positions. Within the honeycreepers, there are 3 nucleotide changes that cause amino acid replacements. Percent total base compositions for all sites are given in table 2.1. The base composition results indicate a striking lack of guanines at third positions in codons. This finding is consistent with that reported for other passerine mtDNAs (Edwards et al. 1991, Helm-Bychowski and Cracraft 1993) and for mammals in general (Irwin et al. 1991).

A distance matrix based on uncorrected pairwise sequence differences and one for distances calculated from the Kimura 2-parameter method are shown in table 2.2. From the uncorrected matrix, the greatest pairwise sequence difference within the Drepanidinae is 7.7% (61 changes) for the

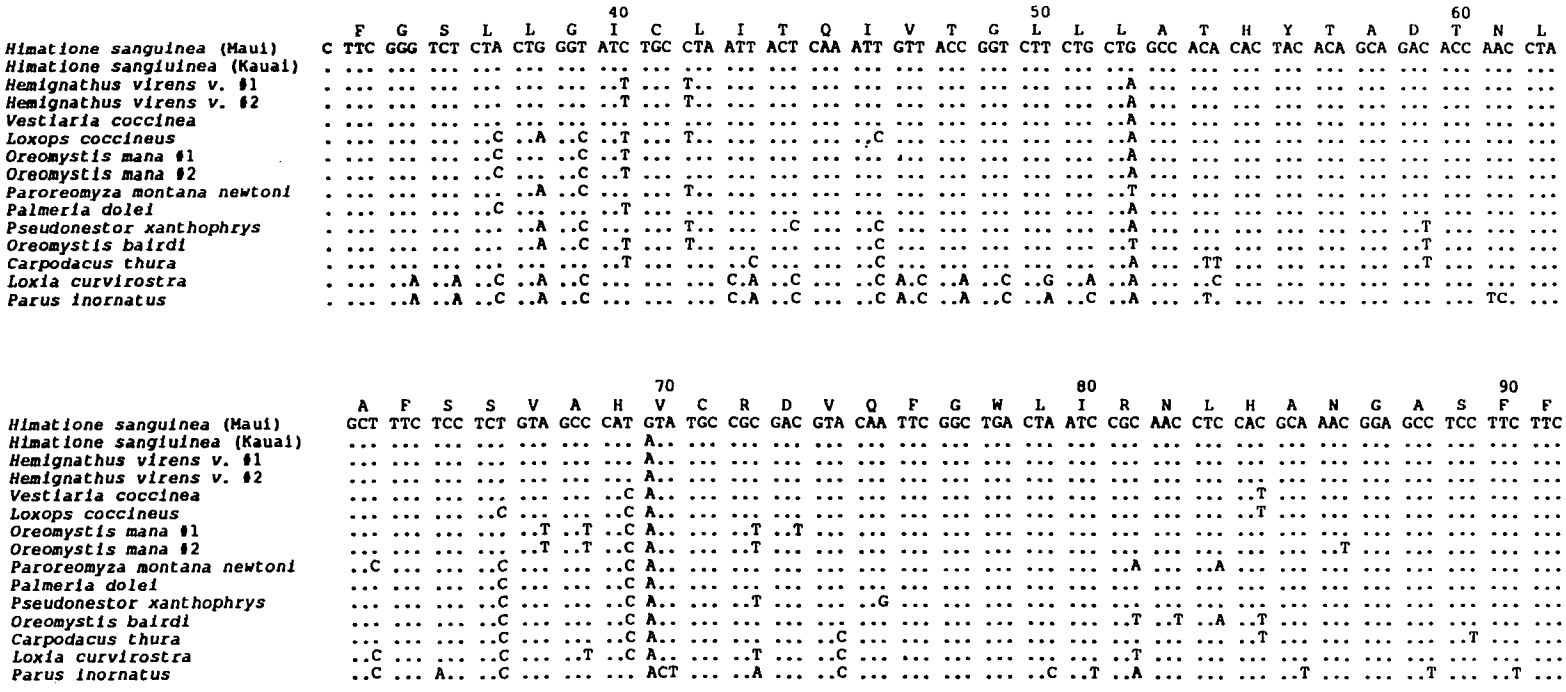

Figure 2.1. Cyt b DNA Sequence Alignment

									100										110										120
	F	I	C	I	Y	L	H	I	G	R	G	I	Y	Y	G	S	Y	L	N	K	E	T	W	N	I	G	V	I	L
Himatione sanguinea (Maui)	TTC	ATC	TGC	ATC	TAC	CTA	CAC	ATC	GGC	CGA	GGA	ATC	TAC	TAC	GGC	TCA	TAC	CTA	AAC	AAA	GAA	ACC	TGA	AAC	ATC	GGA	GTA	ATC	CTC
Himatione sanguinea (Kauai)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hemignathus virens v. #1	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..T	...	...	...	...	...	...	...	...	...	...	...	...
Hemignathus virens v. #2	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	..A	...	..T	...	...	...	...	...	...	...	...	...	...	...	...
Vestiaria coccinea	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...
Loxops coccineus	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...
Oreomystis mana #1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Oreomystis mana #2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Paroreomyza montana newtoni	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Palmeria dolei	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...
Pseudonestor xanthophrys	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..G
Oreomystis bairdi	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	T..	...	...	...	...	...	...	...	...	...	...	...
Carpodacus thura	...	...	...	...	...	..C	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	..T	...	..C	...	...
Loxia curvirostra	...	...	...	...	...	T..	...	...	...	...	...	...	...	...	...	...	..T	...	...	...	...	...	...	...	...	...	..C	...	...
Parus inornatus	...	...	...	...	...	T.T	...	..T	...	...	...	..T	...	...	...	...	...	T..	...	...	...	...	...	..T	...	...	..C	...	..A

										130										140									
	L	L	T	L	M	A	T	A	F	V	G	Y	V	L	P	W	G	Q	M	S	F	W	G	A	T	V	I	T	N
Himatione sanguinea (Maui)	CTC	CTA	ACC	CTC	ATA	GCA	ACC	GCA	TTC	GTA	GGC	TAC	GTA	CTA	CCA	TGA	GGA	CAA	ATA	TCA	TTC	TGA	GGA	GCT	ACA	GTA	ATT	ACA	AAC
Himatione sanguinea (Kauai)	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Hemignathus virens v. #1	...	...	...	...	...	...	..T	..C	...	...	...	...	...	..G	...	...	...	...	..G	..C	...	...	...	...	...	...	...	...	...
Hemignathus virens v. #2	...	...	...	...	...	...	..T	..C	...	...	...	...	...	..G	...	...	...	...	..G	...	...	...	...	...	...	...	...	...	...
Vestiaria coccinea	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Loxops coccineus	...	...	...	...	...	...	..T	..C	...	...	...	...	...	..G	...	...	...	...	...	..G	...	...	...	...	...	...	...	...	...
Oreomystis mana #1	...	...	...	...	...	...	..T	..C	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Oreomystis mana #2	...	...	...	...	...	...	..T	..C	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Paroreomyza montana newtoni	...	T..	...	...	...	...	..T	..C	...	...	...	...	...	..G	...	...	..C	...	...	...	...	...	...	...	...	...	..C	...	...
Palmeria dolei	...	...	...	...	...	...	..T	...	..T	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...
Pseudonestor xanthophrys	...	...	...	...	...	...	..T	..C	...	...	...	...	...	..G	..G	...	...	..	...	...	...	...	...	...	...	..G	...	...	...
Oreomystis bairdi	..T	...	...	..T	...	...	..T	..C	...	...	...	..T	...	..G	..G	...	...	...	...	...	...	...	...	...	...	...	..C	...	..T
Carpodacus thura	...	...	...	..T	...	...	..T	..C	...	..T	...	...	..C	..G	...	...	..C	...	...	...	...	...	..C	..C	...	...	..C	...	...
Loxia curvirostra	T.A	T..	...	...	...	...	..T	...	..T	...	...	..A	...	..C	...	...	...	..C	...	...	...	...	..C	..C	...	...	..C	...	..T
Parus inornatus	...	...	...	...	...	...	...	..C	...	...	...	..A	..T	..T	...	...	...	...	...	...	..G	...	...	..T	...	...	.G.	..C	...	...

Figure 2.1 (Continued)

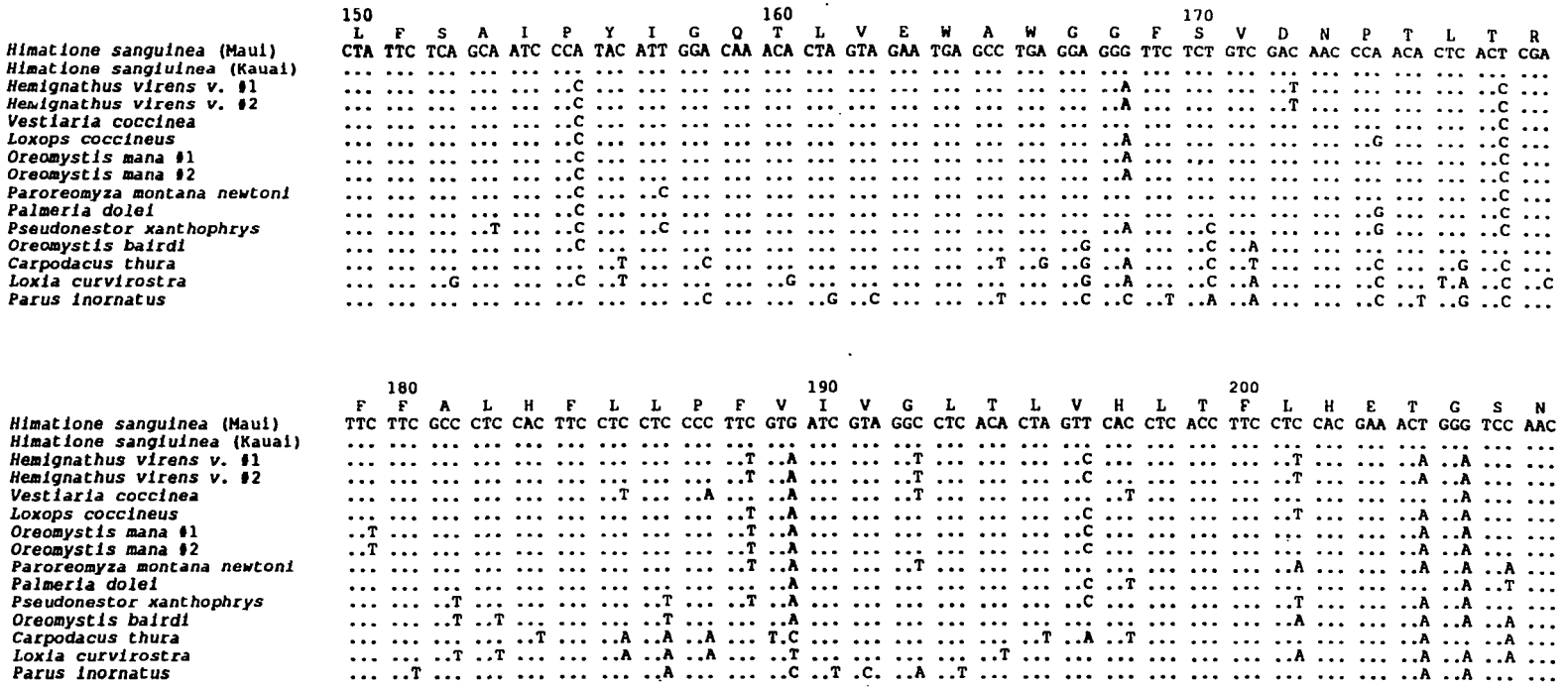

Figure 2.1 (Continued)

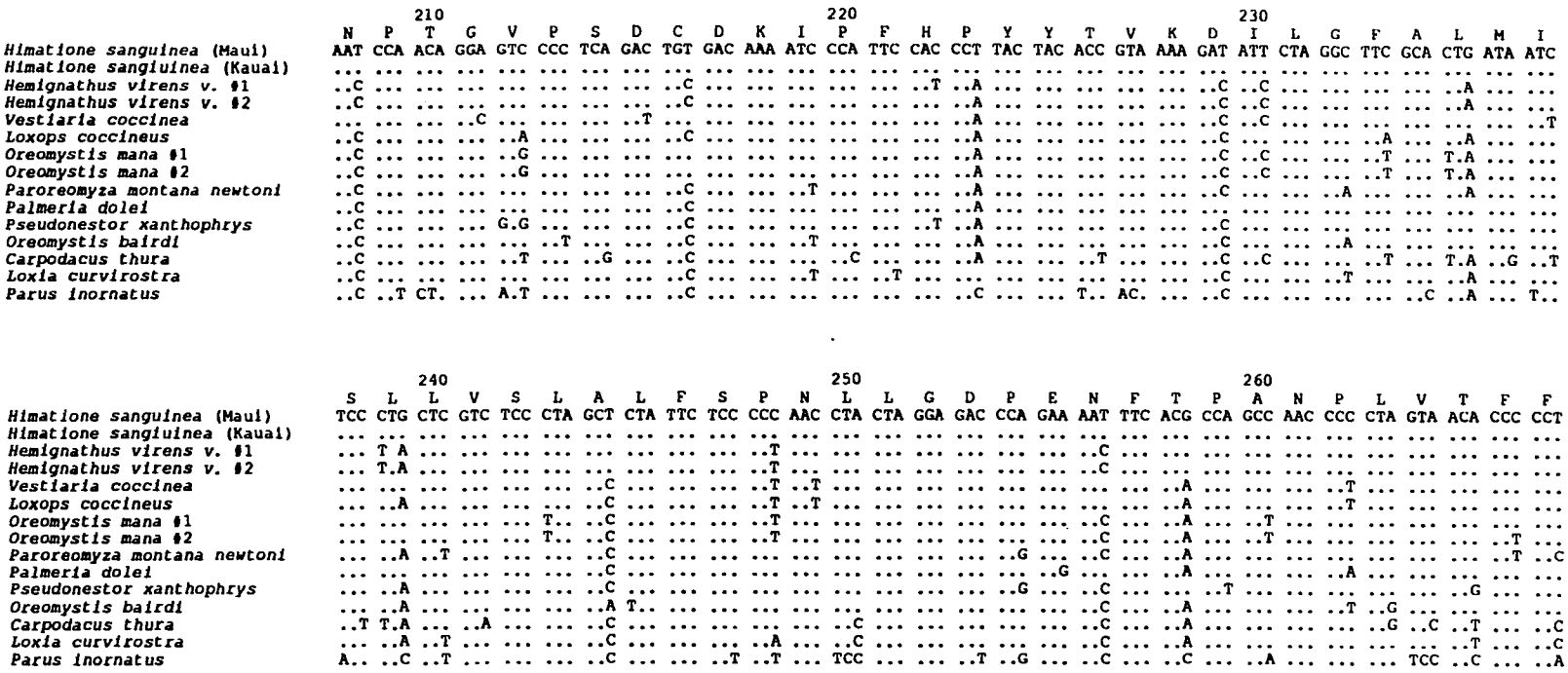

Figure 2.1 (Continued)

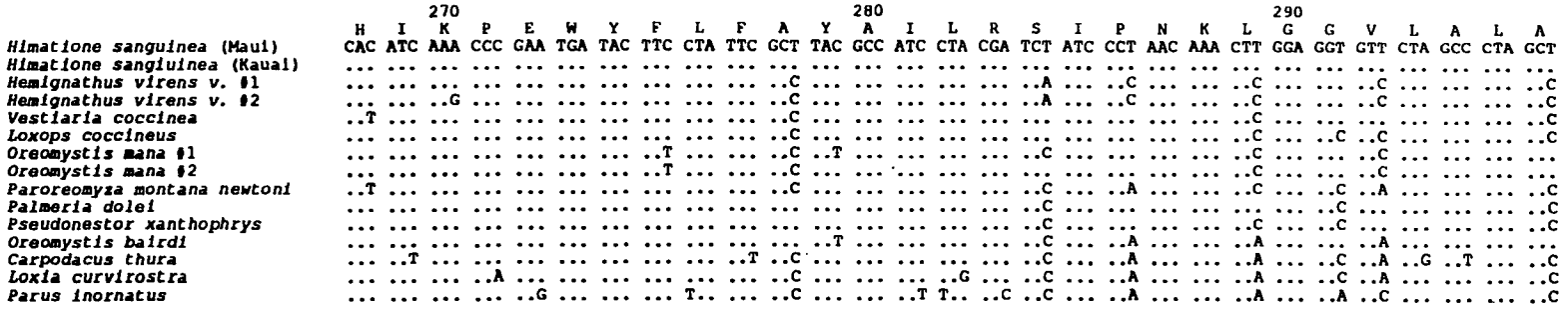

Figure 2.1. DNA sequence alignment for the 790 bp of cytochrome b from the 15 taxa in this study. The nucleotide sequence at the top is for the Apapane (*Himatione sanguinea* [Maui]) individual. The dots indicate an identical base to the reference sequence and the specific base changes are designated. The amino acid sequence is a computer translated version for the Apapane and is given in one-letter code. The numbers correspond to the amino acid positions for the chicken (Desjardins and Morais 1990).

Table 2.1. Base composition for all the sequences in this study. There is a bias against Gs in total base composition and at the second and third positions in codons. Base composition is roughly equal at first positions.

BASE	FIRST	SECOND	THIRD	TOTAL
A	23.6	20.5	37.0	27.1
C	27.5	26.3	49.1	34.3
G	26.5	14.4	2.8	14.6
T	22.4	38.8	11.0	24.0

Table 2.2. Pairwise distances between taxa. The top matrix is for the uncorrected distances. Above the diagonal are absolute uncorrected distances below are mean distances (number of sites different). The lower matrix are the distances from the Kimura 2-parameter analysis setting the transition to transversion ratio to 2.6 to 1.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	*Himatione sanquinea* (Maui)	-	0.001	0.051	0.049	0.034	0.054	0.052	0.051	0.063	0.034	0.063	0.070	0.103	0.108	0.151
2	*Himatione sanquinea* (Kauai)	1	-	0.049	0.048	0.033	0.053	0.051	0.049	0.062	0.033	0.062	0.068	0.101	0.106	0.149
3	*Hemignathus virens v. #1*	40	39	-	0.004	0.049	0.033	0.043	0.043	0.052	0.051	0.052	0.072	0.103	0.100	0.152
4	*Hemignathus virens v. #2*	39	38	3	-	0.048	0.033	0.042	0.042	0.051	0.049	0.053	0.071	0.101	0.099	0.152
5	*Vestiaria coccinea*	27	26	39	38	-	0.051	0.051	0.049	0.061	0.037	0.070	0.077	0.104	0.100	0.156
6	*Loxops coccineus*	43	42	26	26	40	-	0.035	0.034	0.043	0.038	0.038	0.059	0.092	0.090	0.135
7	*Oreomystis mana #1*	41	40	34	33	40	28	-	0.006	0.059	0.046	0.057	0.073	0.100	0.100	0.147
8	*Oreomystis mana #2*	40	39	34	33	39	27	5	-	0.058	0.047	0.058	0.077	0.101	0.101	0.151
9	*Paroreomyza montana newtoni*	50	49	41	40	48	34	47	46	-	0.054	0.051	0.053	0.080	0.099	0.141
10	*Palmeria dolei*	27	26	40	39	29	30	36	37	43	-	0.052	0.066	0.097	0.094	0.151
11	*Pseudonestor xanthophrys*	50	49	41	42	55	30	45	46	40	41	-	0.062	0.099	0.104	0.147
12	*Oreomystis bairdi*	55	54	57	56	61	47	58	61	42	52	49	-	0.090	0.100	0.158
13	*Loxia curvirostra*	81	80	81	80	82	73	79	80	63	77	78	71	-	0.097	0.147
14	*Carpodacus thura*	85	84	79	78	79	71	79	80	78	74	82	79	77	-	0.161
15	*Parus inornatus*	119	118	120	120	123	107	116	119	111	119	116	125	116	127	-

1	*Himatione sanguinea* (Maui)															
2	*Himatione sanguinea* (Kauai)	0.0013														
3	*Hemignathus virens v. #1*	0.0525	0.0512													
4	*Hemignathus virens v. #2*	0.0511	0.0498	0.0038												
5	*Vestiaria coccinea*	0.0350	0.0337	0.0512	0.0498											
6	*Loxops coccineus*	0.0566	0.0552	0.0337	0.0337	0.0525										
7	*Oreomystis mana #1*	0.0539	0.0525	0.0444	0.0430	0.0525	0.0363									
8	*Oreomystis mana #2*	0.0525	0.0512	0.0444	0.0430	0.0512	0.0349	0.0064								
9	*Paroreomyza montana newtoni*	0.0665	0.0652	0.0541	0.0527	0.0638	0.0446	0.0623	0.0610							
10	*Palmeria dolei*	0.0350	0.0337	0.0525	0.0511	0.0377	0.0390	0.0470	0.0484	0.0569						
11	*Pseudonestor xanthophrys*	0.0662	0.0649	0.0539	0.0552	0.0731	0.0390	0.0592	0.0606	0.0528	0.0539					
12	*Oreomystis bairdi*	0.0736	0.0722	0.0763	0.0748	0.0820	0.0624	0.0777	0.0819	0.0551	0.0694	0.0652				
13	*Loxia curvirostra*	0.1126	0.1111	0.1127	0.1111	0.1141	0.1009	0.1098	0.1112	0.0860	0.1069	0.1084	0.0975			
14	*Carpodacus thura*	0.1183	0.1168	0.1095	0.1080	0.1094	0.0978	0.1095	0.1110	0.1078	0.1023	0.1141	0.1094	0.1056		
15	*Parus inornatus*	0.1749	0.1733	0.1771	0.1769	0.1822	0.1558	0.1702	0.1751	0.1629	0.1753	0.1704	0.1855	0.1693	0.1872	

Vestiaria coccinea - *Oreomystis bairdi* pair and for the two *Oreomystis* comparisons. The smallest pairwise difference between a drepanidine and one of the outgroups is 8.0% (63 changes) for the *Paroreomyza* - *Loxia curvirostra* comparison. The observed transition to transversion ratio for all comparisons is 2.6 to 1. Resetting the transition to transversion ratios to 20 to 1 or 1 to 1 did not alter the topologies of the trees produced.

Four phylogenetic analyses support monophyly of the Hawaiian honeycreepers, based on the samples used. The phylogenetic tree from the Neighbor Joining analysis (Figure 2.2) has a total tree length of 381, and the monophyletic node to the honeycreepers is supported at 98% in 1000 bootstrap replications. The minimum PAUP tree (Figure 2.3) has a total tree length equal to the distance tree, and the monophyletic branch to the honeycreepers is supported in 98% (n=1000) of the bootstrap replications. The distance and PAUP trees are topologically similar, although the Neighbor Joining tree places the *C. thura* as the closest outgroup to the honeycreepers, while the PAUP tree places the *Loxia* in that position. The maximum likelihood tree has the same topology as the distance tree and a Ln likelihood value of -3035.82.

The rearranged trees and results of the permutation analyses are shown in figures 2.4 - 2.7. In each case, the first tree is held constant and is the one found in the

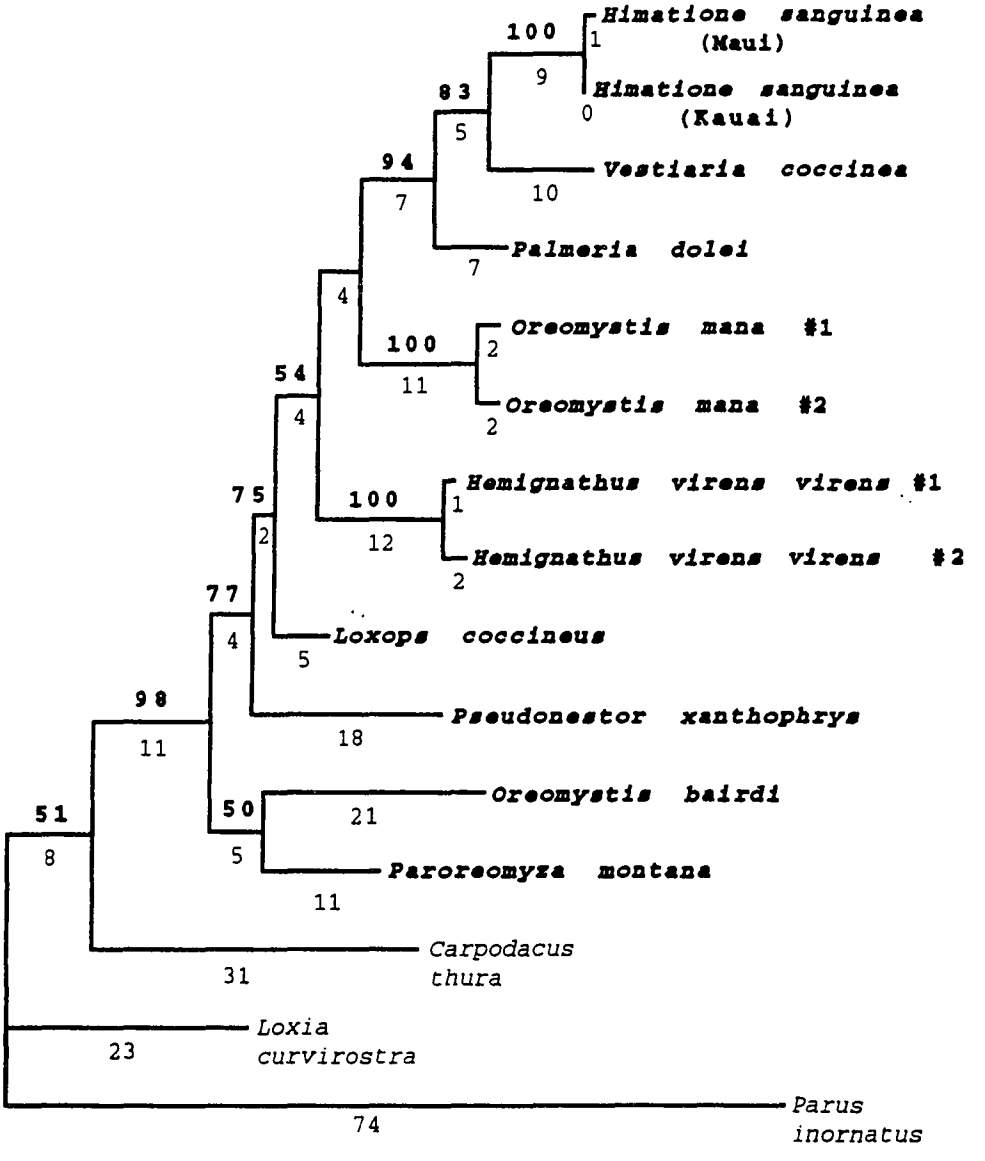

Figure 2.2. Neighbor Joining Tree. The bold names indicate honeycreeper taxa. The bold numbers above the branches are bootstrap values for the node to the immediate right. The numbers below the branches are distances corresponding to number of unique sites on that branch. Treelength is 381, Consistency Index = 0.68, Retention Index = 0.57 and Rescaled Consistency Index = 0.39.

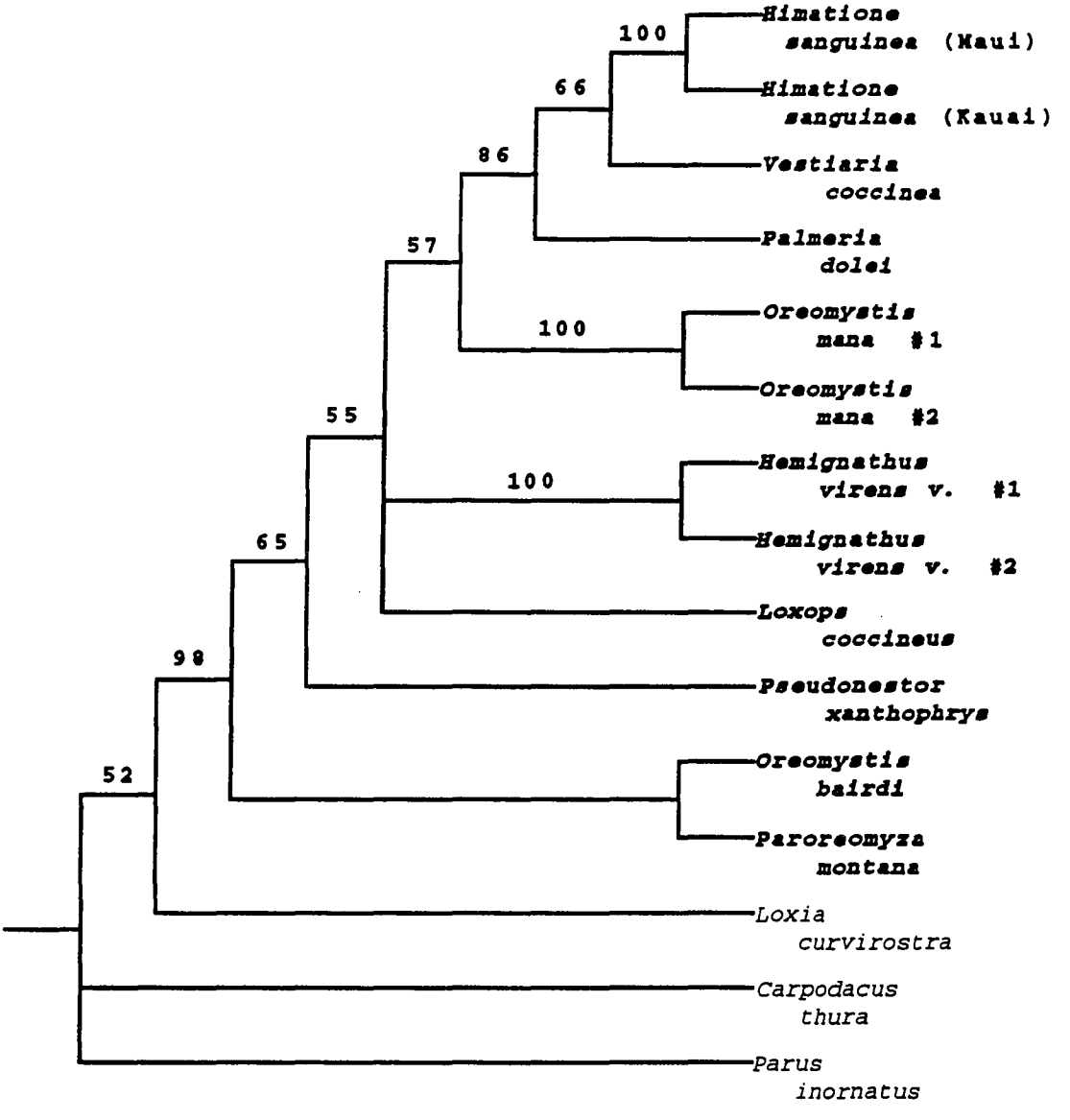

Figure 2.3. PAUP Tree. The bold names are honeycreeper taxa. The numbers are bootstrap values for the node to the right. The tree was bootstrapped 1000 times.

distance and maximum likelihood analyses. The second tree in each figure is the manipulated one. The specific rearrangement that was made can be seen by the dashed line, which represents the branch swap made to arrive at the second tree. These rearrangements each take the hypothesis of monophyly for the honeycreepers, and exchange it for one of non-monophyly.

The first rearrangement (Figure 2.4) was made by exchanging the drepanidine clade that includes the *O. mana* and *P. montana* for the non-drepanidine *Carpodacus thura*. Since these taxa appear closely related, and form a natural unit, it was the first group chosen for rearrangement. The total tree length changes from 381 to 391 after the swap. The distribution of differences for the randomized data for the PTP test is shown in the histogram at the bottom of figure 2.4. The observed difference in tree length (+10) for this permutation is highly significant when compared to the distribution of tree length differences for the randomized data. This is the same tree length difference found for this rearrangement by the DNApars method. Tree 2 is also significantly worse than tree 1 by the Delta Q and maximum likelihood methods. This finding supports inclusion of the *O. bairdi - P. montana* clade within the honeycreepers.

The second permutation (Figure 2.5) is made by swapping out the *Paroreomyza* for the *C. thura*. Tree 3 is the one expected if *C. thura* is more closely related to the

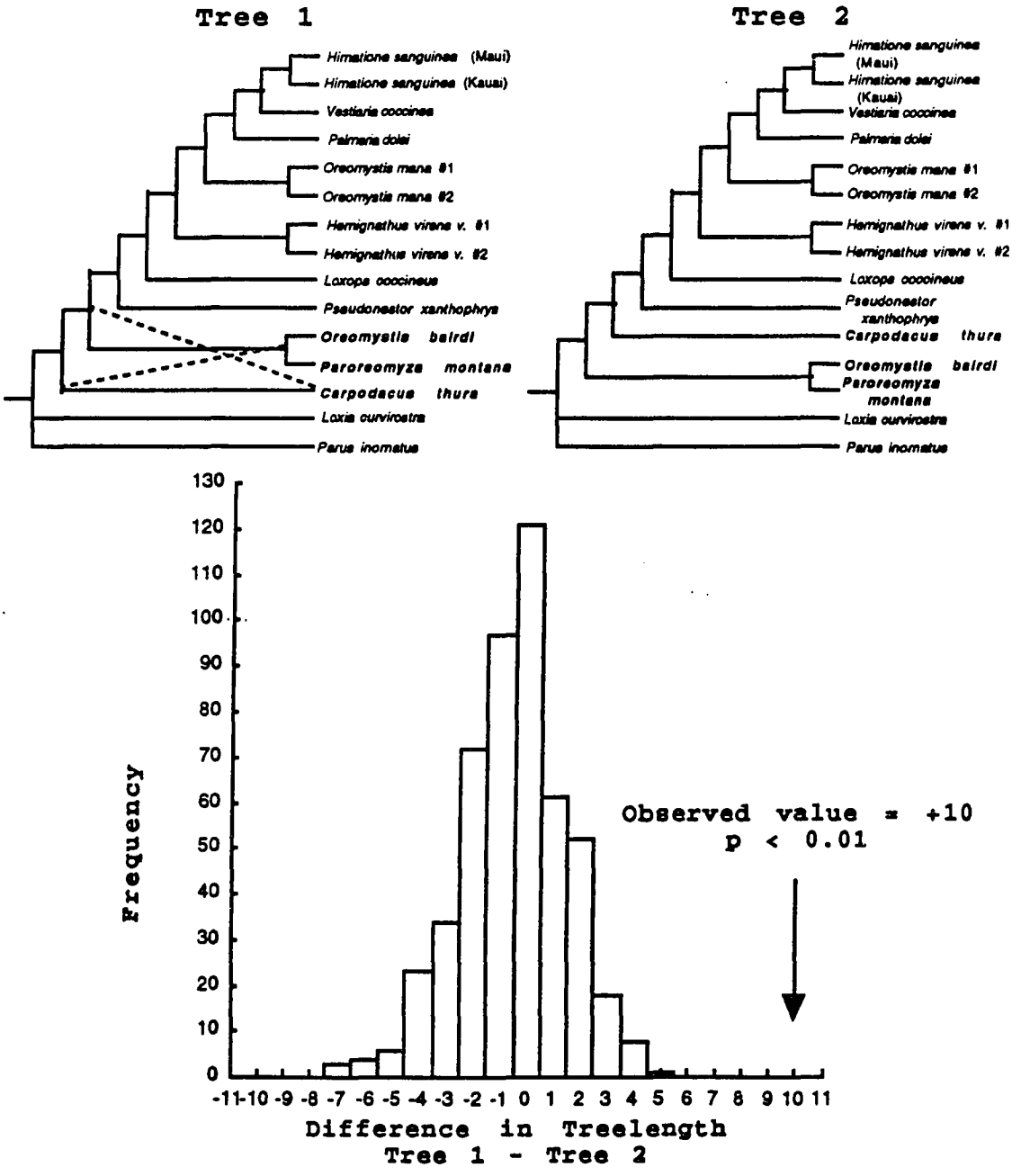

Figure 2.4. PTP analysis for the manipulation moving out the clade of *O. bairdi* and *P. montana* for *C. thura*. The branch swap is indicated as a dashed line on tree 1. Tree 2 is the result of the branch swap. The distribution of tree length differences for this manipulation for the randomized data is shown at the bottom. The arrow indicates the tree length difference for this manipulation on observed data.

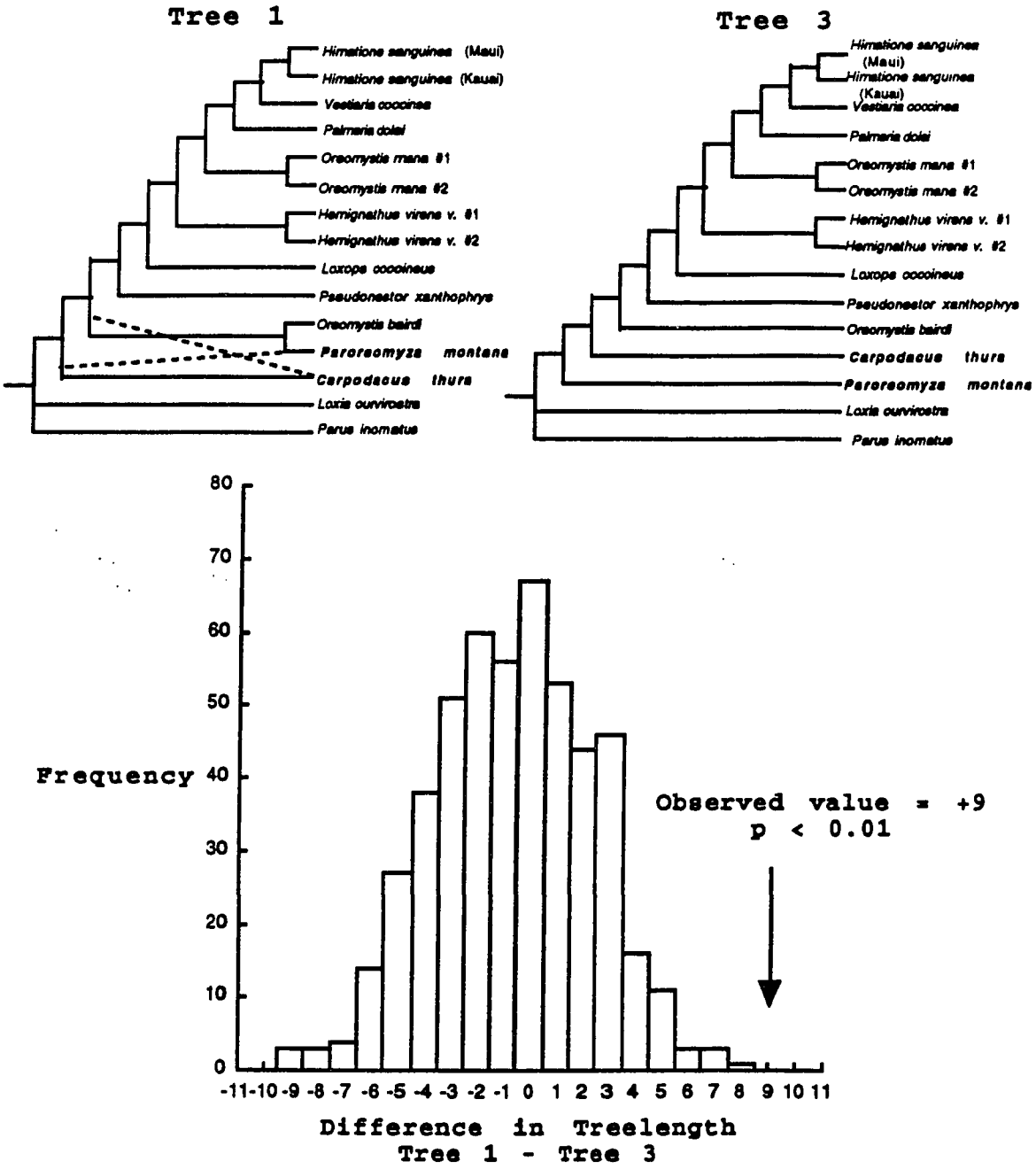

Figure 2.5. PTP analysis for the tree moving the Paroreomyza and the distribution of tree length differences for randomized data.

honeycreepers than is *Paroreomyza*. Tree lengths for the observed data in this rearrangement go from 381 to 390 for both the PTP test and DNApars methods. For the PTP test, the observed tree-length difference, compared to the distribution of differences for the randomized data, again shows high statistical significance. This tree length difference was marginally significant by the Templeton Delta Q method ($p = 0.07$). The manipulation made a significantly worse tree in the maximum likelihood analysis.

The third rearrangement (Figure 2.6) was the reciprocal of the previous one. Here *O. bairdi* was moved out and *Paroreomyza* was left in. For the PTP test, using the observed data, trees with this rearrangement go from 381 to 392. This difference is significant, compared to the randomized data. The treelength difference for this rearrangement by the DNApars method was 14 and this tree was significantly worse by the Templeton Delta Q test and the maximum likelihood test. The results shown in figures 2.5 and 2.6 indicate strong statistical support for including both the Maui and Kauai Creepers in a monophyletic clade with the honeycreepers.

The fourth and final rearrangement (Figure 2.7) took a taxon of the Drepanidinae that is deeper within the clade, the Maui Parrotbill, *Pseudonestor xanthophrys*, and moved it out. This rearrangement was done to give an indication of the relative scale of these differences of total tree

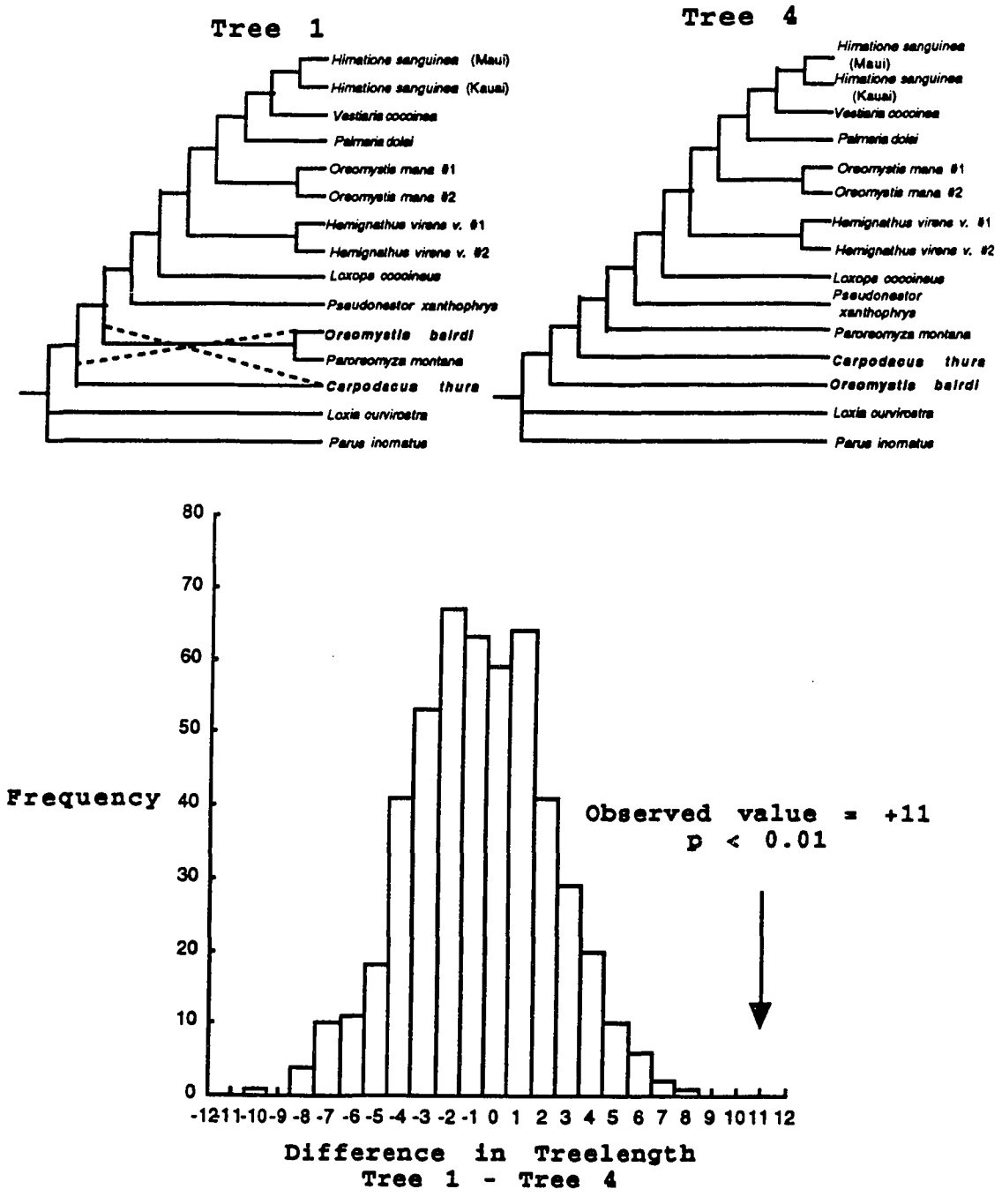

Figure 2.6. PTP analysis for the tree manipulation taking out only the *O. baridi* and the frequency distribution of tree length differences for the randomized data.

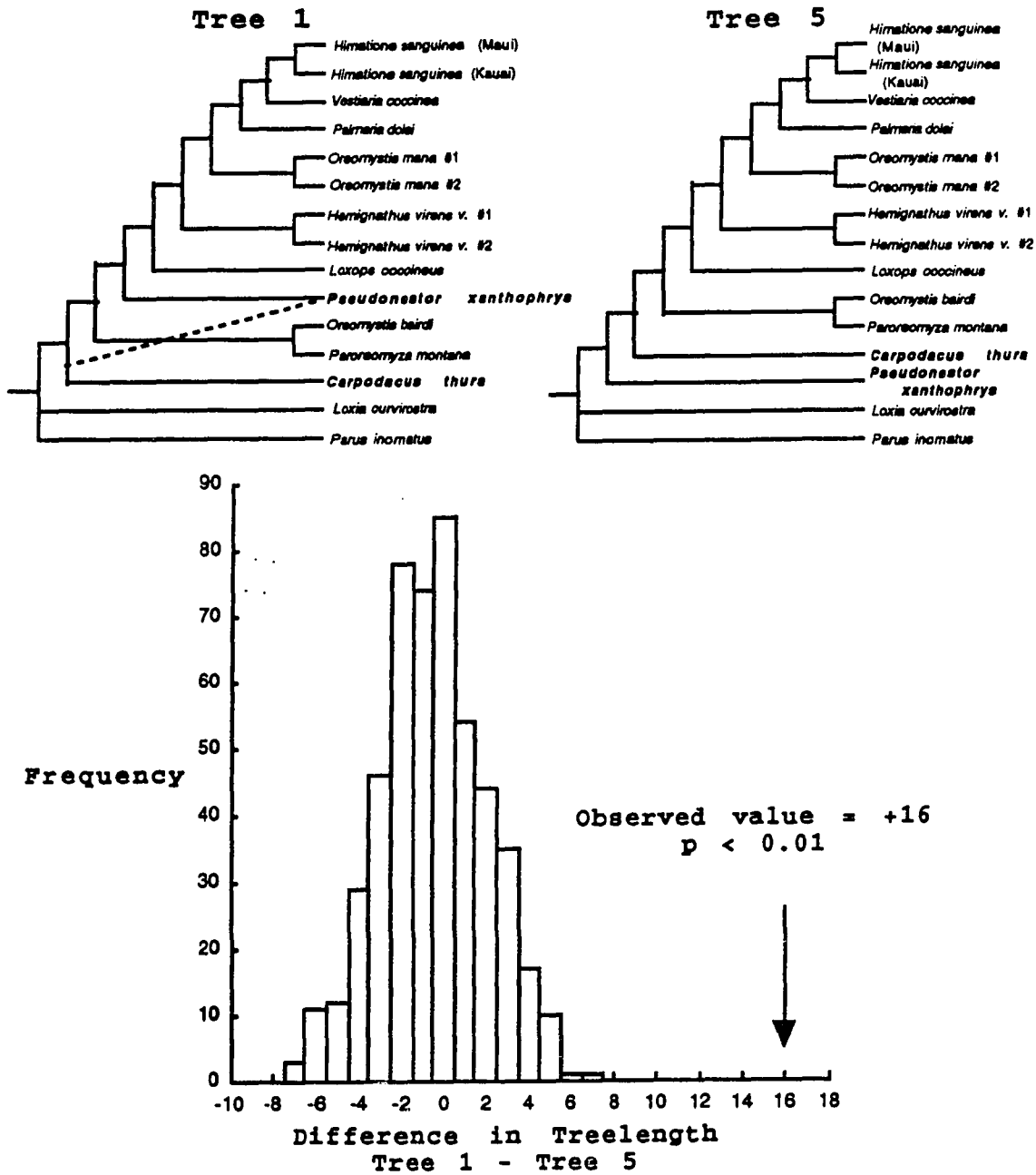

Figure 2.7. PTP analysis for the tree manipulation taking out *Pseudonestor* and the frequency distribution of tree length differences for the randomized data.

lengths. Tree lengths under this rearrangement go from 381 to 397 in the PTP test and to 400 by the DNApars method. For the PTP test, a tree length difference this great was not recorded for the randomized data and is found to be significant. The Delta Q test results indicate that this tree is significantly worse than tree 1.

All trees place intervening taxa between *O. mana* and *O. bairdi*. Forcing the *Oreomystis* congeners together results in a tree 14 steps longer than tree 1 and significantly worse as judged by the Delta Q test. This indicates that using the accepted taxonomy (AOU 1983) of *Oreomystis* as a model results in a significantly worse phylogenetic explanation of the observed DNA sequences. Thus, there is little support from our data for the current classification of the two *Oreomystis* species as congeners. The classification appears to be polyphyletic.

All trees place the Maui Parrotbill (*Pseudonestor xanthophrys*) as derived relative to the *P. montana - O. bairdi* clade. The node making the Maui Parrotbill a derived rather than an ancestral hemignathinine is supported by bootstrap probability value of 77 in the Neighbor Joining tree and 65 in the PAUP tree. These nodes are moderately strong. The permutation analysis made for moving *Pseudonestor* to a basal psittirostrinine position, within the honeycreepers but outside the *P. montana - O. bairdi* clade, resulted in a tree worse than the original at the p = 0.0875

level. The Delta Q test also indicated that this tree was significanlty worse than tree 1.

Discussion

Phylogenetic results presented here strongly support 1) a monophyletic grouping of the Hawaiian honeycreepers including *Paroreomyza montana*, 2) grouping *P. montana* and *O.bairdi* in a basal clade of the insectivorous honeycreepers, 3) polyphyly of the genus *Oreomystis*, and 4) *Pseudonestor* as a derived, rather than ancestral, hemignathinine. High bootstrap values on the node that leads to the honeycreepers for both the distance and parsimony trees would not be expected when using closely related outgroups unless the result represented an accurate phylogeny. Furthermore, the topological agreement of the three methods (distance, parsimony, and maximum likelihood) strengthens the probability that accurate phylogenetic information has been revealed. The results of the permutation analyses by the PTP test, the Delta Q test, and the maximum likelihood tests indicate strong statistical support for including *Paroreomyza* in the Drepanidinae. This finding contrasts with Pratt's (1992a) conclusion. Thus the two major drepanidine synapomorphies that Pratt used to exclude *Paroreomyza* from the honeycreepers, the drepanidine odor and the loss of lingual wings, must have evolved after the split of *Paroreomyza* from the main line of honeycreeper evolution. *Oreomystis* appears to not be a good taxonomic unit, in agreement with Olson and James (1982). Following is a

discussion of the implications of these findings with respect to the evolution of the honeycreepers as a group and to *Oreomystis* as a genus.

Although I did not have a representative from the tribe Psittirostrini, there is no reason to suspect that inclusion of such would have altered these results. Studies that have included psittirostrinine taxa and *Paroreomyza* still support *Paroreomyza* among the basal members of the honeycreepers (Figure 2.8). The tree presented here agrees with the allozyme tree, which placed *P. montana* and *O. bairdi* in a basal clade. Tarr and Fleischer (in press) also found *Paroreomyza* and *O. bairdi* to occupy basal clades, and had a psittirostrinine, *Telespiza cantans*, grouped with *O. bairdi*. *Paroreomyza* was the most basal honeycreeper, in their PAUP tree, and clustered with a non-drepanidine cardueline, *Carpodacus mexicanus*, in their Neighbor Joining tree. The Tarr and Fleischer trees are preliminary, since they only sampled the equivalent of less than 150 bp of mitochondrial DNA sequence and they provided no statistical evidence for the robustness of their phylogenetic conclusions. More work is necessary to resolve the relationships among *Paroreomyza*, *O. bairdi*, and the Psittirostrini honeycreepers, but *Paroreomyza* is clearly a honeycreeper.

The phylogenetic position of *Pseudonestor* as derived relative to *Paroreomyza* and *O. bairdi*, but ancestral relative to the other hemignathinines, indicates convergence with

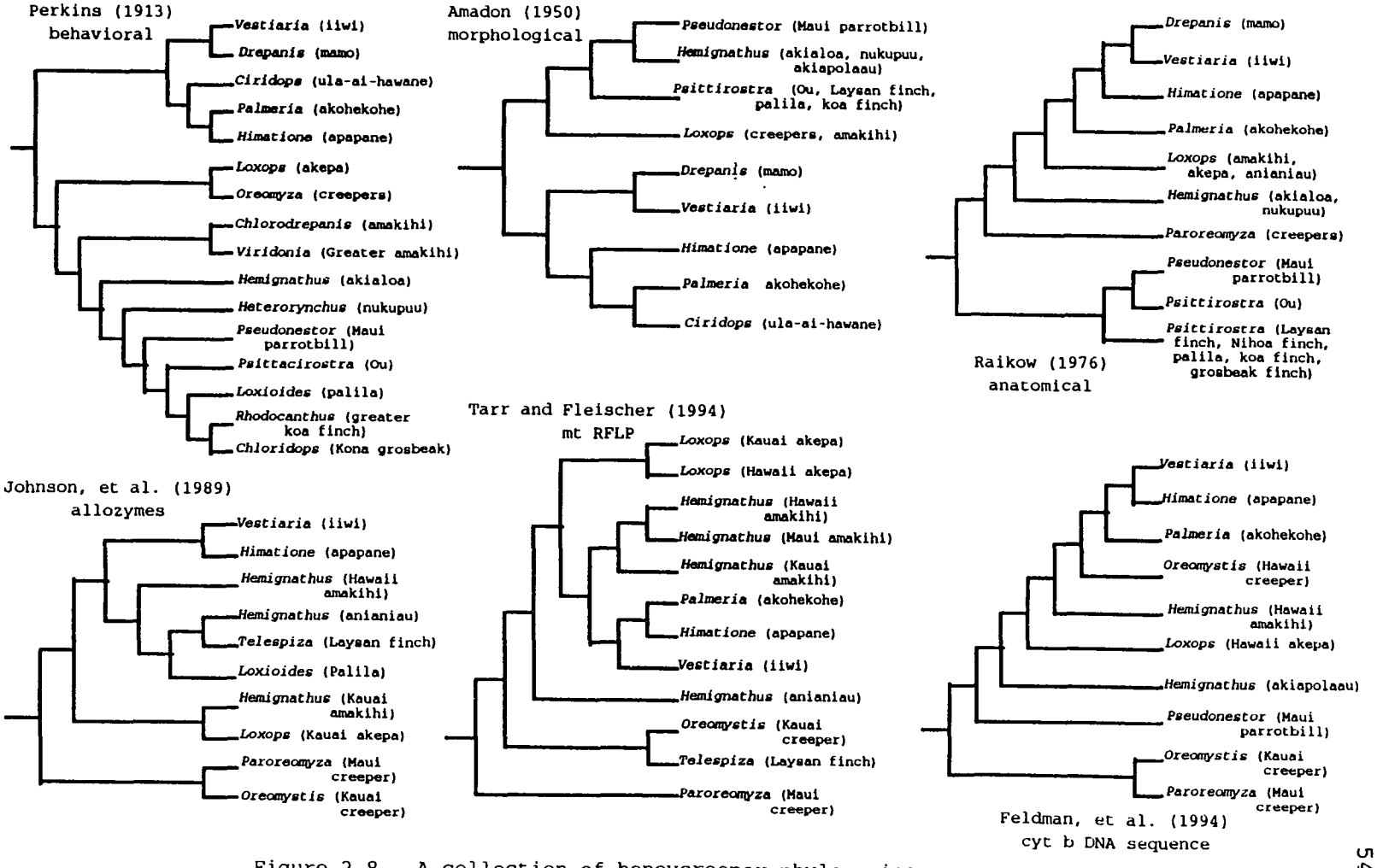

Figure 2.8. A collection of honeycreeper phylogenies.

psittirostrinines in bill morphology. Pratt (1979) argued that secondary thickening of a thin hemignathinine bill and loss of tubular tongue were less parsimonious than retention of a non-tubular tongue and secondary thinning of a thick psittirostrinine bill from a direct psittirostrinine ancestor. It appears now that the non-tubular tongue of *Pseudonestor* is a primitive character retained from the early hemignathinines. The thickened bill, adapted for tearing bark and crushing wood for extracting insects from within, appears to be a specialized modification of a more generalized insectivorous bill. A more thorough phylogenetic analysis that includes additional molecular characters and extinct members of the Hemignathini and Psittirostrini may shed more light on this issue.

Problems with *Oreomystis*

A fundamental result of this study is the finding that the two *Oreomystis* species, *mana* and *bairdi*, (the Hawaii and Kauai Creepers) are phylogenetically distant from one another, supporting the assertion of James and Olson (1982). In fact, the genetic distance between these two taxa is equal to the largest distance within the honeycreepers. Based on this analysis, the closest honeycreeper relative of the Hawaii Creeper is the Common Amakihi. This implies that the current classification of the *Oreomystis* species as congeners

is incorrect and that these species are only superficially related to one another. The basis for this classification in the past has been mainly on behavioral adaptations to insectivory (Pratt 1992a) and morphological characters which are not necessarily phylogenetically informative.

One possible explanation for the *Oreomystis* problem is a hybridization event between a *Hemignathus* - like individual and an *Oreomystis* individual on other islands that resulted in a new subpopulation that is now represented by its descendants recognized as *Oreomystis mana*. A second possibility is that *Oreomystis mana* originated from an early divergence in *Hemignathus* from an older island whose descendants form the present day population of *O. mana* that I sampled. The apparent close relationship of *O. mana* and *bairdi* could be the result of subsequent convergent evolution of *O. mana* and *O. bairdi*. If true, this indicates plasticity in behavioral and anatomical adaptations associated with specialized insectivory. In fact, high levels of behavioral and anatomical plasticity in general may be contributing factors in the evolutionary success of the Hawaiian honeycreepers (Smith et al. in press). The taxonomic position of *O. mana* to other creepers and to the amakihi raises important issues for further field and laboratory studies.

General Implications to Honeycreeper Evolution

I do not share the views of Sibley and Ahlquist (1982) and Johnson et al. (1989) that the molecular phylogenies of the honeycreepers provide evidence for an immediate and explosive radiation into a variety of open niches. Consensus trees (Figure 2.9) show that insectivorous species are ancestral to nectarivorous species. Nevertheless, the intermediate insectivorous species like *Hemignathus virens* and *Loxops coccineus* have tubular tongues (Amadon 1950). This implies that adaptations to consume nectar arose in primarily insectivorous species. Pratt (1992a) reported the occasional use of nectar by *Paroreomyza*, which might be predicted if nectarivory is a later specialization arising from a generalist ancestor. If nectarivory is a recent adaptation in the honeycreepers, it may have been dependent on the maturation of Hawaiian forests. It now becomes of great interest to understand the dates of arrival and radiation of the other non-honeycreeper passerine birds such as the monarchine flycatchers, the frugivorous Hawaiian thrushes (*Myadestes*), and the meliphagid honeyeaters, as well as the plants and arthropods which provided food.

A molecular time scale available in the sequence data provides insight into the age of the honeycreeper taxa sampled and the origin of *Oreomystis mana*. Shields and Wilson (1987) reported a calibration for mitochondrial DNA

divergence of 2% per million years, based on mitochondrial DNA RFLP patterns of two extant goose genera and a fossil date of last common ancestor. There is evidence that cyt b evolves at the same rate as the mitochondrial genome as a whole (Irwin et al. 1991). The 2% per million years calibration can be used to explore systematic and biogeographic questions.

The largest observed pairwise difference within the honeycreepers is 7.7% for the *Vestiaria - Oreomystis bairdi* and for the two *Oreomystis* species. This difference corresponds to a mitochondrial ancestor 3.9 million years old. Based on the ages of the Hawaiian Islands, the island of origin for the mitochondrial DNA ancestor of modern drepanidines sampled could have been Kauai (5.1 MY old), or possibly Oahu (2.6 - 3.7 MY old) if the estimate is slightly off, but not Maui (less than 1.3 MY old) or Hawaii (less than 0.5 MY old) (Clague et al. 1975).

The closest uncorrected pairwise difference for *O. mana* and *H. virens* in this study is 4.2% corresponding to 2.1 MY. Such a date indicates that a hybridization event could not have occurred on the islands of Hawaii or Maui because the ages of those islands are too young, although it might have occurred on either Oahu or Kauai (Clague et al. 1975). The birds must have either island-hopped to the Island of Hawaii, and gone extinct on the others, or gone directly to the Island of Hawaii from either Kauai or Oahu. Complicating the

matter is the fact that fossils of *Oreomystis* have not been found on Maui and fossils of *Paroreomyza* have not been found on the islands of Kauai or Hawaii (Olson and James 1982).

My estimated time of origin of 3.9 MY for a mitochondrial DNA ancestor is slightly older than the 3.5 MY estimate of Tarr and Fleischer (in press) from mtDNA RFLPs which included a psittirostrinine. Tarr and Fleischer apparently took an average from the pairwise comparisons of the drepanidines to their outgroup, *Carpodacus mexicanus*, which may not necessarily be the closest outgroup. Their calculation appears to be giving an average age of the lineages, which is not the same as the date of the last common maternal ancestor of the honeycreepers. My estimate may have been older if I had cyt b sequences from a psittirostrinine. In addition, both estimates may be considered preliminary because they were made from a calibration derived from goose RFLPs (Shields and Wilson 1987) and the rate of divergence may be different for Passeriformes and Anseriformes.

Nonetheless, the mitochondrial estimates are an order of magnitude lower than Sibley and Ahlquist's (1982) DNA hybridization estimate of 15 - 20 million years and roughly half of Johnson et al's. (1989) estimate of 7 - 8 million years based on allozymes. The DNA hybridization study only had intermediate or derived honeycreeper taxa (Common Amakihi, Iiwi and Apapane) and the values were calibrated for

birds whose age of first reproduction is between 2-4 years (Sibley et al. 1988). The honeycreeper age of first reproduction is 1 year (van Riper 1987, Lepson and Freed [in press]) and this may lead to a faster rate of divergence and therefore a shorter estimate of divergence time. In addition, the DNA hybridization method is suspected of giving only a coarse estimate of divergence time (Hillis and Moritz 1990).

The allozyme estimate, which incorporated a psittirostrine and *Paroreomyza*, is calibrated for a straight genetic distance (Nei's D), and the calibration was inferred from other allozyme studies (Johnson et al. 1989). Molecular estimates of time using allozymes and Nei's D have enormous standard errors, and an accumulation of Nei's D of 1.0 for allozymes can range from 0.7 - 18 MY for vertebrates (Hillis and Moritz 1990). The potential large variation associated with the honeycreeper allozyme date of 7 - 8 MY perhaps brings it in line with the mitochondrial estimates.

Resolution of the time of phylogenetic events is crucial for understanding the radiation of the Hawaiian honeycreepers. The extraordinary morphological, behavioral, and genetic diversity of the birds, given the relatively recent geological history and chronological sequence of the main Hawaiian islands, raises significant issues concerning anatomical and behavioral plasticity, adaptation, competition, and rates of change. Further progress on

understanding the evolution of the honeycreepers now depends on incorporating additional taxa, including museum skins and bones of extinct species, data from nuclear loci, rigorous analyses of morphological or behavioral characters, and molecular clocks specifically calibrated for passerines.

Summary

The honeycreeper species examined form a strong monophyletic unit as assessed by bootstrap values and the length of the branch to the Drepanidinae. The use of closely related cardueline finch outgroups in tests of monophyly show strong support for including *Paroreomyza montana* as a member of a basal clade of extant Hawaiian honeycreepers.

Congruence analysis of six trees generated by different methods suggest that the red birds form a clade, the amakihi is in the middle of the tree, and *Paroreomyza* and *Oreomystis bairdi* are members of the most basal clade. It appears that nectarivory is a recent adaptation.

The Hawaii and Kauai *Oreomystis* appear not to be closely related to each other and are much further apart than expected if these species are congeners. This may be explained by an early split of *Hemignathus virens* ancestors and subsequent convergent evolution between the two *Oreomystis* species. Alternatively there may have been hybridization events that occurred between direct ancestors of *Oreomystis mana* and a *Hemignathus virens* - like ancestor. The mtDNA time scale based on observed sequence differences suggests that such an event must have occurred on either Oahu or Kauai rather than Maui or the Island of Hawaii.

Chapter 3

Intraspecific mtDNA Variability of Hawaiian Honeycreepers

Introduction

Understanding how genetic variability is partitioned in natural populations is of fundamental importance for workers interested in identifying mechanisms of evolution. Knowledge of population genetic structure can lead to insights into how the forces causing differentiation, such as mutation, selection, and drift are counterbalanced by the forces tending to homogenize genetic differentiation such as movements of individuals and populations (Slatkin 1987). Furthermore, a genetic understanding of the interplay between gene flow and isolation can give insights into speciation (Mayr 1963). Traditionally, studies of geographic variation in natural populations have been based on morphological comparisons but the degree to which this type of variation is genetically based is rarely tested (Zink et al. 1991). Phenotypic markers, such as allozymes, can give an indication of the relative frequency of alleles among populations, but robust phylogenetic information is often missing.

The use of mitochondrial DNA (mtDNA) has revolutionized studies directed at connecting population structure, biogeographic variation, and phylogenetics (Harrison 1989).

The mitochondrial DNA molecule is useful for phylogenetic and population studies because it has a simple maternal transmission pattern, is haploid, and generally evolves faster than the nuclear genome (Wilson et al. 1985). Most of the early population studies with mtDNA were based on whole genome comparisons of RFLP patterns. These studies contributed to an emerging awareness of the power in taking a phylogenetic perspective on population studies (Wilson et al. 1985, Avise 1986).

The use of DNA sequences (assisted by the development of PCR) has further contributed to the merging of population and phylogenetic studies (Slatkin 1987, Edwards 1993), particularly for groups of closely related species (Avise et al. 1987). These studies make full use of the information available in the mtDNA molecule because the number of characters (nucleotides) that one has access to, is limited only by the amount of sequencing one wants, or needs, for resolving the relationships under study (Felsenstein 1992). The combined use of mtDNA and sequences can allow for the dating of coalescent events, calculation of effective population sizes, and the description of gene flow between populations.

Studies of genetic variability in natural populations are also of fundamental relevance to conservation biology (Frankel and Soule 1981). Mitochondrial DNA is haploid, maternally inherited in most animals, and its effective

population size is 1/4 that of nuclear genes. Thus, it is a more sensitive marker of the effects of bottlenecks and genetic isolation than are nuclear genes (Wilson et al. 1985).

Of equal importance to conservation biologists is the realization that mtDNA can also date past population genetic events (Wilson et al. 1985) and take into account differences in life history, population dynamics, sex ratio, and mating success (Edwards 1993). For populations that have declined in range and census size, estimates of genetic diversity can perhaps give an indication of the degree to which a population is at risk of extinction (Lande and Barrowclough 1987, Lande 1988). For endangered species, with fragmented distributions, measures of genetic diversity and the determination of phylogenetic relationships between remaining populations can help to indicate if populations were subdivided in the past and should now be maintained as separate breeding units (Avise and Nelson 1989).

For avian studies, mtDNA RFLP systems have been used to assess population differentiation in relation to potential geographic barriers to gene flow in continental species (Zink et al. 1991, Ball et al. 1988, Ball and Avise 1992). These studies often focus on species whose ranges were fragmented by natural and anthropogenic barriers. Few studies have been directed specifically at island populations. Ironically, it is island systems with sedentary species that have the

greatest potential for revealing geographic and genetic population structure due to the presence of oceanic barriers to dispersal and gene flow.

This study is about mtDNA sequence diversity and population structure in the Hawaiian honeycreepers. The honeycreepers are a monophyletic group (Chapter 2) of closely related birds that are variable in their degree of morphological differentiation among islands (Amadon 1950). Some taxa even show ecological differentiation within islands (Kern and van Riper 1984). In addition, honeycreeper populations vary greatly in census size and distribution patterns both laterally and elevationally (Scott et al. 1986). A hierarchical approach, one that assesses genetic variability at several scales, has the potential to reveal much about the evolutionary dynamics of the Hawaiian honeycreepers, especially when coupled with morphological data for the same populations. Adding a phylogenetic perspective to these studies has the potential of revealing patterns of island colonization, divergence, or hybridization that might be common among taxa.

Single Location Study at Hakalau

The single-location analysis was conducted on birds living at the Pua Akala tract of the Hakalau Forest National Wildlife Refuge on the Island of Hawaii (Figure 3.1). This

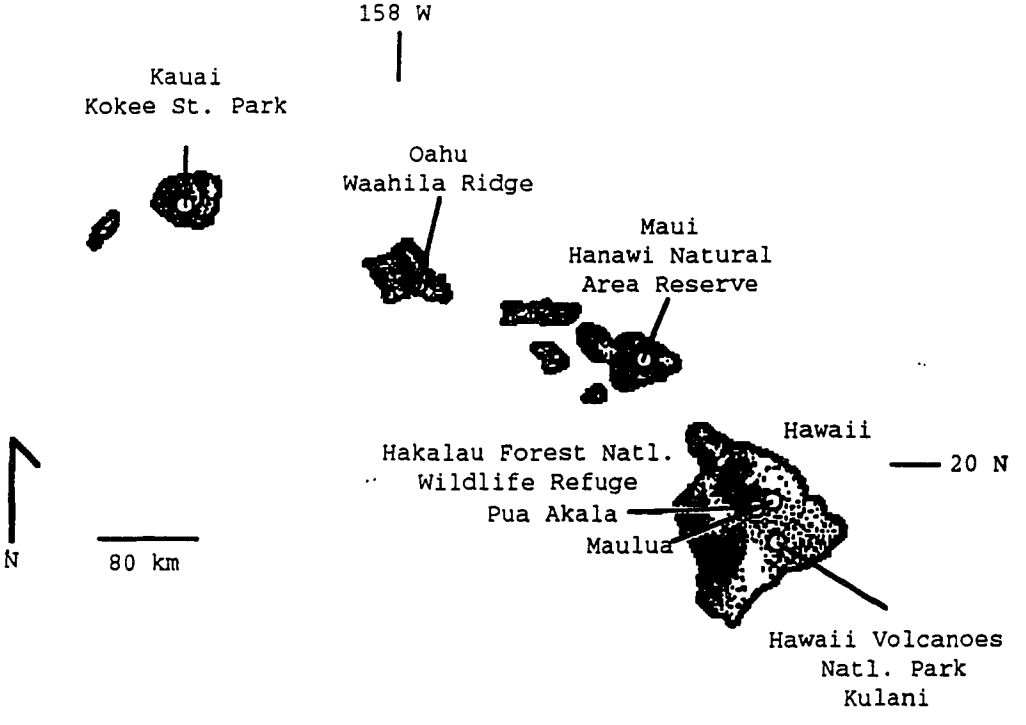

Figure 3.1. Sampling locations.

location, at 1900 m elevation on the east slope of Mauna Kea, is home to the largest remaining assemblage of Hawaiian honeycreepers (6 species) on the Island of Hawaii (Scott et al. 1986). The Pua Akala site is at the top of the Hamakua forest that encircles the eastern third of Mauna Kea from 300m to 2000m elevation.

The Pua Akala location, in the Hamakua study site of Scott et al. (1986), is an excellent location to examine the genetic effect of habitat fragmentation and dynamic decline on small populations. The six honeycreeper species that occur at Pua Akala vary in population size and in distribution pattern on Mauna Kea and elsewhere on the island of Hawaii. Pua Akala is the center of distribution for three endangered species on Mauna Kea, the Akiapolaau (*Hemignathus wilsoni*), Hawaii Creeper (*Oreomystis mana*), and Akepa (*Loxops coccineus*) (Scott et al. 1986). Population densities of these species decrease sharply away from Pua Akala both elevationally and laterally within elevational contours (Scott et al. 1986). Estimated census sizes of these species in the Hamakua district are 900 (+/- 200) for the Akiapolaau, 10,000 (+/- 1600) for the Hawaii Creeper, and 7,900 (+/- 1,800) for the Akepa (Scott et al. 1986). Additional population centers are on Mauna Loa and Hualalai, and are separated from Pua Akala by distances of 25 and 20 km respectively (Scott et al. 1986).

Pua Akala is thus an isolated location of a recently fragmented distribution. The decline surrounding Pua Akala is less than 100 years old and is thought to be related to disease and habitat degradation including deforestation and the introduction of feral ungulates (Warner 1968, van Riper et al. 1986, Scott et al. 1986). The destruction of bird habitat has severely reduced the ranges of the endangered honeycreepers. On the Island of Hawaii, the Akiapolaau now occupies only 7.5% of its original range, the Akepa only 9.7%, and the Hawaii Creeper only 14.8% (Scott et al. 1986).

In contrast, three other species of Hawaiian honeycreepers, the Apapane (*Himatione sanguinea*), the Iiwi (*Vestiaria coccinea*), and the Common Amakihi (*Hemignathus virens*) have higher population sizes in the Hamakua study site and do not show the same steep declines away from Pua Akala tract which are manifested by the endangered species. Within the Hamakua district there are an estimated 174,000 (+/- 2200) Common Amakihi and 299,200 (+/- 10,560) Iiwi (Scott et al. 1986). The 1,080,000 (+/- 25,000) Apapane on the Island of Hawaii form a continuous distribution band from Hamakua through Puna and Kau to Kona (Scott et al. 1986). The Hamakua population is also contiguous with populations on Mauna Loa, but the ranges of these species are only 30 - 40 % of their estimated ranges prior to Western contact (Scott et al. 1986).

Populations of the endangered Akiapolaau, Hawaii Creeper, and Akepa at Pua Akala were examined for genetic evidence of the influence of decline in population numbers and habitat fragmentation around them in relation to the other honeycreepers. Smaller scale, single-location studies were also undertaken on birds at Hanawi Natural Area Reserve on Maui and the Na Pali Kona Forest Reserve on Kauai. Maui species included the Maui Creeper (*Paroreomyza montana*) and the Akohekohe (*Palmeria dolei*). From Kauai, small populations of the Kauai Amakihi (*H. v. stejnegeri*) and the Anianiau (*H. parva*) were also sampled.

Multiple Locations on the Island of Hawaii

The Maulua Tract of the Hakalau Forest National Wildlife Refuge, 13 Km away from the Pua Akala tract was also studied. The Maulua tract of Hakalau, is a high density area for Common Amakihi, Iiwi, and Apapane, but Hawaii Creeper and Akiapolaau are rare and the Akepa is absent (Scott et al. 1986). This comparison was useful for examining a geographically close population on the same volcano for the presence of novel haplotypes and to assess potential effects of gene flow or genetic drift.

For the multiple location, single island comparison, I concentrated on the Common Amakihi because of the potential for finding greater mitochondrial variability in these birds

than in the other species of honeycreepers. This was based on the findings that the Common Amakihi occurs at lower elevations and inhabits a wider range of habitats than do the other species of honeycreepers (Scott et al. 1986). A diversity of mtDNA RFLP haplotypes have also been reported for this species (Tarr and Fleischer 1994). In addition, populations of Common Amakihi sampled from different sites on the Island of Hawaii have shown potential genetically-based differences in response to induced malaria challenge (van Riper et al. 1986) and in nest building behaviors (Kern and van Riper 1984).

An expanded single island comparison was made for populations of Common Amakihi inhabiting the two major volcanoes, Mauna Kea and Mauna Loa, on the Island of Hawaii. This study was relevant for examining the possibility of genetic isolation between populations of Common Amakihi living on the different volcanoes. This potential isolation is based on the older age of Mauna Kea and the possibility that older, more divergent populations of Common Amakihi living there. The multiple location, single island study deals with population structure and potential gene flow at a larger scale than the Pua Akala - Maulua study.

Multiple Island Comparison of mtDNA Diversity

A multiple island comparison of intraspecific mtDNA diversity has the potential to reveal inter-island

phylogenetic relationships, historical patterns of movements between the islands, and colonization directions. A multiple island study was conducted on three species of honeycreepers that are relatively abundant and widely distributed: the Amakihi, Iiwi, and Apapane.

These three species vary in degree of interisland morphological and behavioral differentiation (Amadon 1950, Freed et al. 1987) and on this basis might be expected to differ in degree of interisland mitochondrial variation as well. The Apapane and the Iiwi are monomorphic and behaviorally similar across all of the main Hawaiian islands (Amadon 1950, Pratt et al. 1987) while the Amakihi varies morphologically and behaviorally between islands and even within the island of Hawaii (Kern and van Riper 1984).

The interisland differentiation of the Amakihi is reflected in their current classification as subspecies from Hawaii (*H. v. virens*), Maui (*H. v. wilsoni*), Oahu (*H. v. chloris*), and Kauai (*H. v. stejnegeri*) (AOU 1983). The suggestion has been made that the Kauai Amakihi should be classified as a separate species (Pratt 1989). The genetic correlate of this classification for the Amakihis was addressed by a phylogenetic study of individuals from these islands. In this chapter, the name "Common Amakihi" will be used to refer to *H. v. virens*, *H. v. wilsoni* and *H. v. chloris*. The name "Kauai Amakihi" will be used to refer to *H. v. stejnegeri*.

Methods

Sampling of Species and Locations

Birds were caught in pole-based and aerial mist nets between December 1988 and May 1993 on the islands of Hawaii (Hakalau Forest National Wildlife Refuge; Pua Akala and Maulua tracts, and Volcanoes National Park; Kulani), Maui (Hanawi Natural Area Reserve), Kauai (Na Pali Kona Forest Reserve), and Oahu (Waahila Ridge) (Figure 3.1). A total of 99 individuals were sampled including 15 Apapane of which 11 were from Hawaii, 2 from Maui, and 2 from Kauai; 15 Iiwi, including 11 from Hawaii, 2 from Maui, and 1 from Kauai; 45 Amakihi including 16 from Pua Akala, 4 from Maulua, 5 from Kulani, 14 from Maui, 5 from Kauai, and 1 from Oahu. Endangered species sampled from a single location at Pua Akala included 7 Hawaii Akepa, 4 Hawaii Creeper, and 2 Akiapolaau. Species sampled from a single location at Hanawi, included 2 Akohekohe (*Palmeria dolei*), 4 Maui Creeper (*Paroreomyza montana*), and 1 Maui Parrotbill (*Psuedonestor xanthophrys*). From Kauai, 3 Anianiau (*Hemignathus parva*) and 1 Kauai Creeper (*Oreomystis bairdi*) were sampled.

A phylogenetic study of the honeycreepers using a 790 bp fragment of the mitochondrial cytochrome b (cyt b) gene indicated that the honeycreepers are monophyletic (Chapter 2). In that study, there was no interspecific heterogeneity

of sequences detected, i.e., all of the sequences sorted with one particular species or taxonomic unit although some intraspecific sequence differences were noticed for the Common Amakihi, Apapane, and Hawaii Creeper. From the results of the phylogenetic study, I decided that the cyt b gene might be useful for detecting population genetic structure within these species. Cyt b DNA sequence has proven useful for detecting intraspecific population variability in other groups of passerine birds (Edwards and Wilson 1990, Johnson and Cicero 1991, Taberlet et al. 1992).

PCR and DNA Sequencing

Blood samples (50-100 µl) were taken by brachial vein puncture with a 26 gauge syringe needle, using sterile techniques from mist-netted birds, which were later released after being banded. Total DNA was extracted after the method of Quinn and White (1987). A 307 bp fragment of the mitochondrial cyt b gene was amplified through PCR using conserved primers #006 (L-strand, chicken #14991): 5'-AAA AAG CTT CCA TCC AAC ATC TCA GCA TGA TGA AA-3' and #005 (H-strand, chicken #15297): 5'-AAA CTG CAG CCC CTC AGA ATG ATA TTT GTC CTC A-3' (Kocher et al. 1989, Desjardins and Morais 1990). The primer #006 was biotinylated and used for separation by magnetic beads and subsequent single-stranded sequencing. For double strand PCR, 2 µl of a 1:10 dilution of the DNA

extracted from bird blood was added to 5 µl of 10X PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.01% gelatin and 2 mg/ml BSA), 5 µl each primer (10 mM), 8 µl dNTPs (1.25 mM each) and 0.25 µl (1.25 units) of Taq DNA polymerase (Perkin-Elmer Cetus/ABI) in a total volume of 50 µl. The reactants were overlain with mineral oil and subjected to 25 cycles of PCR at 92°C, 40 seconds, 48°C, 2 minutes, 72°C, 45 seconds followed by an extension cycle of 10 minutes at 72°C in a Perkin-Elmer Cetus DNA Thermal Cycler. A 5 µl aliquot was electrophoresed on a 2% agarose 1X Tris Borate EDTA (TBE) gel, stained in ethidium bromide, and visualized under UV light.

DNA from successful amplifications was sequenced by the biotin solid-phase method using magnetic beads (Dynal Corp.). The magnetic beads were prepared for binding to the biotin labeled DNA by washing 20 µl of the beads twice in 20 µl Binding and Wash (BW) buffer (2X = 10 mM Tris-HCl pH 7.5, 1.0 mM EDTA, 2.0 M NaCl). For both washes, the beads were vortexed 5 seconds, collected on a magnet, and the BW buffer removed. After the second wash, the beads were centrifuged and resuspended in 45 µl BW buffer. The DNA was bound to the beads by adding 45 µl of the PCR and slowly rotating it on a mixer for 30 minutes. The immobilized DNA was collected by placing the tube in a magnet and removing the supernatant. The bound DNA was washed once with 45 µl BW buffer. The bound

double stranded DNA duplex was denatured by adding 8 µl of fresh 0.1 M NaOH and incubating 10 minutes at room temperature. The tubes were placed back in the magnet and the supernatant (containing the NaOH and the non-biotinylated single strands) removed. The stripped strand was saved but usually not sequenced because yield was routinely too low to give a strong signal on sequencing gels. The bound, denatured single strands were washed once with 50 µl 0.1 M NaOH, once with 40 µl BW buffer and once with 50 µl TE. Eight µl of the bound DNA was removed and sequenced by the Sequenase method (USB Corp.) priming with the non-biotinylated primer (#005) used in the PCR. The sequencing reaction products were electrophoresed on a 6% acrylamide, 7 M Urea sequencing gel, fixed, dried and exposed to Fuji RX X-ray film for 18 hours. For all the taxa in this study a 250 bp contiguous portion of the cyt b gene was used. Only unambiguous sequence reads are reported.

Population Structure Analysis

Single Location Comparison

It was initially assumed that birds caught at Pua Akala represented a single breeding population. The distribution of individuals by haplotype within species was examined to indicate degree of mitochondrial diversity in these

populations. The number of nucleotide differences between haplotypes was assessed to provide a measure of the relative degree of lineage diversity within the populations. Lineage diversity among species was also compared by examining the relationship between number of haplotypes detected and number of individuals sampled.

Multiple Location Analysis

Population structure analyses for the Common Amakihi and Apapane were calculated for the aligned cyt b mtDNA sequences using the methods of Hudson et al. (1992) and the program "Heap Big Alignment" (Palumbi pers. comm.). This method calculates,

$$Fst = 1- Hw/Hb,$$

where Hw is mean number of differences between different sequences sampled from the same subpopulation, and Hb is the mean number of differences between sequences sampled from two different subpopulations (Hudson et al. 1992). From the Fst equation, Nm can be obtained by the equation,

$$Nm = 1/2(1/Fst-1),$$

where N is the number of individuals in each subpopulation and m is the fraction of migrants in each subpopulation in each generation (Wright 1951). These equations are appropriate for maternally transmitted haploid mtDNA where N

is the effective number of females in each generation (Hudson et al. 1992).

The Amakihi were analyzed for mitochondrial population structure at three scales; 1) as 3 demes consisting of the Islands of Hawaii, Maui, and Kauai, 2) as 2 demes consisting of the Islands of Maui and Hawaii, and 3) a single island (Hawaii) population consisting of three demes (Pua Akala, Maulua, and Kulani). The Apapane were treated as 3 demes consisting of the Islands of Hawaii, Maui, and Kauai. The Iiwi were considered as three demes consisting of the Islands of Hawaii, Maui, and Kauai.

For the Amakihi and Apapane, distributions of pairwise differences between haplotypes were calculated and tested against a Poisson distribution. The goodness of fit of these distributions to a Poisson was analyzed using the Kolmogorov-Schmirnoff (KS) one parameter test (Sokal and Rohlf 1981). A distibution of pairwise differences that is similar to a Poisson can be an indication that the population is an exponentially growing one (Rogers and Harpending 1992). However, this observation is also consistent with other models of population growth dynamics, such as recent demographic events that have forced coalescent events into a narrow time frame (Slatkin and Hudson 1991). It was therefore useful to do this test for the honeycreeper populations, since most if not all of them have had their

population sizes drastically reduced in historical times (Scott et al. 1986).

The Amakihi populations were analyzed 5 times, once for Hawaii, Maui, and Kauai together, once each for Maui, Hawaii, and Kauai separately and once by combining the Maui and Hawaii populations. Distribution of pairwise differences for the Apapane were calculated once treating all the islands together as a single population.

Phylogenetic Analysis of mtDNA Cyt b Sequences

A phylogenetic analysis of mtDNA lineages was done for the Amakihi to examine interisland allelic relationships and attempt to reconstruct patterns of colonization from allelic genealogies (Takahata 1992). Members of the genus *Hemignathus* (the Hawaii, Maui, Oahu, and Kauai Amakihi, one Akiapolaau, and one Anianiau) were analyzed for phylogenetic structure using distance (Neighbor Joining) (Saitou and Nei 1987) and parsimony (PAUP) (Swofford 1991) methods. The non-amakihi hemignathine species (the Akiapolaau and Anianiau) were included as outgroups for the analysis. These phylogenetic analyses were not done on the other species sampled because the numbers of individuals were too small and the number of nucleotide differences too low to make meaningful phylogenetic trees.

For the *Hemignathus* individuals, sequences were read, aligned by eye, and entered into the computer program MacClade (Maddison and Maddison 1992) for sorting and ordering. A sequence file was saved using the PHYLIP exporting option in MacClade. For the Neighbor Joining analysis, a sequence file was bootstrapped 400 times using the Seqboot program in Phylip (Felsenstein 1989) and then distances were calculated using the program DNAdist. For tree construction, transition to transversion ratios were first set equal and then weighted 3 transitions to 1 transversion. The Anianiau was designated as the outgroup taxon. In the parsimony analysis, a heuristic search was done using the tree bisection-reconnection (TBR) swapping algorthim weighting transitions over transversions by 3 to 1 and by equal weighting. A consensus tree was found from the 27 shortest trees using the strict, majority rule (50%) option, and bootstrapped 400 times using the heuristic method.

Results

There were a total of 42 variable sites for the 99 individuals sequenced for 250 bp of cyt b. Of the 42 sites, 36 occurred at third codon positions, 6 at first, and none at second (Figure 3.2). A total of 31 unique haplotypes were detected. All the species contained unique sets of haplotypes not shared with members of other species, as predicted by the phylogenetic analysis from chapter 2. The overall transition to transversion ratio for the 99 individuals was 2.6 to 1 (31 to 12).

For the 45 Amakihi, 17 variable sites were found, of which 13 were transitions and 4 were transversions (TS to TV ratio = 3.25 to 1). Sixteen of 17 substitutions were at third positions. The group of 45 Amakihi from Hawaii, Maui, Kauai, and Oahu contained 15 different haplotypes.

There were 3 sites within intraspecific comparisons where nucleotide changes caused amino acid replacements. All the other intraspecific changes were silent. The replacement changes occurred in #518 Apapane (position #15091, where a G to A transition caused a Valine to Methionine amino acid replacement), #582 Common Amakihi (position #15222, a C to A transversion caused an Asparagine to a Lysine amino acid replacement), and in M63 Maui Creeper (position #15049, a G to C transversion caused an Alanine to a Lysine amino acid replacement). The intraspecific nucleotide changes in #518

				1111111111111111111111111111111111111111	
				5555555555555555555555555555555555555555	
				0000000000000000001111111111111111222222222	
				0001112344688999990011233445678880111123334	
				3692684089917013690817958140510691036727 80	HT
1.	Apapane	Maui	M30	AGTCCATTGGCTCTGACTGCCAACCCCCCCCCCCCCACCCCAC	X
2.	Apapane	Hawaii	69	..................A.....................	L
3.	Apapane	Hawaii	89	..................A.....................	L
4.	Apapane	Hawaii	104	..................A.....................	L
5.	Apapane	Hawaii	112	..................A.T....................	M
6.	Apapane	Hawaii	206	..................A.T....................	M
7.	Apapane	Hawaii	398	..................A.....................	L
8.	Apapane	Hawaii	424	..................A.T....................	M
9.	Apapane	Hawaii	481	..................A.T....................	M
10.	Apapane	Hawaii	506	..................A.....................	L
11.	Apapane	Hawaii	518	..............A...A.T....................	N
12.	Apapane	Hawaii	608	..................A.....................	L
13.	Apapane	Maui	M11	..................A.....................	L
14.	Apapane	Kauai	K19	..................A.....................	L
15.	Apapane	Kauai	K13	..................A.....................	L
16.	Iiwi	Maui	M109	........A........CA.......T.............T	Y
17.	Iiwi	Hawaii	68	........A........CA.......T.............T	Y
18.	Iiwi	Hawaii	74	........A........CA.......T.............T	Y
19.	Iiwi	Hawaii	92	........A........CA.......T.............T	Y
20.	Iiwi	Hawaii	188	........A........CA.......T.............T	Y
21.	Iiwi	Hawaii	464	........A........CA.......T.............T	Y
22.	Iiwi	Hawaii	612	........A........CA.......T.............T	Y
23.	Iiwi	Hawaii	U7	........A........CA.......T.............T	Y
24.	Iiwi	Hawaii	U9	........A........CA.......T.............T	Y
25.	Iiwi	Hawaii	U27	........A........CA.......T.............T	Y
26.	Iiwi	Hawaii	U31	........A........CA.......T.............T	Y
27.	Iiwi	Hawaii	U33	........A........CA.......T.............T	Y
28.	Iiwi	Maui	M157	........A........CA.......T.............T	Y
29.	Iiwi	Maui	M179	........A........CA.......T.............T	Y

Figure 3.2. Cytochrome b DNA sequence.

							HT
					1111111111111111111111111111111111111111		
					5555555555555555555555555555555555555555		
					0000000000000000001111111111111111222222222		
					0001112344688999990011233445678880111123334		
					3692684089917013690817958140510691036727 80		HT
1.	Apapane	Maui	M30		AGTCCATTGGCTCTGACTGCCAACCCCCCCCCCCCCACCCCAC		X
30.	Iiwi	Kauai	K24		........A.........CA.......T.............T		Y
31.	Common	Amakihi	Hawaii	51	...TT...A.........A..........T....A.T.....		F
32.	Common	Amakihi	Hawaii	76	...TT...A.........A..........T....A.T.....		F
33.	Common	Amakihi	Hawaii	516	...TT...A.........A..............A.T.....		G
34.	Common	Amakihi	Hawaii	519	...TT...A.........A..........T....A.T.....		F
35.	Common	Amakihi	Hawaii	522	...TT...A.........A..............A.T.....		G
36.	Common	Amakihi	Hawaii	541	...TT...A.........A..........T....A.T.....		F
37.	Common	Amakihi	Hawaii	544	...TT...A.........A..........T....A.T.....		F
38.	Common	Amakihi	Hawaii	546	...TT...A.........A..............A.T.....		G
39.	Common	Amakihi	Hawaii	578	...TT...A.........A..........T....A.T.....		F
40.	Common	Amakihi	Hawaii	605	...TT...A.........A..............A.T.....		G
41.	Common	Amakihi	Hawaii	444	...TT...A.........A..........T....A.T.....		F
42.	Common	Amakihi	Hawaii	374	...T....A.........A..........T....ACT.....		H
43.	Common	Amakihi	Hawaii	575	...T....A.........A..........T....A.T.....		I
44.	Common	Amakihi	Hawaii	582	...TT...A.........A..........T....A.T.A...		J
45.	Common	Amakihi	Hawaii	623	...TT...A.........AG.........T....A.T.....		K
46.	Common	Amakihi	Hawaii	700	...TT...A.........A..............A.T.....		F
47.	Common	Amakihi	Hawaii	U8	...TT...A.........A..............A.T.....		F
48.	Common	Amakihi	Hawaii	U36	...TT...A.........A..............A.T.....		F
49.	Common	Amakihi	Hawaii	U43	...TT...A.........A..............A.T.....		F
50.	Common	Amakihi	Hawaii	U51	...T....A.......T.A..........T....A.T.....		W
51.	Common	Amakihi	Hawaii	KP401	...TT...A.........A..............A.T.....		F
52.	Common	Amakihi	Hawaii	KP405	...TT...A.........A..............A.T.....		F
53.	Common	Amakihi	Hawaii	KP488	...TT...A.........A..............A.T.....		F
54.	Common	Amakihi	Hawaii	KP515	...TT...A.........A..............A.T.....		F
55.	Common	Amakihi	Hawaii	KP543	...TT...A.........A..............A.T.....		F
56.	Common	Amakihi	Maui	M5	...TT...A.........A..............A.T.....		F
57.	Common	Amakihi	Maui	M6	...T....A.......T.A..........TT...........		O

Figure 3.2 (Continued)

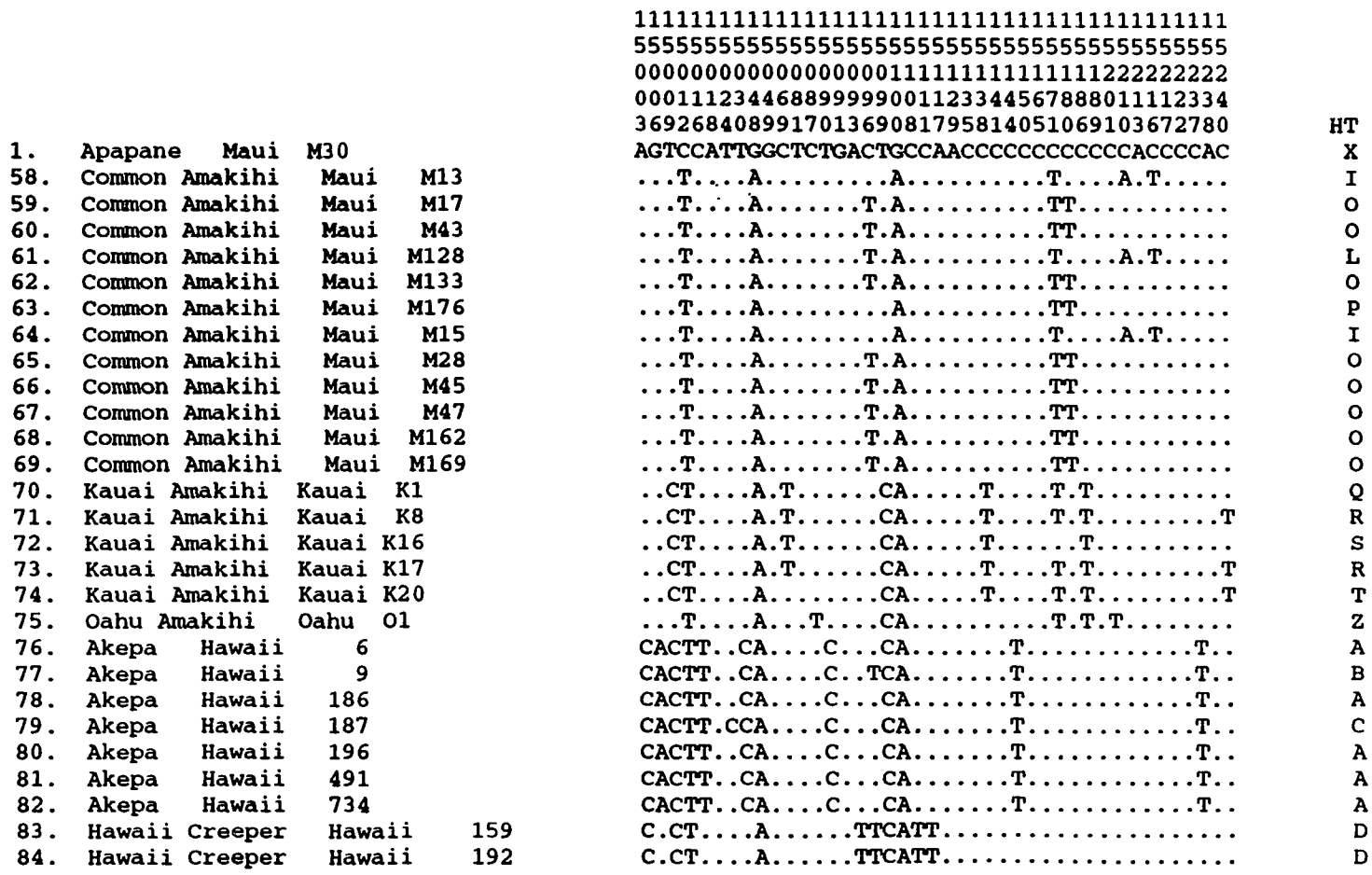

Figure 3.2 (Continued)

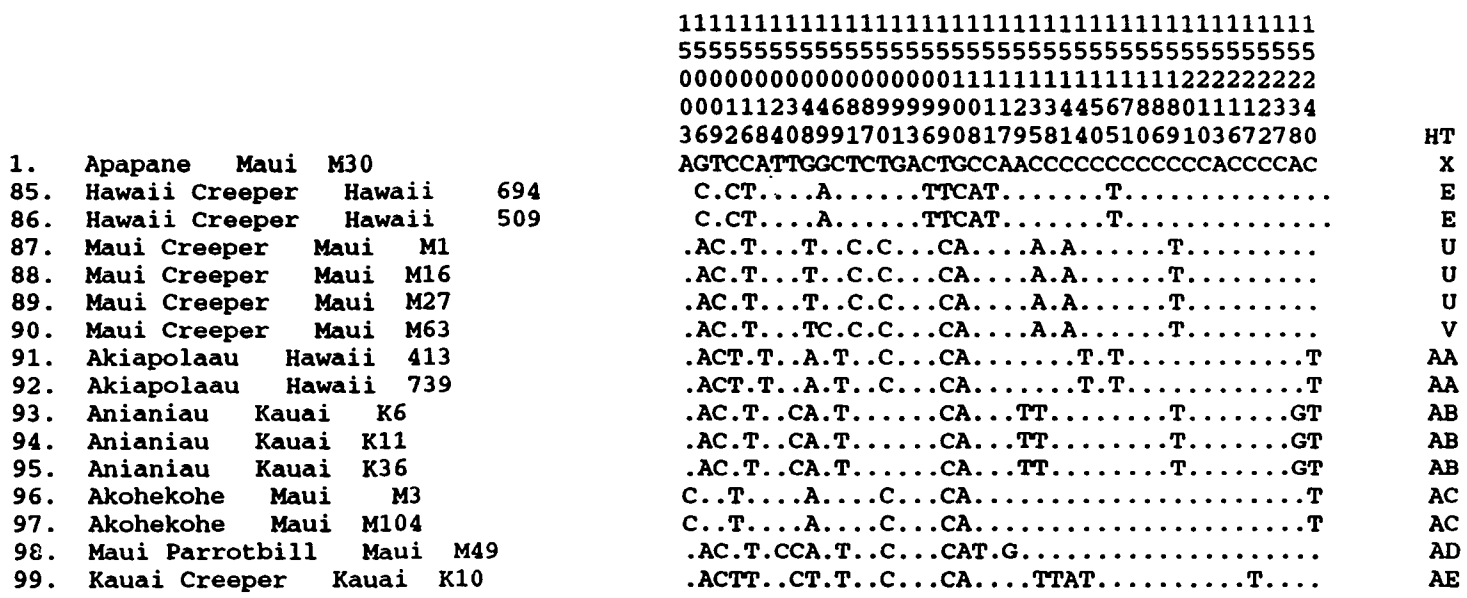

Figure 3. 2. Cytochrome b sequence for the 99 individuals in this study. All individuals were sequenced for 250 bp and the 42 variable sites are given. The numbers at the top identify the nucleotide positions and should be read from the top down, for example the first position is # 15,003 in the chicken mitochondrial genome (Desjardins and Morais 1990). The honeycreepers are aligned to the top sequence for #1 Apapane Maui M30. The haplotypes are designated by the letters to the right of the sequence (HT).

Apapane and #582 Common Amakihi that caused amino acid replacements occurred at 1st positions of codons. The change in M63 Maui Creeper that caused an amino acid replacement occurred at a third position of a codon.

A summary of the sequence results grouped by species is shown in table 3.1. Data are arranged to show sample sizes, substitution ratios, uncorrected percent differences, and number of haplotypes detected. In contrast to the high diversity of haplotypes seen in the Amakihi, a total of 15 Iiwi sequenced from three islands were identical for the nucleotide sites compared. Of the 15 Apapane sequenced from Hawaii, Maui, and Kauai, 4 haplotypes were detected. Among the 4 Maui Creepers, 2 haplotypes were detected, and the 3 Anianiau had a single haplotype. Among the endangered species examined, the 7 Akepa contained 3 haplotypes, the 4 Hawaii Creepers had 2, and the 2 Akiapolaau and 2 Akohekohe each had a single haplotype.

Single Location Analyses

The results of the single site study at Pua Akala indicate that there is variability in the cytb sequences analyzed but that detection of this variability is strongly dependent on the numbers of individuals that were sampled (Figure 3.3). A regression using sample size and number of haplotypes for the Akiapolaau, Hawaii Creeper, Akepa and

Table 3.1

Cytochrome b diversity in Hawaiian honeycreepers. Birds were sampled from the Islands of Hawaii, Maui, Oahu and Kauai and are designated in the table as H, M, O, and K respectively. The total number of haplotypes detected and the number of bases (sites) that were different within each species for the 250 bp of cyt b DNA sequence are given. TS:TV is the number of transitions to transversions. 3-2-1 refers to the positions in the codons where substitutions occurred. The % max difference is the maximum pairwise difference for each intraspecific comparison.

	Apapane	Iiwi	Amakihi	Akepa	Hawaii Creeper	Maui Creeper	Akiapolaau	Anianiau	Akohekohe
# Birds	15	15	45	7	4	4	2	3	2
Islands	H, M, K	H, M, K	H, M, O, K	H	H	M	H	K	M
# Haplotypes	4	1	15	3	2	2	1	1	1
# Sites Different	3	0	17	2	2	1	0	0	0
TS:TV	3:0	-	13:4	2:0	2:0	0:1	-	-	-
3-2-1	1-0-2	-	16-0-1	2-0-0	2-0-0	0-0-1	-	-	-
% Max Difference	1.2	0	6.8	0.8	0.8	0.4	0	0	0

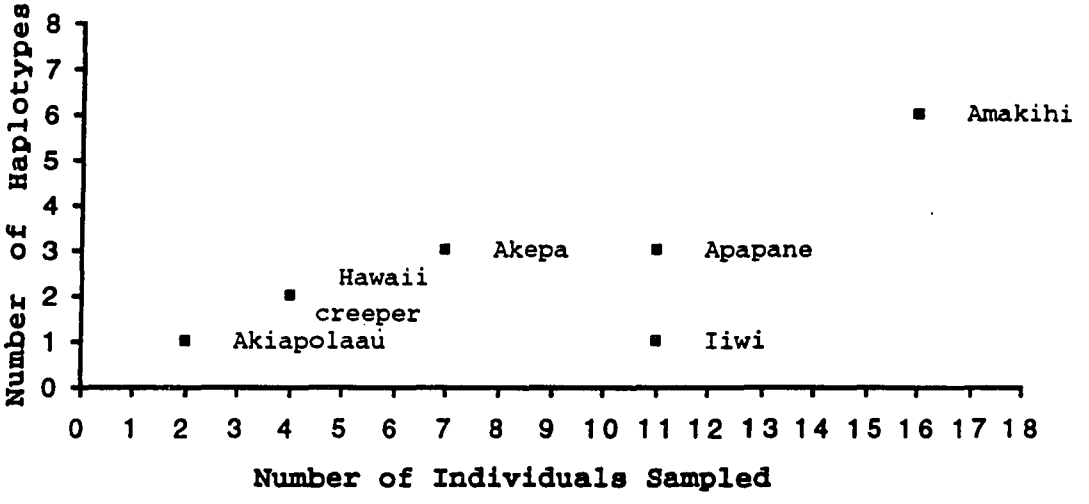

Figure 3.3. Number of individuals sampled versus number of haplotypes detected for Pua Akala birds.

Common Amakihi (the species that are low dispersers) indicates that sample size accounts for variability in number of haplotypes (R^2 = 99.4%, p = 0.002). This relationship disappeared when the Iiwi and Apapane were included (R^2 = 42.0%, p = 0.1012) and might indicate that the Iiwi and Apapane populations are more recently descended from a mitochondrial coalescent than are the other honeycreeper species. The four species showing the increasing number of haplotypes with sample size relationship are less recently evolved than the Iiwi and Apapane, occupying basal or non-terminal branches on the phylogenetic tree (Chapter 2).

Detection of haplotype diversity in the Pua Akala birds sampled is consistent with the pattern of recapture and dispersal for these species (Baldwin 1953). Recapture data indicate great differences in pattern among the Apapane, Iiwi, Common Amakihi, and Akepa (Lepson and Freed, pers. comm.). The Akepa appears to be sedentary and the Common Amakihi are consistent with the general avian pattern of greater dispersal of juveniles than adults (Greenwood and Harvey 1982). Recapture rates of the Iiwi and Apapane have the most variable pattern. Stable Pua Akala adult breeding populations, observed over Lepson and Freed's study period, would require birds dispersing from elsewhere mostly for Apapane, and in decreasing order for Iiwi, Common Amakihi, and Akepa.

Mitochondrial sequence diversity was detected for the Akepa, the Hawaii Creeper, Common Amakihi, and Apapane at Pua Akala. The distibution of individuals at Pua Akala by haplotype is shown in figure 3.4. Three haplotypes were detected for the Akepa and five of the seven individuals sampled share a single haplotype, designated type A. Two other Akepas had unique haplotypes. The four Hawaii Creeper sampled were equally split between the two haplotypes detected, types D and E. The 16 Common Amakihi were distibuted into 6 haplotypes with type F having 8 individuals, type G having 4 and haplotypes H, I, J and K each having a single individual. The Apapane from Pua Akala fell into 3 haplotypes, 3 individuals have type L, 2 have type M and one has type N. Rare haplotypes, i.e., single individuals, become more evident as sample size increases.

The differences between the haplotypes detected at Pua Akala are shown in figure 3.5. Four species had haplotypes that differed by 2 bases, while the Common Amakihi had haplotypes that differed by 3 bases (that species was sampled more extensively than any other). In pairwise comparisons of the three Akepa haplotypes, two, types A and B, differed by a single base and one pair, B and C, differed by 2 bases. The two Hawaii Creeper haplotypes differed by 2 bases. Pairwise comparisons of the 6 Common Amakihi haplotypes showed that 4 pairs differed by a single base, 9 pairs differed by 2 bases and 2 pairs differed by 3 bases. Two of the three Apapane

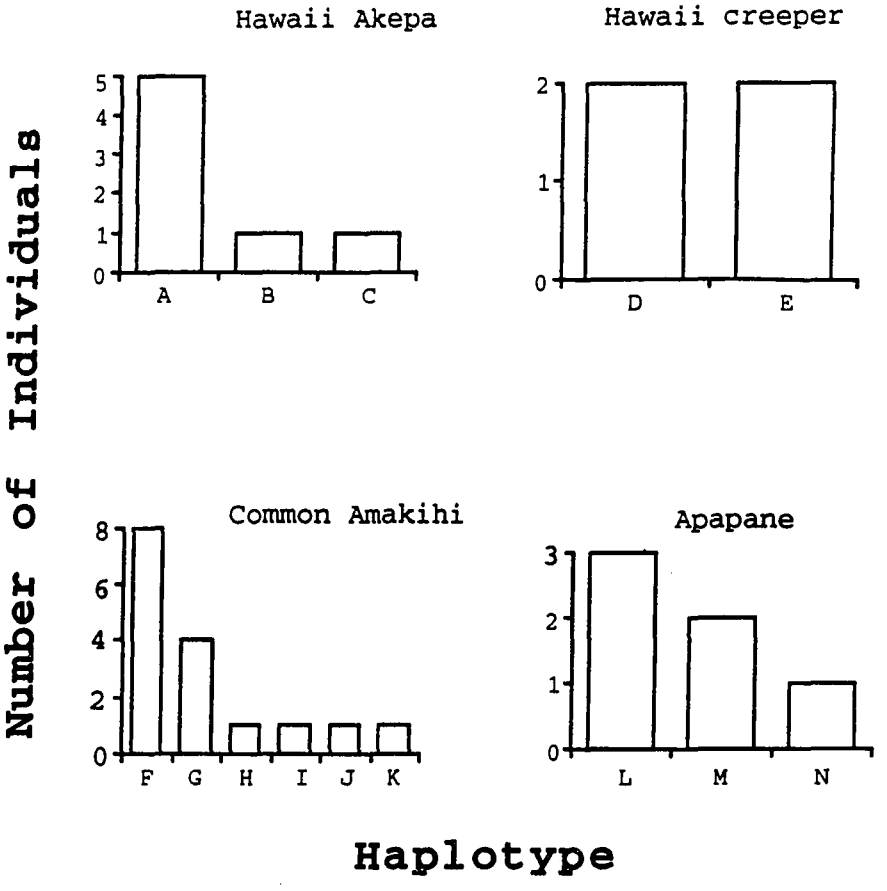

Figure 3.4. Distribution of haplotypes for four populations at Pua Akala. Haplotype identities, designated by letters, are the same as those used in the sequencing figure (Figure 3.2).

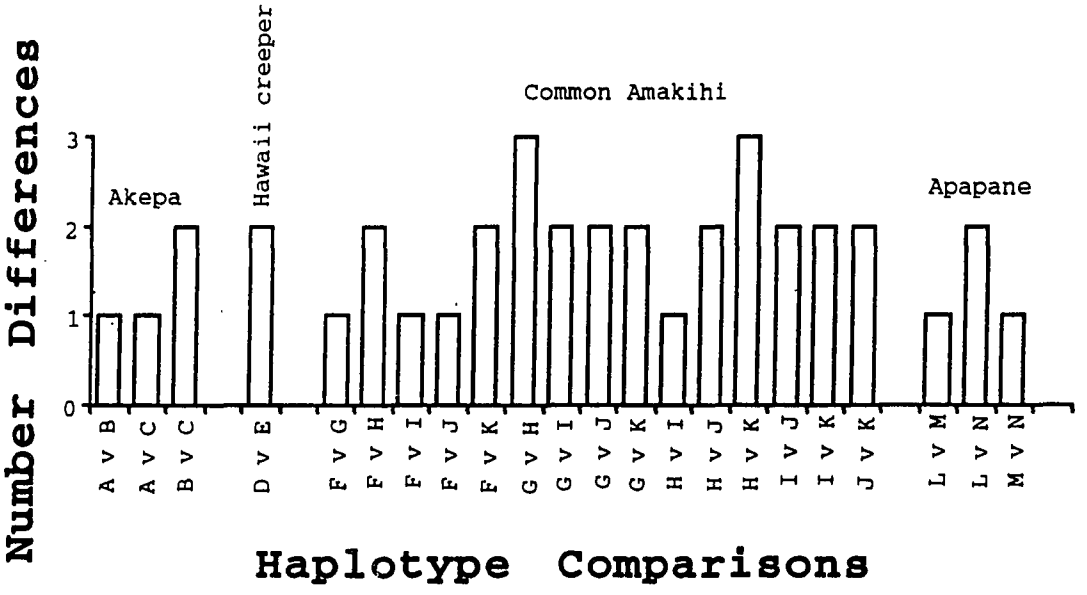

Figure 3.5. Number of differences between haplotypes for the Akepa, Hawaii Creeper, Common Amakihi and Apapane in the single site study at Pua Akala. The letters at the bottom refer to haplotypes in pairwise comparisons against each other and are the same as those shown in figure 3.2.

haplotypes differed by a single base and one pair differed by 2 bases.

Three other populations surveyed from single sites showed mitochondrial variation over the region of cyt b sequenced. These were the Maui Creeper, the Kauai Amakihi, and the Maui Common Amakihi (Figure 3.6). The five Maui Creeper examined fell into 2 haplotypes, 3 individuals in the U type, and 1 in the V type. The Maui Creeper is one of the oldest species of honeycreepers (chapter 2) and might be expected to have more ancient lineages preserved. The 5 Kauai Amakihi fell into 4 haplotypes, Q, R, S and T. The R haplotype had 2 individuals and the other 3, a single individual each. The diversity of haplotypes found for Kauai Amakihi may also reflect the potential great age of the population since Kauai is the oldest of the main islands (Clague et al. 1975). The 14 Maui Common Amakihi comprised 5 haplotypes, F, O, I, L, and P. The O haplotype had 9 individuals, 2 were of the I haplotype and a single individual was in each of the F, L, and P haplotypes. The greater number of haplotypes found for the Maui Common Amakihi compared to the Kauai Amakihi may reflect the greater sample size for the Maui study.

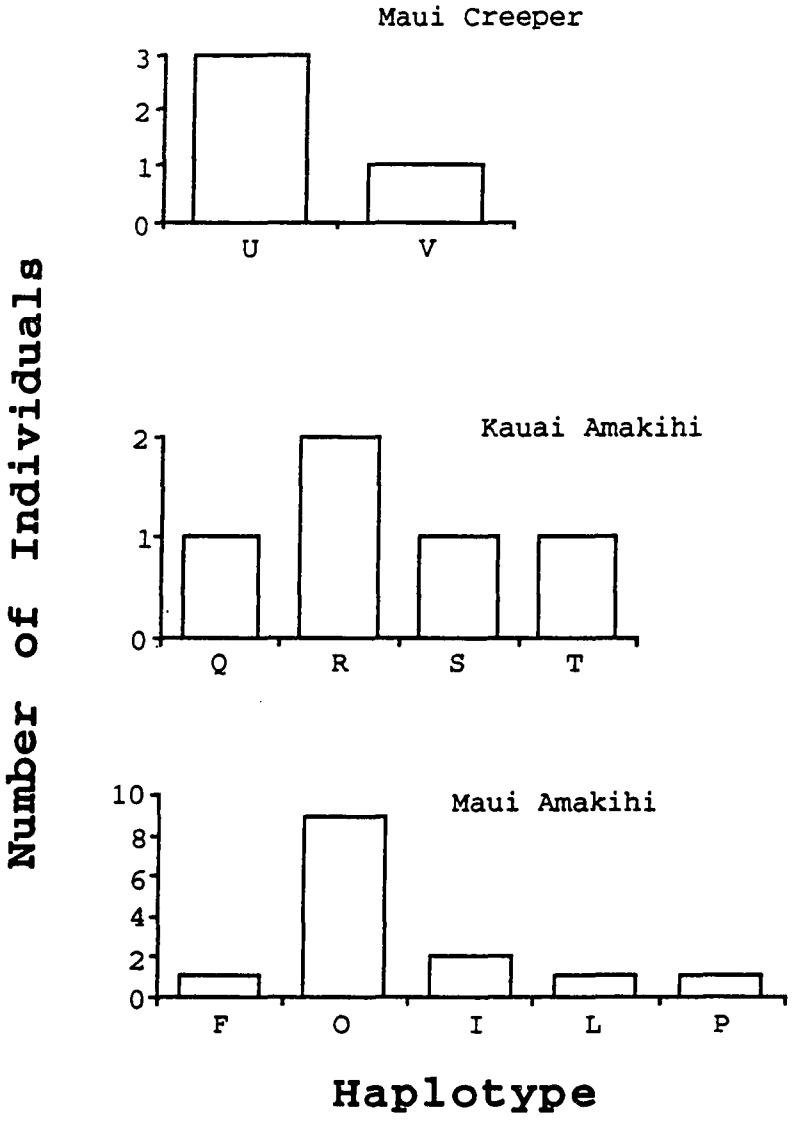

Figure 3.6. Distribution of haplotypes for 4 single site populations of the Maui Creeper, Kauai Amakihi and Maui Amakihi.

Within Island Comparisons

The comparison of Pua Akala and Maulua Common Amakihi resulted in an additional haplotype found at the Maulua site not detected in Common Amakihi from Pua Akala (Figure 3.7). The most common haplotype, F, found for the birds at Maulua (n = 3) was also the the most common haplotype for the Pua Akala birds (n = 8), and the only haplotype found at Kulani. Haplotype G, found for 4 individuals at Pua Akala, was not seen in the Maulua birds nor were any of 4 haplotypes represented by single individuals at Pua Akala. However a new haplotype, W, was found for a Maulua bird and not detected in the Pua Akala birds sampled. This indicates that sampling from populations relatively close to the main Pua Akala population can result in detection of additional mitochondrial haplotypes for the Common Amakihi. This also indicates that the Pua Akala population was not adequately sampled, leading to the prediction that if more individuals had been sequenced, this haplotype found at Maulua would have been detected at Pua Akala too. The variation found in populations of Common Amakihi at Hakalau Forest National Wildlife Refuge was not seen at Kulani, but this again may reflect a bias of small sample size.

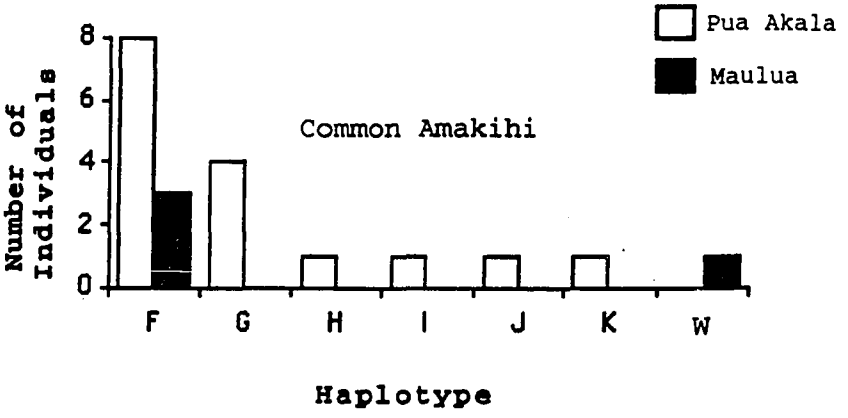

Figure 3.7. Distibution of haplotypes for Common Amakihi from two sites, Pua Akala and Maulua. Most individuals from both sites are haplotype F. One haplotype, W, was detected in a bird from Maulua and not in the Pua Akala population.

Between Island Comparisons

Mitochondrial Population Structure Results

Estimates of Fst and Nm for the Common Amakihi are shown in figures 3.8 - 3.10. Average intrademe nucleotide distance calculations for the 44 Common Amakihi from Hawaii, Maui, and Kauai (Figure 3.8) indicate that the Maui population is the most mitochondrially diverse (0.83% average intrademe nucleotide distance). Kauai (0.56%) contains slightly more intrademe diversity than the Island of Hawaii (0.43%) yet has the smallest sample size. The Hawaii - Maui interdeme distance was 1.7 % or about 1/2 the distance between the Hawaii - Kauai (3.7%) and the Maui - Kauai (3.2%) comparisons. Based on this information alone it would appear that the Kauai population is the most genetically distinct, and the Maui and Hawaii populations are more closely related. The average diversity between demes (2.9%) is much greater than the average diversity within the demes (0.61%) indicating that most of the genetic diversity in the Common Amakihi species at the 3-island scale is partitioned between islands and this is reflected in the high Fst (0.789) and the low Nm (0.134) values.

High levels of differentiation between Common Amakihi populations were still found when the Hawaii and Maui birds were analyzed for population structure (Figure 3.9). The

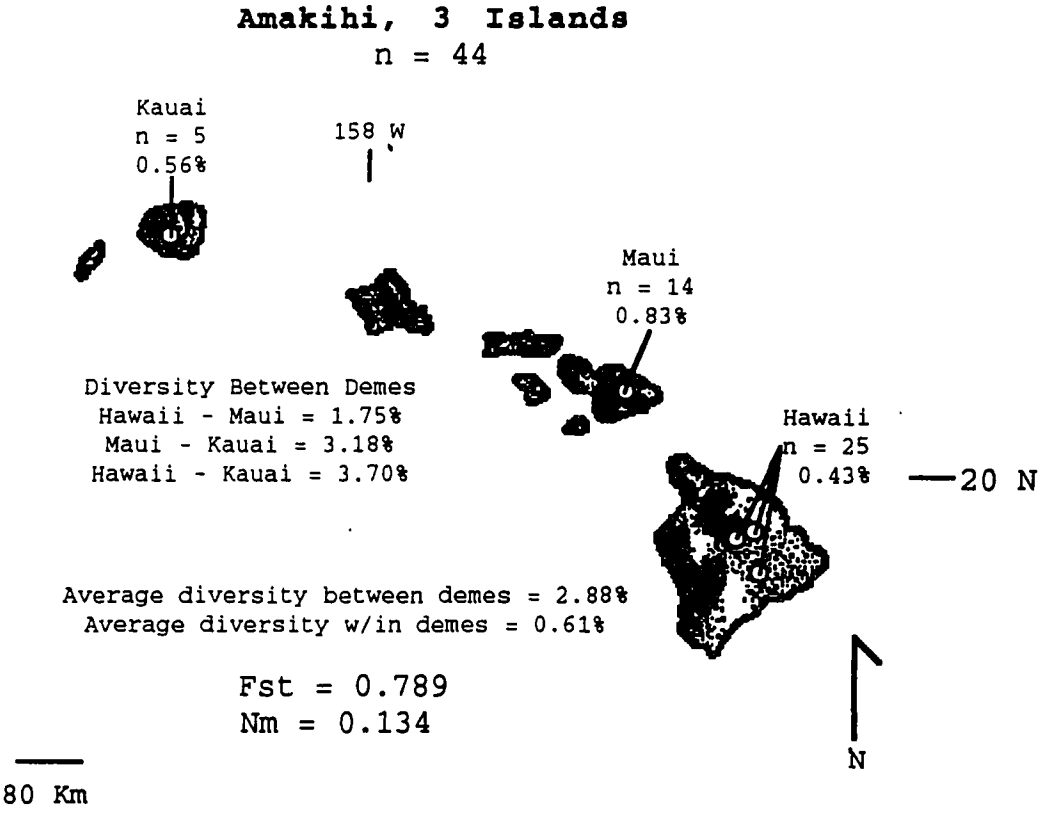

Figure 3.8. Amakihi sampled from 3 islands and analyzed for gene flow by the method of Hudson et al. (1992) using the program "Heap Big Alignment".

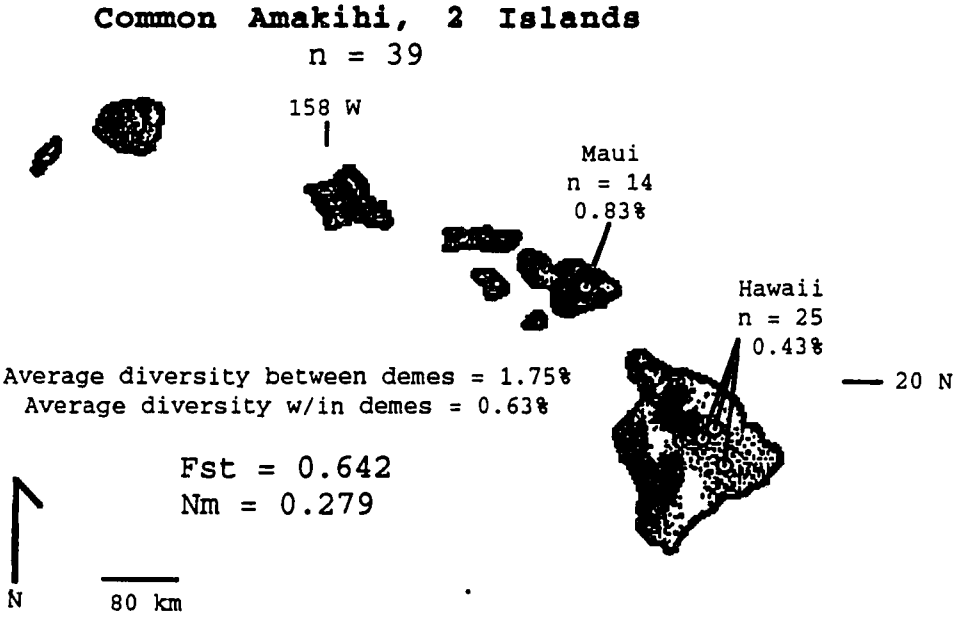

Figure 3.9. Common Amakihi analyzed for mtDNA sequence differentiation between Maui and Hawaii.

average intrademe nucleotide distance for the 14 Maui Common Amakihi was 0.83% and for the 25 Hawaii Common Amakihi was 0.43%. The average diversity between the demes (1.75%) was nearly 3 times the average diversity within the demes (0.63%) and was reflected in a high Fst (0.642) and low Nm (0.279) value. The Fst value for the Hawaii - Maui comparison was lower than that for the three island analysis, suggesting that Common Amakihi are less differentiated at the 2-island scale than they are at the 3-island scale.

Results from the single island comparison for Common Amakihi showed less differentiation among populations compared to analyses on the 2 or 3 islands. Intrademe distances within the Hawaii populations at Pua Akala, Maulua, and Kulani were 0.43%, 0.61% and 0.0% respectively (Figure 10). Interdeme distance calculations for the 3 Hawaii demes showed that the distances of Hawaii populations to each other (0.54%, 0.40%, 0.30%) were less than any other pairwise deme comparison for Common Amakihi. The average diversity between demes for the 3 deme Hawaii Common Amakihi comparison was 0.42% and the average diversity within the demes was 0.35%. The Fst for the 3 deme, Hawaii Common Amakihi comparison is 0.169 and the Nm is 2.453. Thus, the Common Amakihi populations that are closest geographically also have the lowest Fst and highest Nm values. This suggests that the closer the Common Amakihi populations are to one another

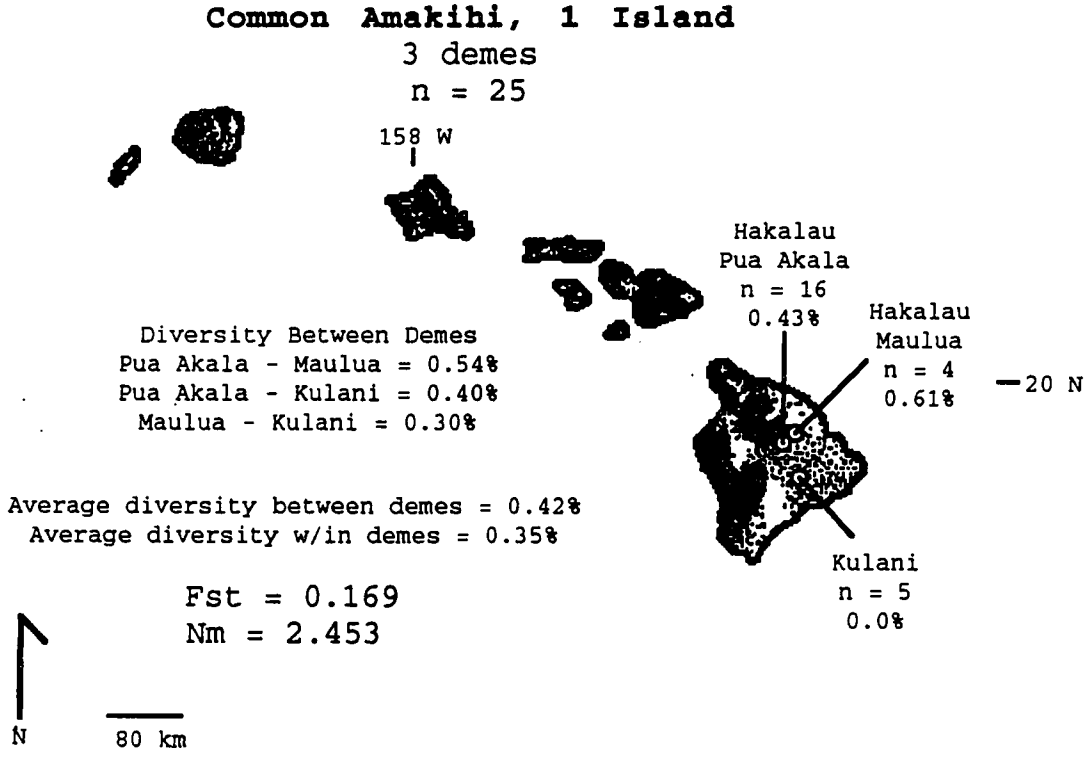

Figure 3.10. Single island analysis of mtDNA sequence differentiation for 3 populations of Common Amakihi on the Island of Hawaii.

geographically, the less genetically differentiated the populations are from one another.

The geographic structure of mitochondrial genetic diversity found for the Common Amakihi was not found for the Apapane and Iiwi. The intrademe distances for 15 Apapane from Hawaii, Maui, and Kauai range from 0.0% for 2 Kauai birds to 0.4% for 2 Maui birds to 0.29% for 11 Hawaii birds. The interdeme distances for the Apapane were 0.20% for the Maui - Kauai comparison, 0.22% for the Hawaii - Kauai comparison, and 0.42% for the Hawaii - Maui comparison. The average diversity between demes of Apapane (0.28%) was close to the within-deme diversity (0.23%) and is reflected in a low Fst value (0.175) and high Nm value (2.358). These values should be viewed as preliminary, however, because of the low numbers of individuals sampled from Kauai and Maui where more haplotypes may be detected with greater effort, or they may reflect comparisons from recently evolved species that have not had sufficient time to diverge.

The Iiwi from Hawaii, Maui, and Kauai analyzed in this study are genetically indistinguishable (Table 3.1) by the cyt b mtDNA sequence used. This may reflect the fact that only a short (250 bp) segment of cyt b mtDNA sequence was analyzed. However, further limited analysis of an adjacent 500 bp fragment of cyt b sequence for Iiwi (n = 4) shows only a single polymorphic site (Feldman, unpublished result).

This indicates that even when more genetic information is used, the Iiwi still appear closely related.

Frequency distributions of pairwise differences were calculated for the Amakihi and Apapane, the two widely dispersed taxa showing intraspecific polymorphism in this survey (Figure 3.11). The joint haplotypes from 44 Amakihi from Hawaii, Maui, and Kauai produced a frequency distribution of pairwise differences with 2 major peaks. A Poisson curve was fit to this distribution and the K-S test applied. The distribution of pairwise differences for the 44 Amakihi from Hawaii, Maui, and Kauai are significantly different from a Poisson (KS $\alpha = 0.01$).

The distribution of pairwise differences for a subset of 39 Hawaii and Maui Common Amakihi was also significantly different from a Poisson (KS $\alpha = 0.01$). The results of the Maui comparison for 14 birds (Figure 3.11) was similar to that for the Hawaii and Maui populations together. The bimodal distribution of pairwise differences for the Hawaii and Maui birds taken together is reflected in the phylogenetic tree structure that indicates two large clades from these islands. The distribution of pairwise differences for the Maui Common Amakihi is significantly different from a Poisson (KS $\alpha = 0.01$) suggesting that the Maui population is not exponentially growing (Rogers and Harpending 1992). In contrast, the distribution of pairwise differences for the Kauai Amakihi, Hawaii Common Amakihi, and

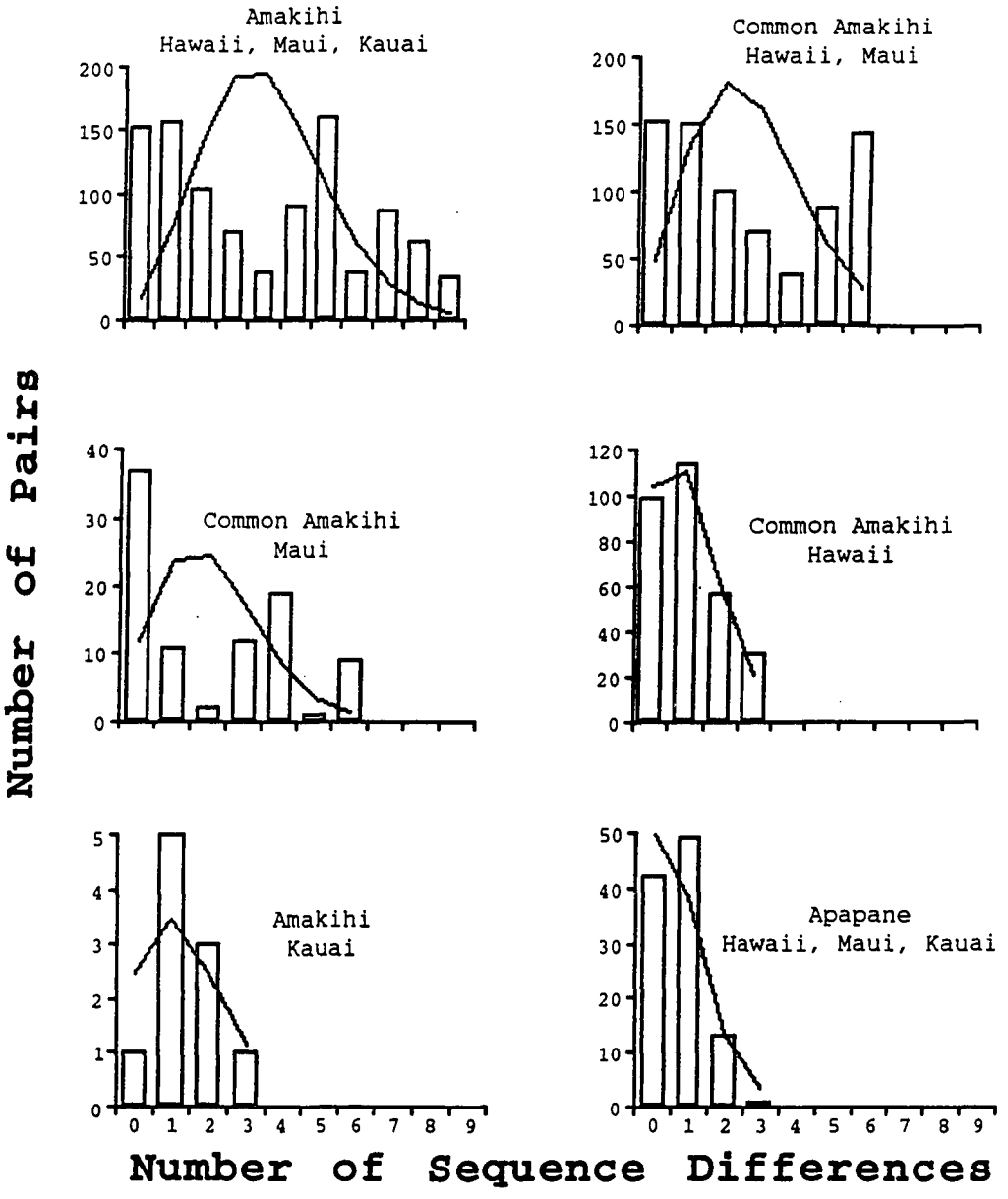

Figure 3.11. Distributions of pairwise nucleotide differences for 6 populations of Hawaiian honeycreepers. Number of differences is indicated by height of the bars and a Poisson curve is fit to each distribution. The Amakihi from Hawaii, Maui and Kauai (top left), Hawaii and Maui (top right) and Maui (middle left) are significantly different from a Poisson (KS a=0.01). The Common Amakihi from Hawaii (middle right), Kauai (bottom left) and the Apapane (bottom right) are not significantly different from a Poisson (KS a= 0.01).

the Apapane from Hawaii, Maui, and Kauai, are not significantly different from a Poisson (Figure 3.12). Since there is no evidence that the populations of Hawaiian birds sampled are growing exponentially, this observation is more consistent with the explanation that distributions of pairwise comparisons can be similar to a Poisson because recent demographic events have forced the population to a coalescent in a narrow time frame (Slatkin and Hudson 1991, Rogers and Harpending 1992).

Inter-island Phylogenetics of the Amakihi

The Neighbor Joining tree for the *Hemignathus* genus (Figure 3.12) indicates that the 5 Kauai Amakihi are phylogenetically distinct and form a unique clade. The single Oahu Common Amakihi is also genetically distinct relative to the Hawaii and Maui Common Amakihi. A cluster of 9 identical Maui birds is distinct from the largest lineage group in the tree composed of 4 Maui and 25 Hawaii birds. No lineages from Kauai and Oahu appear in the Hawaii or Maui clades or vice versa. The PAUP tree (Figure 3.13) unites 10 of the 14 Maui Common Amakihi in a clade and places the 5 Kauai and the single Oahu bird in a basal position. The largest clade in the PAUP tree consists of 4 Maui and 25 Hawaii birds. There was no difference in tree topoplogy when

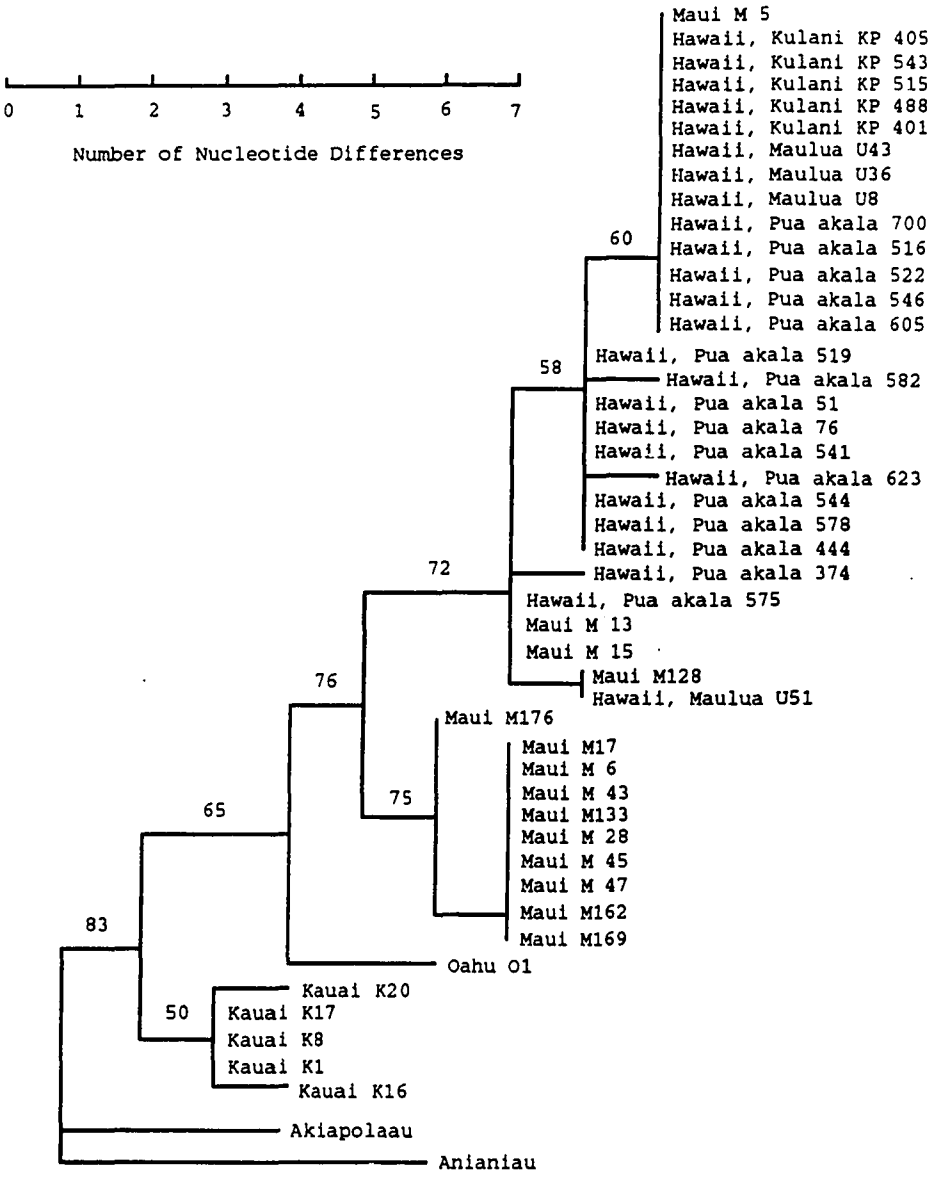

Figure 3.12. Neighbor Joining tree for 47 *Hemignathus* individuals for 250 bp cyt b. With the exception of the Anianiau and the Akiapolaau all the individuals in this tree are Amakihi. The Amakihi sampling island and locations are given in the taxon labels. The tree was bootstrapped 400 times and the percent bootstrap values are given on each node. For this tree transitions were weighted over transversions by 3:1. The Anianiau was designated as the outgroup taxon.

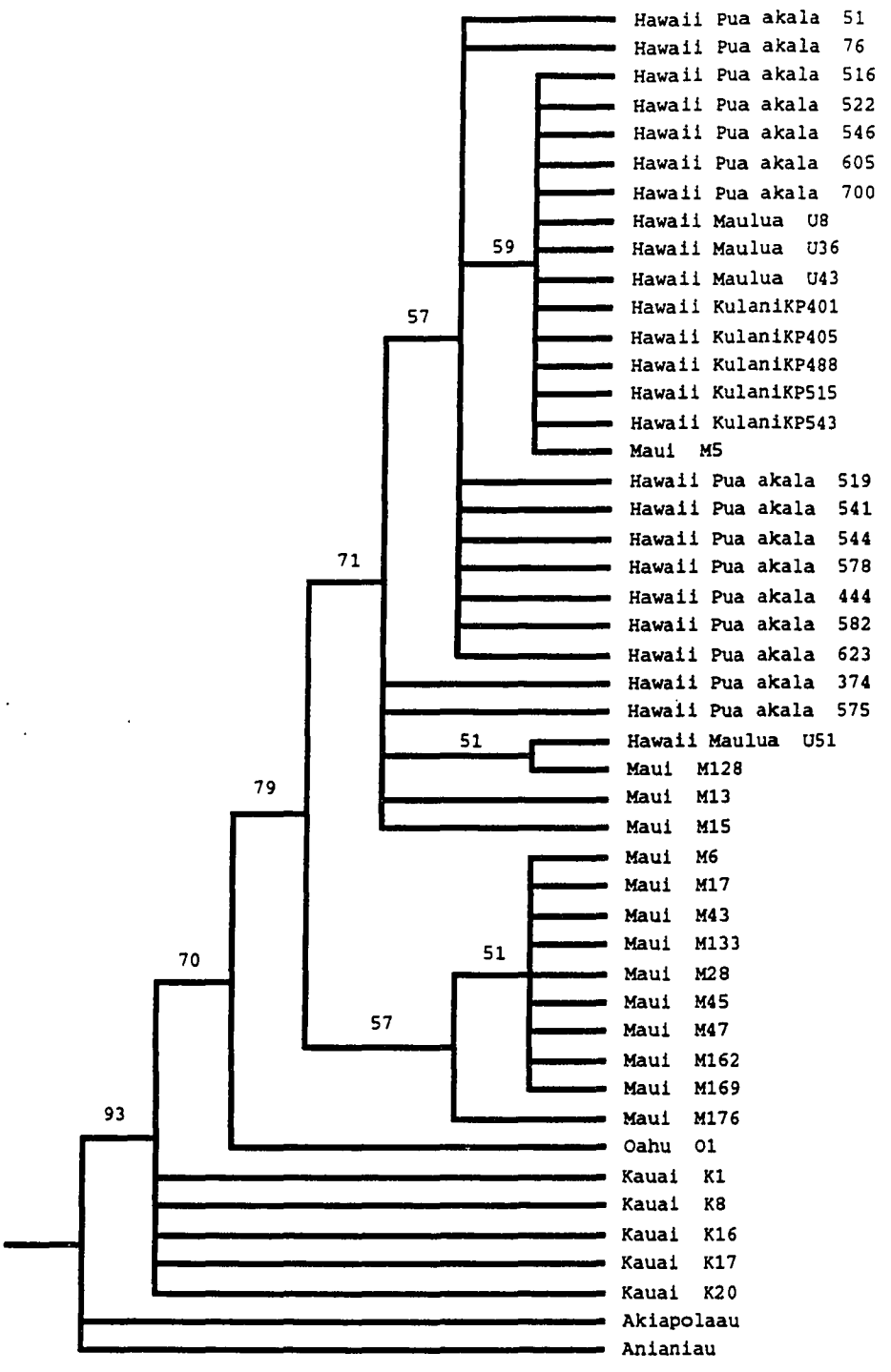

Figure 3.13. PAUP consensus of the 27 shortest trees for 47 *Hemignathus* individuals by heuristic TBR branch swapping. All individuals are Amakihis except the Anianiau and the Akiapolaau. Transitions were weighted over transversions by 3:1. Numerical values are bootstrap frequencies for 400 replications.

the transition to transversion ratios were reset to 1 to 1 or 4 to 1.

Bootstrap values on the Neighbor Joining tree of greater than 50% suggest a founding pattern consistent with movemnet from older islands (Kauai and Oahu) to younger ones (Maui and Hawaii). This pattern is even stronger in the PAUP tree, were the Kauai Amakihi form an undifferentiated cluster of basal lineages, leading to strongly supported nodes from which all other Amakihi lineages radiate.

Discussion

The short segment of cyt b used in this study has revealed a wealth of genetic information on mtDNA diversity in natural populations of Hawaiian honeycreepers. Intraspecific sequence diversity was detected in individuals from single locations, multiple locations on a single island, and on multiple islands. The mitochondrial sequence diversity found in the honeycreeper species has relevance for understanding population substructuring, historical versus current gene flow, and conservation efforts. The implications of findings to these topics are discussed below.

The combination of results from the *Hemignathus* phylogenetic study, the Amakihi mitochondrial population structure analysis, and the distribution of pairwise differences from haplotype analyses indicate that the Kauai Amakihi is genetically distinct from the Hawaii and Maui forms. This finding correlates well with observed high morphological variability of the Amakihi, especially when individuals from Kauai and Oahu are compared to those from Maui and Hawaii (Pratt et al. 1987). Furthermore, the levels of genetic diversity found between the Kauai Amakihi, Oahu, Maui, and Hawaii Common Amakihi correspond well with subspecific taxonomic rank of these birds. The level of interdeme nucleotide diversity reported here between the Kauai - Maui and Kauai - Hawaii Amakihi (> 3%), compared to

the Maui and Hawaii diversity, supports the recommendation that the Kauai Amakihi should be classified as a separate species from the Maui and Hawaii forms (Pratt et al. 1987, Tarr and Fleischer 1994).

This study expands on the RFLP analysis of Tarr and Fleischer (1994). They sampled 35 individuals from Kauai, Oahu, Maui and Hawaii for mitochondrial RFLP variation. Their phylogenetic trees also indicate that the Oahu and Kauai Amakihi are genetically distinct from the Maui and Hawaii taxa. However they found no evidence for shared lineages between island populations in the Amakihi. The finding by DNA sequence of shared haplotypes in Common Amakihi from Maui and Hawaii, not detected by the RFLP study, can probably be attributed to the higher level of resolution of the cyt b DNA sequence study over the RFLP study.

While the high Fst and low Nm values for the Amakihi at the 3 and 2 island scales are consistent with the morphological differentiation between these populations, the shared lineages between the Hawaii and Maui populations indicate the potential for gene flow. It also appears based on the phylogenetic results and the pairwise distribution of differences, that the Maui Common Amakihi population is composed of two groups. One is endemic to Maui and the other is composed of shared lineages between Maui and Hawaii. This may reflect the founding of Hawaii by only a subset of Maui mitochondrial lineages. Although Common Amakihi have high

philopatry, the Maui and Hawaii populations are not yet sufficiently separated geographically or in time to isolate the 2 island populations from one another.

The pattern of island colonization of the Amakihi has been hypothesized as reflecting a series of double invasions (Amadon, Bock 1970). This pattern involves colonization by founders from an older island to a younger one, genetic divergence from the original population by drift and then a second invasion by a new founding population from the original island. The secondary contact leads to competition and character displacement (Tarr and Fleischer 1994). This mechanism of speciation has been proposed as a general one for island birds and applied to discussions of speciation in the Galapagos Finches (Lack 1947, Grant 1981).

The phylogenetic results for the Amakihi from my study are consistent with a pattern of colonization from the older islands to the younger one. The phylogenies suggest that the Kauai birds are older than the Maui birds and that the Maui clade is older than the Hawaii clade. The Hawaii birds appear derived from a Maui population and the Maui birds from Kauai founders. The split in the Maui birds indicates that there is a high level of divergence in this population but this alone is not evidence that one clade was derived from an independent colonization than the other one.

Tarr and Fleischer (1994) produced a mtDNA RFLP tree from their study of 35 Amakihi, but their trees could not

resolve the relationship between Amakihi from the older islands (Kauai and Oahu) and the younger ones (Maui and Hawaii), and thus do not provide further genetic insight to the matter. The issue of double colonization for the Amakihi could be resolved by extensive sampling from different locations on Kauai, Oahu, Maui and Hawaii, coupled with standard morphological data from the same birds.

Comparisons of morphological characters for Common Amakihi between Maui and Hawaii suggest that gene flow between these islands was historical, not current. If haplotypes are found on both islands due to migration, then morphological characters of one island population should occassionally appear in the other. This is not the case, at least with respect to bill size measurements where there is a significant difference in bill lengths between the Maui and Hawaii populations with virtually no overlap between groups (Amadon 1950, S. Fretz [pers. comm.]). At least at the level sampled here, there is no evidence to suggest that gene flow in the Maui and Hawaii Common Amakihi is ongoing.

Low Fst and high Nm value for the Apapane correlates well with observed lack of morphological differentiation of these birds across the main Hawaiian islands, contrasting sharply with the genetic isololation by distance detected in the Amakihi (Figure 3.14). But low numbers of individuals sampled from Kauai and Maui may also generate the results, so

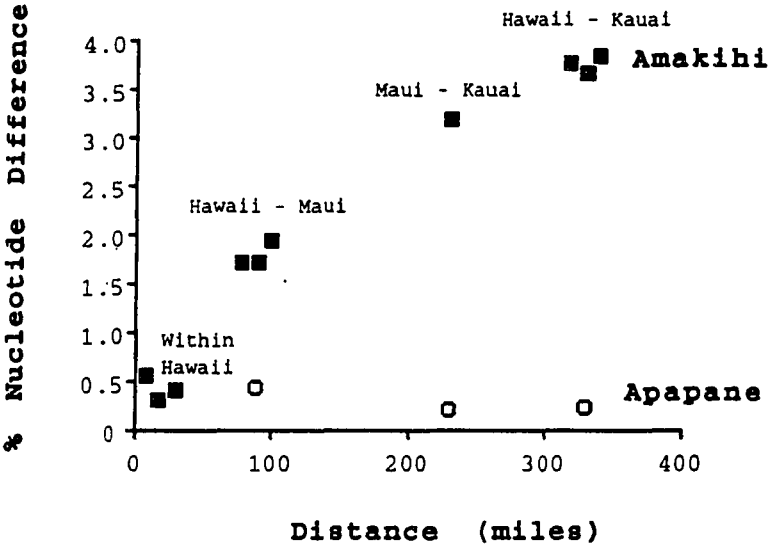

Figure 3.14. Average interdeme per cent nucleotide difference versus geographic distance for the Amakihi and Apapane. The Amakihi were sampled from three populations on Hawaii (Pua Akala, Maulua and Kulani), one on Maui (Hanawi) and one on Kauai (Kokee). The Apapane were sampled from Pua Akala Hawaii, Hanawi Maui, and Kokee Kauai. Genetic distance increases with geographic distance for the Amakihi but not for the Apapane.

the analysis of Apapane should be seen as only a preliminary one.

The Iiwi are genetically indistinguishable using 250 bp cyt b DNA sequence. Because of the low sample size (15 individuals) and short stretch of sequence used, this finding should be taken as a preliminary one, and ecological and evolutionary inferences should be made cautiously. However, this finding may correlate well with the lack of morphological differentiation and recent origin of the group (Chapter 2). The lack of genetic differentiation in the Iiwi also correlates well with observations of high migration and dispersal for the Iiwi (MacMillen and Carpenter 1980).

Results of these studies for the Apapane and the Iiwi may indicate each has a single panmictic population. This may not be the general case, however, and, again, results of this study should be taken with caution until other gene regions have been examined. The lack of detection of mitochondrial genetic diversity in the Iiwi could be the result of using a gene fragment that does not accumulate mutations fast enough to show population differentiation for this species, even though it may exist. For most comparisons involving passerines, the cyt b gene is not useful for resolving relationships less than 1 MY old (Arctander 1991). The use of a faster evolving region, such as the control region, might show the accumulation of population specific

mutations that could be used to define Iiwi (or Apapane) population structure.

A lower level of variation for these species, that might reflect their recent evolution, could exist because they diverged or went through a bottleneck too recently to have differentiated. Analysis of these populations with other loci, either those with higher mutation rates or those undergoing sexual recombination, will be neccessary to choose between these broad alternatives.

It is intriguing to consider the extinct Laysan Apapane (*H. s. freethi*) in terms of the role selection might play in morphological divergence. The Apapane from the main islands are not morphologically different. The Laysan Apapane, in contrast, was smaller and had a dusky, more orange plumage. The morphological variation of the Laysan Apapane relative to the main islands may reflect the fundamentally different ecosystem that the founders encountered and susequent evolution in isolation. The lack of morphological divergence of the main island Apapane may be the result of strong selection for nectarivory in similar ecosystems.

Analysis of pairwise difference distributions from sequence data can indicate past episodes of population growth and reduction (Rogers and Harpending, 1992). A distribution of pairwise differences that fits a Poisson is consistent with the hypothesis that the population has been growing exponentially in size or has been forced through a recent

coalescent (Slatkin and Hudson 1991). The latter explanation appears to be the case for the three honeycreeper populations that show agreement with a Poisson distribution. This observation is also expected to be reflected in a star phylogeny for these populations (Slatkin and Hudson 1991).

The simulations of Rogers and Harpending (1992) also showed that reductions in population size can leave a characteristic signature in the distribution of pairwise differences by forming an L-shaped curve with high values at the left, reflecting recent divergence from a common ancestor and high values in the right tails implying an excess of large pairwise differences. The distributions in figure 3.12 appear to show an L-shaped pattern, i.e., while they are unimodal and Poisson distributed, they have most of their values at the low end of the distribution. They do not, however, have high values at the upper ends of the distributions. This may be attributed to low sample size.

A problem in this study, and a general one in population genetics, is the difficulty to distinguish between current and ongoing gene flow (Slatkin and Maddison 1989). This problem can be addressed by overlaying a phylogeny onto population structure information and inferring the most parsimonious explanation of migration events that could explain the phylogenetic pattern (Edwards 1993).

For the Amakihi, the combination of high population structure between islands and strong phylogenetic separation

of Kauai to Maui and Hawaii and between most of Maui and Hawaii argues for the lack of gene flow between the islands. Under the low, or no, gene flow scenario, maintainance of lineages in Maui that occur in Hawaii may be a relict of a prior larger population and slow evolution of the cyt b gene. It is possible that not enough changes have accumulated in this fragment to show the differentiation in these lineages. The prediction is that a faster evolving region would show changes, indicating that these are really not the same lineages but rather lineages that have diverged more recently than the cyt b substitutions are tracking. If gene flow is truly ongoing between Maui and Hawaii, we would expect that no matter what region, or chronometer was used, we would still detect the same haplotypes occurring at Maui and Hawaii. Detection of these haplotypes would not be a function of the technique used (Avise et al. 1988) but would reflect the true biological situation.

In this study, sample size accounts for the ability to detect haplotypes in all of the hemignathinines. This raises the question of what constitutes a sufficient sample size for documenting population structure. One would expect to see a leveling off in the ability to detect new haplotypes as more individuals were sequenced. This level could be approximated by sequencing more individuals for these species and plotting number of haplotypes versus number of individuals sampled. If the relationship between species holds, one would expect

to see a flattening of this curve, indicating that after a certain number of individuals were sequenced, it becomes less important to sequence more because the ability to detect new haplotypes decreases (Stoneking and Cann 1989). This would indicate the number of individuals that have to be sampled in order to have a certain level of confidence in detecting the rare haplotypes and estimating Nm. However, even greater numbers of individuals would have to be sequenced to estimate the distribution of haplotypes within the population with greater precision.

The results from the species sampled from single populations indicate that mitochondrial genetic diversity exists in populations of the Hawaii Creeper, Hawaii Akepa, Maui Creeper, Apapane, Kauai Amakihi, and Common Amakihi. This finding is especially significant for the endangered Hawaii Creeper and Hawaii Akepa because it indicates that, despite the relatively low census sizes, these two populations at Pua Akala still retain genetic diversity. This may indicate that there has been preservation of older lineages in the Pua Akala Hawaii Creeper and stands as evidence against a potential genetic bottleneck for this species due to low census size (Scott et al. 1986), although a bottleneck could have reduced Hawaii Creeper diversity to only the two lineages detected. The finding of diversity in the short segment of cyt b in the Akepa suggests that it may be a useful mitochondrial marker for studying systematics in

this species (Pratt 1989) and investigating phylogenetic relationships between Akepa from Hawaii, Oahu, and Kauai.

No mitochondrial genetic diversity was discovered for the two Akiapolaau from Hawaii, the three Anianiau, from Kauai, or the two Akohekohe from Maui. However, small numbers of individuals were sampled from these species and only one haplotype is not unexpected if one haplotype predominates in the population. However, the detection of only a single haplotype in the Akiapolaau and Akohekohe sampled may also reflect the current low census sizes of these endangered species and recent population genetic bottlenecks.

The maximum levels of intraspecific percent nucleotide difference found in this study ranged from 0.0% to 6.8%. This level of diversity falls within the range of the maximum between-lineage percent nucleotide difference reported from other avian studies using this region of cyt b (Table 3.2). These results indicate that the short (even less than 300 bp) fragment of cyt b holds considerable information for avian population genetic studies. The ease with which it amplifies and sequences insures that it will become an important tool for future studies of intraspecific avian population genetic structure.

It is difficult to relate the findings of this study to censused population size and life history because the sample

Table 3.2

Intraspecific cytochrome b DNA sequence diversity in bird studies. The honeycreepers fall within the range reported for other bird studies using this region of cyt b.

Species	# Base Pairs Sequenced	% Difference (Maximum or Range)	Reference
Hawaiian Honeycreepers	250	0.0 - 6.8	This study.
Sparrows	288	1	Johnson and Cicero (1990).
Murres	397	2.4 - 6.2	Birt-Friessen et al. (1992).
Babblers	282	0.4 - 2.1	Edwards and Wilson (1990).
Alcids	307	2.4	Friesen et al. (1993).
Dunlins	302	2.6	Wenik et al. (1993).

sizes are too small and colinear. However, the Hawaii Akepa and Hawaii Creeper are on the interspecific line that relates the number of lineages detected with sample size. This indicates that despite strong declines in population density for these species away from Pua Akala, the core population at Pua Akala has not yet been affected to a point that a decline in mitochondrial genetic diversity has occurred. This result might be expected since these are sedentary species with little dispersal. It would be interesting to look at populations around the Pua Akala population, in more disturbed areas, for comparison with this one. These other populations might have been more strongly influenced by what has been happening around them on Mauna Kea and elsewhere on the island. One implication of this finding is that mitochondrial diversity may still exist in a population as greatly reduced as the Hawaiian crow (*Corvus hawaiiensis*) (NRC 1992), now known from only a single location on the Island of Hawaii. Results presented here indicate that even for endangered species, the 250 bp fragment of cyt b is a useful genetic marker for gaining mitochondrial information about honeycreeper population structure.

Summary

The cyt b DNA sequences used in this study provide information on intraspecific population genetic relationships in the Hawaiian honeycreepers. Single site populations at Pua Akala for the Common Amakihi, Apapane, and the endangered Akepa and Hawaii Creeper are mitochondrially diverse, with multiple haplotypes present even in relatively small samples. Diversity was also detected in the single site study of Maui Creeper, Maui Common Amakihi and Kauai Amakihi. Phylogenetic analysis of the Amakihi indicates that this species shows a major mitochondrial genetic break between Kauai and Maui, lending support to the assertion of some systematists (Pratt et al. 1987) that the two are separate species. Two Common Amakihi populations exist on Maui and one of them has maternal genetic lineages that also appear on Hawaii. Bill length data suggest that gene flow was historical and is not ongoing. Thus, populations on different islands have not had sufficient time to experience lineage sorting to separated the populations at the cyt b DNA sequence level. The Oahu Common Amakihi is genetically distinct, and further studies should focus on determining its status as a separate species. The pattern of differentiation for the Amakihi may be a common one for philopatric Hawaiian birds and should be tested by looking at other species that vary morphologically across the islands such as the Akepa, Akialoa, and Nukupuu.

Future progress in the analysis of population genetic diversity now depends on greater sampling efforts at key locations on different islands, as well as exhaustive sampling at a single location on a single island.

Chapter 4

A PCR Test for Avian Malaria in Hawaiian Birds

Introduction

The Hawaiian avifauna has experienced more extinction than has any other major group of island birds (Atkinson 1977, Olson and James 1982, Scott et al. 1988). Initial extinctions, based on fossil evidence (Olson and James 1982), were associated with direct exploitation of birds and alteration of habitat by humans that first arrived around 700 AD (Holt 1985, Hunt and Holsen 1991). A second wave of extinction, of birds known historically, was initiated with the arrival of Europeans in 1778 (Ralph and van Riper 1985). Habitat alteration became more extensive and a great variety of birds and mammals were introduced that were potential competitors and predators of endemic forms. During the last 150 years, 21 of the 59 historically known passerine species and subspecies of Hawaiian birds, mostly the Hawaiian honeycreepers (Drepanidinae), have gone extinct and many others have become endangered (van Riper et al. 1986, Pyle 1990). The extinction and decline between 1850 and 1930 coincided with the introduction of avian poxvirus, malaria, and a suitable vector (Warner 1968, van Riper et al. 1986).

The distribution of surviving native Hawaiian passerines is consistent with the disease model of extinction. These birds, especially the endangered species, persist mainly in forests above 1500 m, including those that are disturbed, despite the presence of more pristine forests at lower elevation (Warner 1968, Scott et al. 1986). The paucity of native birds at low and mid-elevations is associated with the presence of the introduced mosquito, *Culex quinquifasciatus*, a vector of the malarial parasite *Plasmodium* (Warner 1968, van Riper et al. 1986). This model of distributional incompatibility of native birds and introduced mosquitoes is the basis for the establishment of high elevation refuges in Hawaii (Scott et al. 1985).

The susceptibility of native birds to *Plasmodium* is also consistent with the disease model of extinction. In a series of challenge experiments with *Plasmodium*, using standard giemsa stain and light microscopy (Garnham 1966), van Riper et al. (1986) found that native Hawaiian birds tolerated infection and resisted challenge less than introduced Red-Billed Leiothrix (*Leiothrix lutea*), Japanese White-Eye (*Zosterops japonicus*), and Canary (*Serinus canarius*). The greater susceptibility of the Hawaiian birds to malaria is assumed to reflect the evolution of these birds in the absence of this disease and its vectors (Warner 1968, van Riper et al. 1986).

However, the disease model is tempered by evidence of variability within species in response to experimental infection with malaria. Van Riper et al. (1986) found that 40-60% of 3 species of forest birds, Apapane (*Himatione sanguinea*), Iiwi (*Vestiaria coccinea*), and Common Amakihi (*Hemignathus virens v.*) tolerated the challenge (contracted the disease but survived). Individuals from one low elevation population of Common Amakihi even resisted the challenge (no detectable establishment of the disease). Observations of Apapane and Common Amakihi at low elevations are consistent with the experimental findings, but the absence of Iiwi at these elevations is not. No challenges have been performed with native birds that are endangered and restricted to upper elevation forests.

The relevance of the disease model and the indication of variability of susceptibility within species suggest a need for a test that could detect avian malaria at a subclinical level. Such a test would allow for a finer scale analysis of disease prevalence in Hawaiian birds and for delimitation of the range of *Plasmodium* in Hawaiian forests. It would also allow for the development of a rigorous distinction between tolerance and resistance by defining levels of infection lower than that typically found by light microscopy with histological stain. For example, it might be possible to distinguish between tolerance of low levels of infections and the complete clearance of *Plasmodium* from a bird's system. A

test that involves genetic detection of a parasite also allows for the analysis of genetic diversity of *Plasmodium* in Hawaiian birds.

In this study I report the development of a PCR (Polymerase Chain Reaction) based test for avian malaria that is more sensitive than the standard smear method and most DNA blot based tests. The level of sensitivity of the test was quantified by QC-PCR (Quantitative Competitive PCR). I used the test to assess the prevalence of malaria in populations of native and introduced birds in high elevation forests on the islands of Hawaii and Maui that are protected areas for endangered birds.

Methods

The basis for the test is the detection of a *Plasmodium* rDNA fragment, that differs in length from the bird's rDNA, in avian blood samples. *Plasmodium* has an 18S rRNA with a unique insertion of about 200 nucleotides (Gunderson et al. 1987, Neefs et al. 1990, Waters and McCutchan 1989) (Fig. 4.1). This insertion varies both in size and sequence and is a useful marker for distinguishing between *Plasmodium* species (Waters and McCutchan 1989, Snounou et al. 1993). PCR primers were designed to eukaryotic conserved regions (Neefs et al. 1990) spanning the insertion. The length of the DNA fragment, including the primers, is 580 bp from *Plasmodium* and 380 bp from vertebrates. PCR amplified DNA from malaria infected individuals is expected to have two bands, one from *Plasmodium* at about 580 bp and one from the bird at about 380 bp.

Blood samples were taken from birds captured in mist-nets. A small puncture was made in the wing vein with a sterile 26 gauge needle. An average of 50 µl of blood was collected in heparinized microhematocrits. Birds were then banded and released. The sample was quickly transferred to an eppendorf tube filled with 0.5 ml TEN buffer (10 mM Tris, 10 mM NaCl, 2 mM EDTA pH 8.0). This tube was then kept cool for the rest of the day and placed in a propane freezer or liquid nitrogen in the field at night. Thawing occurred

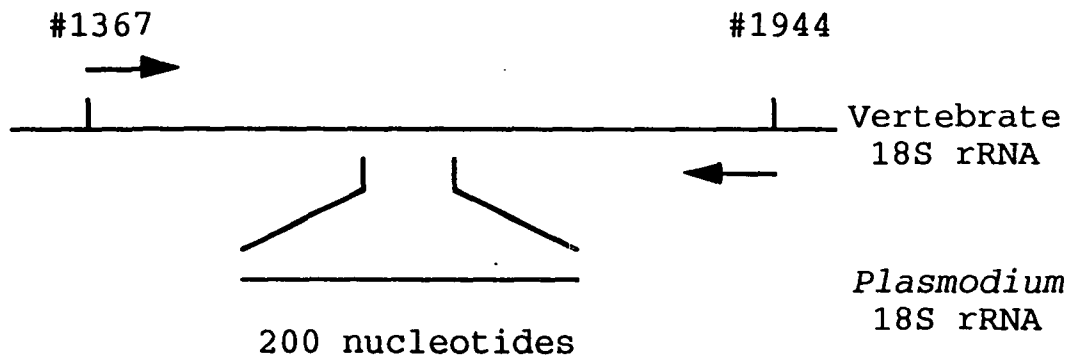

Figure 4.1. Insertion in *Plasmodium* 18S rRNA relative to vertebrates. Numbers refer to nucleotide numbers for *Plasmodium berghei* (Gunderson et al. 1987). The locations of the PCR primers are indicated by the arrows.

during the transfer from the field to the laboratory, whereupon the sample remained frozen until processed.

Total DNA was extracted (Quinn and White 1987) and added to a 25 µl PCR containing 50 mM KCl, 10 mM Tris-HCl pH 8.3, 30 mM $MgCl_2$, 0.01% gelatin, 1.25 mM each dNTP, 1 mM primer #90 (5'-GCATGGCCGTTTTTAGTTCGTGAAT-3'), 1 mM primer #89 (5'-TATCTTTCAATCGGTAGGAGCGACG-3'), and 0.625 units Taq polymerase (ABI/Perkin-Elmer Cetus). Approximately 40 ng DNA was amplified through 30 cycles of PCR at 94°C 40 sec., 48°C 2 min., 72°C 45 sec., followed by an extension at 72°C 10 minutes. One half the PCR was electrophoresed on a 2% agarose gel in 1X Tris-borate (TBE) buffer, stained with Ethidium bromide, and visualized under UV light (Figure 4.2); the other half was reserved for a second electrophoresis as necessary.

The operational employment of the PCR screen is as follows. Batches of samples were screened in groups of 18 or 36 plus a negative and a positive control. A master mix of common reagents was prepared consisting of 10X PCR buffer, dNTPs, primers, and Taq DNA polymerase. The total volume of the common reagent pool was adjusted with sterile ddH_2O to 24 µl per reaction and aliquotted in 23 µl volumes to sterile 0.5 ml eppendorf tubes. Extracted DNA samples were diluted 1:10 and 2.5 µl used for each PCR. The DNA samples were added using a sterile positive displacement pipette with a fresh tip for each reaction to avoid cross contamination of

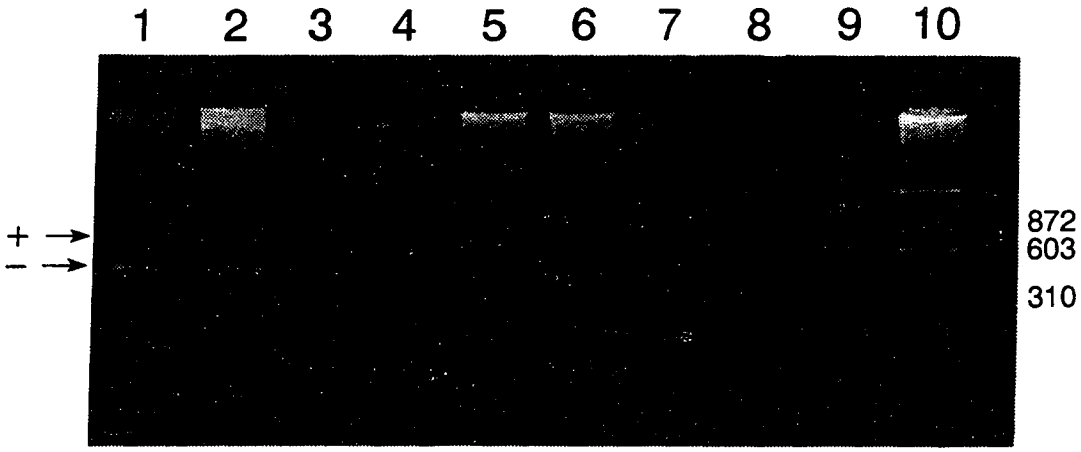

Figure 4.2. A diagnostic PCR gel. Lanes 1-7 are tested samples, lane 8 is a positive control (*P. gallinaceum*) DNA, and lane 9 is a negative control (ddH_2O). The arrow indicates the presence of *Plasmodium* rDNA bands at 580 bp and bird rDNA bands at 380. Lanes 1 and 2 contain positive test samples.

samples. The PCR profile was 30 cycles at 94°C 40 sec., 48°C 2 min., 72°C 45 sec., followed by an extension at 72°C 10 minutes. The samples were electrophoresed as described above and scored positive if bands were seen at 380 bp and at 580 bp, negative if only a single band was seen at 380 bp, and ambiguous if the reaction produced extra bands, no bands, or a smear (Figure 4.2). Reactions that produced ambiguous results could have come from DNA samples whose concentrations were either too low or high to produce clean PCR products or that contained degraded DNA. Amplifications that produced ambiguous results were repeated by adjusting the DNA content in the PCR sample. Only samples that produced unambiguous positive or negative results were counted in the prevalence screens.

These DNA fragments were identified as follows. A portion of each was sequenced from both primers and compared to *Plasmodium relictum*, *P. gallinaceum*, and *P. elongatum*. The fragments were sequenced by the direct double-strand method (Casanova et al. 1990) using detergents to stabilize the denatured strands (Bachmann et al. 1990). Sequences from the 580 bp and the 380 bp bands were compared to the Genbank/EMBL sequence database using the FastA program in the UWGCG (University of Wisconsin Genetics Computer Group) package.

The sensitivity of the PCR test relative to standard smearing and staining (Garnham 1966) was determined in two sets of experiments. First, 30 samples initially screened positive by thin film blood smears were then screened by PCR to determine the reliability of the test. Second, I used the PCR test in blind fashion on a set of native and introduced birds from around 1300 m elevation on Mauna Loa. The samples were collected and screened by smear by Dr. Carter Atkinson of the National Wildlife Health Research Center. Atkinson examined each slide by light microscopy at 400X for 10 minutes and estimated that 25,000 cell were inspected. Cells were examined in a local area of the slide near the feathered edge of the smear. The first set of samples of 15 birds was diagnosed by smear before PCR and was known to include both smear-positive and negative birds. PCR tests on the second sample set of 27 birds were conducted without knowledge of diagnosis by smear. All PCR tests included a positive control (total DNA extracted from blood samples of ducklings experimentally infected with *P. gallinaceum* by M. Skoldager at the Baltimore Zoological Society) and a negative control (sterile ddH_2O) to ensure that in a particular run, *Plasmodium* was detected when known to be present and not detected when known to be absent.

Two additional experiments were conducted to determine the absolute limit of detection of the PCR test. A dilution series experiment, using pure *Plasmodium* DNA, measured the

lowest absolute level of *Plasmodium* detectable by the PCR. The initial concentration of *P. gallinaceum* genomic DNA was 1.0 ng/µl based on an OD_{260} reading made in a Hitachi U-1100 spectrophotometer. This concentration was expressed as the equivalent number of *Plasmodium* organisms by dividing by the diploid *Plasmodium* genome weight, 0.118 pg DNA/organism (Cavalier-Smith 1985). *Plasmodium* genomes vary in rDNA copy number from 4 in the human parasite, *P. falciparum*, to 6 in the avian parasite, *P. lophorae* (Langsley et al. 1983, Unnasch and Wirth 1983). For this study, the avian malaria figure of 6 copies in the haploid genome (12 in the dipoid genome) was used to define copy number of the 18S rDNA genes in the reaction. Therefore, one µl of the original undiluted sample of *Plasmodium* DNA contained the equivalent of 8474.58 *Plasmodium* organisms and 101,694.96 copies of the rDNA gene. The dilution series started at 5084.75 and ended at 254.24 copies per reaction. This range is equivalent to 423.7 to 21.19 individual *Plasmodium* parasites per reaction (Table 4.1). A second dilution series ended in a concentration of *Plasmodium* organisms equivalent to 2.2 organisms per reaction.

QC-PCR (Quantitative Competitive-PCR) (Piatak et al. 1993a, 1993b) experiments measured the sensitivity threshold of the PCR test to amplify *Plasmodium* rDNA in the presence of competing bird DNA. The starting concentration of bird DNA was determined from an OD_{260} reading of 269.5 ng/µl. Using 2X

Table 4.1

Plasmodium Dilution Series

Dilution	Number of organisms per µl	Number µl used	Number of organisms per reaction	Number of rDNA targets per reaction
undiluted	8474.58	1.0	8474.58	101,694.96
1:50	169.49	2.5	423.73	5084.75
1:100	84.75	2.5	211.86	2542.37
1:200	42.37	2.5	105.93	1271.19
1:300	28.25	2.5	70.62	847.46
1:400	21.19	2.5	52.97	635.59
1:500	16.95	2.5	42.37	508.47
1:600	14.12	2.5	35.31	423.73
1:700	12.11	2.5	30.27	363.20
1:800	10.59	2.5	26.48	317.80
1:900	9.42	2.5	23.54	282.49
1:1000	8.47	2.5	21.19	254.24
1:10000	0.85	2.5	2.12	25.42

the genome size of 1.25 pg DNA/cell reported for the chicken (Cavalier-Smith 1985), the starting concentration of bird DNA in the reactions was the equivalent of 107,800 bird blood cells/µl. The diploid genome number of 18S rDNA copies in the bird cell is 400 (McClements and Skalka 1977), so multiplying 107,800 bird blood cells/µl by 400 copies of the gene gives 43,120,000 copies of the bird rDNA/µl in the undiluted sample. In the QC-PCR experiments, the starting concentration of the *Plasmodium* DNA was diluted from 5084.74 to 254.24 rDNA copies per reaction while the concentration of bird DNA was held constant at 10,780,000 rDNA copies per reaction. This range is the equivalent of 423.73 to 21.19 *Plasmodium* parasites in a constant background of 26,950 bird blood cells (Table 4.2). The QC-PCR test was repeated five times.

I used the PCR test to estimate the prevalence of *Plasmodium* in birds at elevations above 1700 m. Birds were sampled at Hakalau Forest National Wildlife Refuge on the Island of Hawaii (n = 532 individuals from 14 species) and at Hanawi Natural Area Reserve on the Island of Maui (n = 122 individuals from 6 species). Each of these sites was established for protection of endangered forest birds, the presence of which has been interpreted as evidence of safety from mosquito-borne disease in these sites (Scott et al. 1986). The Hakalau samples came from two subsites. The Pua Akala tract is the core area of distribution of endangered forest birds on Mauna Kea. In contrast, the Maulua tract,

Table 4.2

QC-PCR Experimental Set-up. For each reaction 2.5 microliters of the dilution was used. A dash indicates that component was not added to the reaction. NC is the negative control, neither *Plasmodium* nor bird DNA was added to that reaction.

Lane	*Plasmodium* dilution	Bird dilution	Number *Plasmodium* rDNA targets per reaction	Bird rDNA targets per reaction	Number *Plasmodium* organisms per reaction	Number bird cells per reaction	Number *Plasmodium* organisms per 10,000 bird cells
1	1:50	-	5084.74	-	423.73	-	-
2	1:50	1:10	5084.74	10,780,000	423.73	26,950	157.23
3	1:100	1:10	2542.37	10,780,000	211.86	26,950	78.62
4	1:200	1:10	1271.19	10,780,000	105.93	26,950	39.31
5	1:300	1:10	847.46	10,780,000	70.62	26,950	26.20
6	1:400	1:10	635.59	10,780,000	52.97	26,950	19.65
7	1:500	1:10	508.47	10,780,000	42.37	26,950	15.72
8	1:600	1:10	423.73	10,780,000	35.31	26,950	13.10
9	1:700	1:10	363.20	10,780,000	30.27	26,950	11.23
10	1:800	1:10	317.80	10,780,000	26.48	26,950	9.83
11	1:900	1:10	282.49	10,780,000	23.54	26,950	8.73
12	1:1000	1:10	254.24	10,780,000	21.19	26,950	7.86
13	-	1:10	-	10,780,000	-	26,950	-
14	NC	NC	NC	NC	NC	NC	NC

with comparable habitat, has few endangered forest birds and is considered a distributional anomaly that may be caused by "invisible" factors such as disease (Scott et al. 1986). Samples were obtained from Pua Akala between 1988-1993, with most months represented. Samples were obtained from Maulua during June-August 1992, and from Hanawi during February and July 1992.

Results

Proof of the PCR Test

Sequence alignments for the *Plasmodium* DNA from an Apapane, *P. relictum*, *P. gallinaceum*, and *P. elongatum* are shown in figure 4.3, along with sequences from the human (*P. falciparum*) and the rodent (*P. berghei*) parasites from published results (Neefs et al. 1990, Gunderson et al. 1987). The fragment was sequenced from both primers, #89 and #90, but the reads did not overlap and left a gap of about 40 bp. This region contains a high level of secondary structure and was impossible to read on the gels. A short segment of the 380 bp fragment from the Apapane was sequenced to positively identify it as the bird rDNA.

A 317 bp segment of sequence from the 580 bp fragment amplified from Apapane #71 was compared to the Genbank/EMBL database. The best 12 matches were to *Plasmodium* 18S rRNA sequences. Sequence from the 380 bp band from the same Apapane was also compared to the database. The best matches were to 18S rRNA from human, mouse, rabbit, and rat (94% over 100 bp) and there was no match to a bird rRNA, indicating that there was not one in Genbank when these searches were made. There was no match (for the top 41 scores) to a *Plasmodium* 18S rRNA. For the 78 bp compared, the avian rDNA differed from the *Plasmodium* at 21 sites. These results

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                                                                                                                   100
P. (apapane)  TCTAACACAA G-AAGTTTAA GGCAACAACA GGTCTGTGAT GTCCTTAGAT GAACTAGGCT GCACGCGTGC TACACTGATA TATATAACGA GTTATTAAAA
P. rel.       ....-.C... .-........ .......... .......... .......... .......... .......... .......... .......... ..........
P. gall.      .......... .......... .......... .......... .......... .......... .......... .......... .......... ..........
P. elong.     -......... .......... .......... .......... .......... .......... .......... .......... .......... ....C.....
P. berg.      .......... .......... .......... .......... .......... AT........ .......... .......... .G..A..... ..GC...--.
P. fal.       .......... .......... .......... .......... .......... .......... .......... .......... .......... ...T......

                                                                                                                   200
P. (apapane)  GTATATCTTT GTATATATAT ATTCTGTATA TATAT----- -ACAAAAGAT ATAATTTTCC TCCACTGAAA AGTGTAGGTA ATCTTTATCA ATATATATCG
P. rel.       .......... .......... ....G..... .....C---- --....G... .......... .......... .......... .......... ..........
P. gall.      .....CGAA. .......... T..A.A.... ..C..-TTTG T.-------- -......... .......... .......... .......... ..........
P. elong.     .....CAAA. A.C....... T..A.A...- ---------G T.T.CTTTG. ...C...... .......... .......... .......... ..........
P. berg.      ..T...A.C. ..--GCT..G G---...-.. A.GCC-TATG TT-TC.GT.. ...T...... .......... .......... .......... ...C......
P. fal.       ....GC--.. A...T.G... C..G-A.GCT .....-TTTG C.-------- -..C...... ...G...... G.C....... .......... ..........

                                                                                    275
P. (apapane)  TGATGGGGAT AGATTATTGC AATTATTAAT CTTGAACGAG GAATGCCTAG TAAGCATGAT TCATCAGATT GTG
P. rel.       .......... .......... .......... .......... .......... .......... .......... ...
P. gall.      .......... .......... .......... .......... .......... .......... .T........ ...
P. elong.     .......... .......... .......... .......... .......... .......... .--------- ---
P. berg.      .......... .......... .......... .......... .......... .......... .......... ...
P. fal.       .......... .......... .......... .......... .......... .......... .......... ...
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Figure 4.3. Sequence from primer #89

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                                                                                                                        100
P. (apapane) TCGATAACGA ACGAGATCTT AACCTGCTAA TTAGCGGTAA ATACAACATA TTCTTAAGTA AATAAGAATA TAAGA-TAAA AATAACAAAT -AAGAGAATA
P. rel.      .......... .......... .......... .......... ......T... ..T...T... ...G...T.. ..G..G.... .G.T...G.. T.G.....A.
P. gall.     C......... .......... .......... .......... .......... .......AC. ..G....... ..-..-.... .......... -...T...A.
P. elong.    .......... .......... .G........ .......... .....C.... ..TC..
P. berg.     .......... .......... .......... .......CG. G...TCT... .C...T.T.G -----.G.G. .TG.TT.TG. CG.T-T.TG. GGGC.T.T.G
P. fal.      .......... .......... .......... .......... G....CT.,. ..T...TT.G ..ATT..... ..G--G...T T..-...TG. TT.TTC.G.G
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                                               134
P. (apapane) TATGAT---- -GTCTTCT-- ----TCCTGT TCTT
P. rel.      ...T.GAGAT A..TA.A.AT ATAG.TACA. CA.C
P. gall.     ...T.G---- -.ATA.T.-- ----.T-.AA .A.C
P. berg.     -..T.-ATCA AT.GG.T.AC CTTT.G..C. .T..
P. fal.      .-.C.AATTA G.ATA.T.TT ----.TT.A. .AA-
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Fig. 4.3 (Continued) DNA sequence results for *Plasmodium* from infected Apapane (#71) and representative *Plasmodium* species from primer #90. Periods in the sequence indicate identity to the top sequence, gaps are indicated by dashes. Between the ends of the primer reads there is a gap of approximately 40 bp. This region contained high secondary structure and was impossible to read on the gels.

indicate that the 580 bp band was amplified from the *Plasmodium* and the 380 bp band from the bird, confirming that the design of the test gave the expected results.

Sensitivity of PCR Test

Every bird in the set of 30 smear positive birds gave the expected 580 bp band after PCR amplification. The set included species in two passerine families, the Apapane (*Himatione sanguinea*, Fringillidae) and the Omao (*Myadestes obscurus*, Muscicapidae), and the non-passerine Cape Penguin (*Spheniscus demersus*). The test is thus reliable across a variety of bird taxa, although some variability exists for certain species. For example, Japanese White-Eyes (*Zosterops japonicus*, Zosteropidae) have occasionally given extra bands, indicating that they may have duplicated rDNA genes, pseudogenes of rDNAs, additional parasites with variably sized rDNA fragments, or PCR artifacts.

The PCR test was more sensitive than the smear-based tests. For both of the mixed samples of forest birds from mid-elevation (1,300 m - 1,400 m), the PCR test showed more positive birds than did the smear test (Table 4.3). For the non-random sample, the PCR test significantly increased the "prevalence" of *Plasmodium* estimated by smear by 67% (Fisher exact test, $p=0.01$).

Table 4.3

Prevalence of malaria diagnosed by PCR and blood smear. Blood samples collected and smears analyzed using standard techniques (Garnham 1966) by Carter Atkinson, National Wildlife Health Research Center, Hawaii Volcanoes Field Station. Samples were collected from study site at 1400 m elevation in the Hawaii Volcanoes National Park, Hawaii. [1]Set A collected between Sept 6, 1991 and Nov. 15, 1991, Fisher exact test, p=0.01. [2]Set B collected between Jan. 23, 1992 and Feb. 10, 1992, Fisher exact test, p=0.5.

	Set A[1]		Set B[2]	
Test	+	-	+	-
Smear	9 (60%)	6 (40%)	6 (22%)	21 (78%)
PCR	15 (100%)	0 (0%)	7 (26%)	20 (74%)

Results from the dilution series experiments are shown in figure 4.4. The PCR test was able to amplify *Plasmodium* rDNA through the entire dilution series. The lowest level of 254.24 copies of rDNA per reaction is equivalent to 21.19 *Plasmodium* parasites in the reaction. A second dilution experiment (not shown, because the gel reproduction was poor) resulted in a positive at the level of 25.42 copies of rDNA per reaction or the equivalent of 2.2 organisms per reaction.

Results from the QC-PCR experiments are shown in figure 4.5. The detection limit of *Plasmodium* parasites per 10,000 bird blood cells in 5 separate QC-PCR experiments was 78.62, 39.31, 15.72, 15.72, and 7.86. The mean detection limit of these experiments is 31.45 *Plasmodium* parasites per 10,000 bird blood cells (Figure 4.6).

A comparison of smear and PCR tests, in terms of genetic equivalents of number of red blood cells (RBC) screened, is derived as follows. Passerine birds are assumed in this model to have a RBC count of 4 x 106 cu mm (Ronald et al. 1968), a leucocyte and thrombocyte count totalling 55 x 103 mm^3 (Sturkie and Griminger 1976), and a haploid genome size of 1.25 pg DNA (Cavalier-Smith 1985). The PCR test uses 2.5 µl of a solution with DNA concentration of 26.95 ng/µl. The test thus screens 67.395 ng DNA, or 26,949 cell equivalents, of which 26,572 are RBC equivalents.

The extent of a slide that this represents requires estimation of the volume and density of RBCs on the slide.

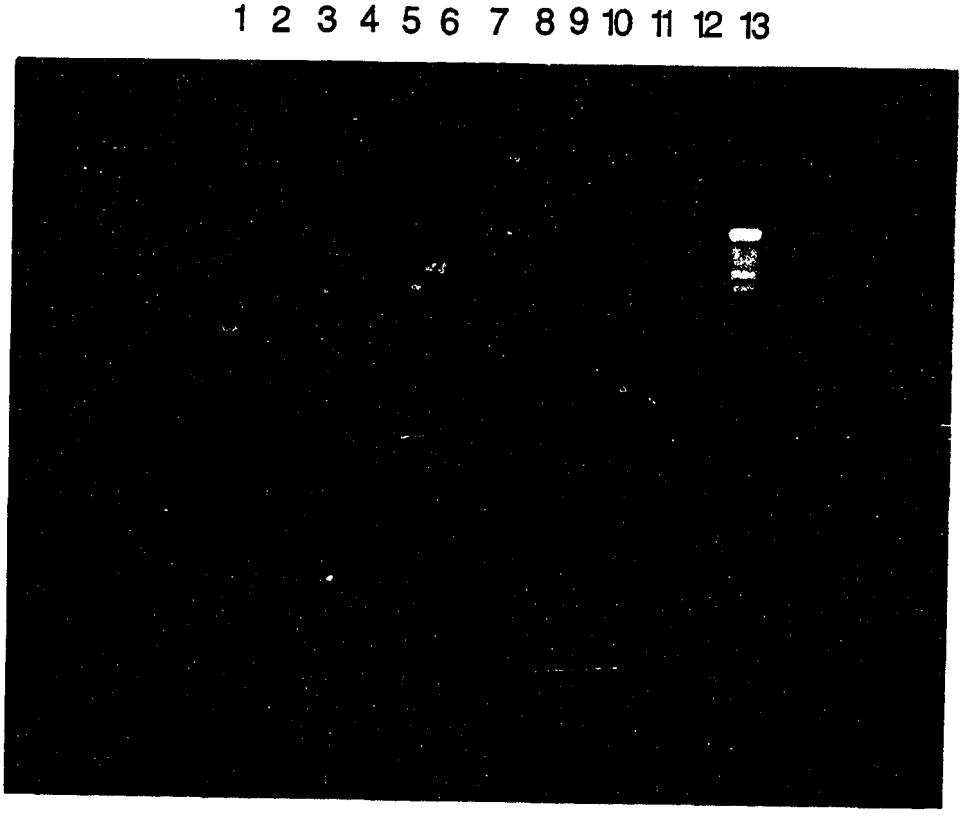

Figure 4.4. *Plasmodium* dilution series. Initial concentration of *Plasmodium* DNA in the starting reaction was decreased from right to left. From left to right the equivalent number of *Plasmodium* organisms in the reactions were 1) 423.7, 2) 211.87, 3)105.93, 4) 70.62, 5) 52.97, 6) 42.37, 7) 35.31, 8) 30.27, 9) 26.48, 10) 23.54, 11) 21.19 and 12) negative control.

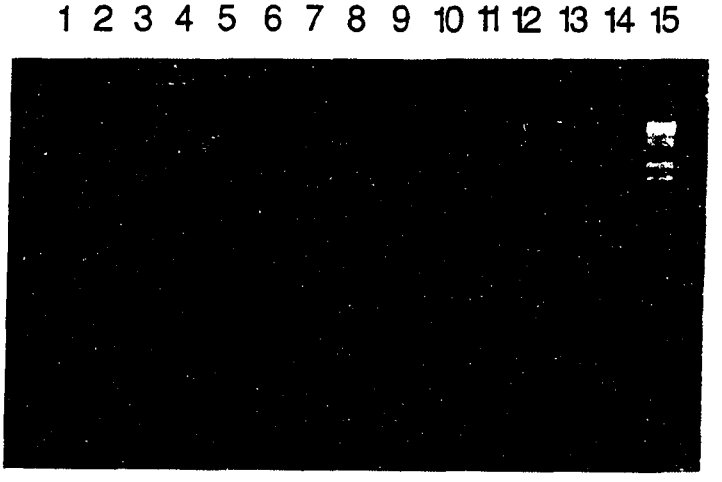

Figure 4.5. Results of the QC-PCR experiment. The equivalent number of bird cells was held constant in all the reactions at 26,950 while the concentration of *Plasmodium* DNA was decreased from right to left. The *Plasmodium* concentrations in each lane were the same as in figure 4.4. Dilution information is given in Table 4.2.

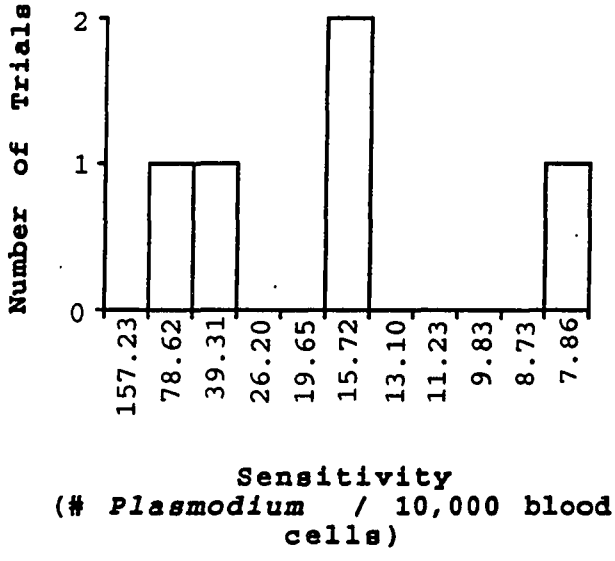

Figure 4.6. Repeatibility of the QC-PCR experiments. The number of trials that resulted in a detection endpoint is given on the Y-axis. Sensitivity increases from left to right and is reported as the number of *Plasmodium* cells per 10,000 bird blood cells in the reaction. The mean limit of detection for the 5 trials is 31.45 *Plasmodium* organisms per 10,000 bird blood cells.

Assuming that a smear occupies 687.5 mm^2 of a slide (50% of the non-frosted portion of a standard 55 x 25 mm slide), and has a height of 15×10^{-3} mm (the longest dimension of 10 mm of the avian RBC [Hodges 1979] times 1.5 to represent a monolayer), the resulting volume of blood on the slide is 10.31 mm^3 with 41.25×10^6 RBC/slide. The PCR test thus covers about 0.065% of such a smear or a random area equivalent to 0.449 mm^2.

Surveys of Birds at Upper Elevations

The PCR test revealed *Plasmodium* in birds sampled from both the Pua Akala and Maulua tracts of Hakalau Forest National Wildlife Refuge on Hawaii (Table 4.4) and from Hanawi Natural Area Reserve on Maui (Table 4.5). Infected Apapanes were detected in all 3 sites, with highest prevalence at Hanawi (11.8%) and comparable prevalence at Hakalau (10.0%). Infected Common Amakihis were detected at Hakalau (2.6%) and Hanawi (1.9%). At Hanawi, *Plasmodium* was also detected in the Maui Creeper (*Paroreomyza montana*) (3.0%) and the Crested Honeycreeper (*Palmeria dolei*) (20%). The only other infected bird was an endemic Omao (*Myadestes obscurus*) from Pua Akala (3.8%). Despite a large sample size from Hakalau, none of the 206 Iiwis tested positive. The

Table 4.4

Malaria screening results for Hakalau Forest National Wildlife Refuge, Pua Akala and Maulua tracts on the Island of Hawaii. The total percent positives and the number of positive individuals over the total number of individuals screened are given. Hakalau is at an elevation of 1,800 to 1,900 m.

Species	Percent positive	Number +/Number tested
Honeycreepers		
Apapane	10.0	9/90
Common Amakihi	2.6	2/76
Iiwi	0.0	0/206
Akepa	0.0	0/47
Akiapolaau	0.0	0/2
Hawaii Creeper	0.0	0/13
Other Endemics		
Elepaio	0.0	0/13
Omao	3.8	1/26
Introduced		
Japanese White-Eye	0.0	0/44
House Finch	0.0	0/6
Rd-bill Leiothrix	0.0	0/6
Pheasant	0.0	0/1
Turkey	0.0	0/1
Northern Cardinal	0.0	0/1
Totals	2.1	11/532

Table 4.5

Malaria screening results for Hanawi Natural Areas Reserve, Maui. Although fewer birds were sampled from July 1992 captures, more positive individuals were detected. There was no Maui Parrotbill caught from the July 1992 birds sampled.

Species	Feb. 1992		July 1992	
Honeycreepers	percent +	n+/nT	percent +	n+/nT
Common Amakihi	0.0	0/30	4.5	1/22
Apapane	13.3	2/15	0.0	0/2
Iiwi	0.0	0/7	0.0	0/7
Maui Creeper	3.2	1/31	0.0	0/2
Crested Honeycreeper	0.0	0/2	33.3	1/3
Maui Parrotbill	0.0	0/1	-	-
Introduced				
Red-Billed Leiothrix	0.0	0/10	66.7	2/3
Japanese White-Eye	0.0	0/6	0.0	0/2
Totals	2.9	3/102	9.8	4/41

overall prevalence at Pua Akala was 1.8%, and at Maulua it was 2.6%.

Temporal distribution of positive birds reveals two patterns (Figure 4.7). First, the prevalence of *Plasmodium* was concentrated around the wetter months of the year, from November through May at Pua Akala. Second, the concentration of prevalence at Pua Akala during the first half of the sampling period, and especially around December 1988, suggests that an epizootic may have occurred at that time.

Screening results on birds from Hanawi Maui also supports the occurrence of an epizootic. Birds were caught in February and July 1992. From the batch of February 1992 birds, 2.9 % of the 102 birds tested were positive. From the July 1992 set, 41 birds were tested and 9.8 % of them were positive. An increased number of species tested positive from the blood samples taken in July as well. In February, Apapane and Maui Creeper tested positive, in July, Common Amakihi, Crested Honeycreepers, and Red-Billed Leiothrix tested positive.

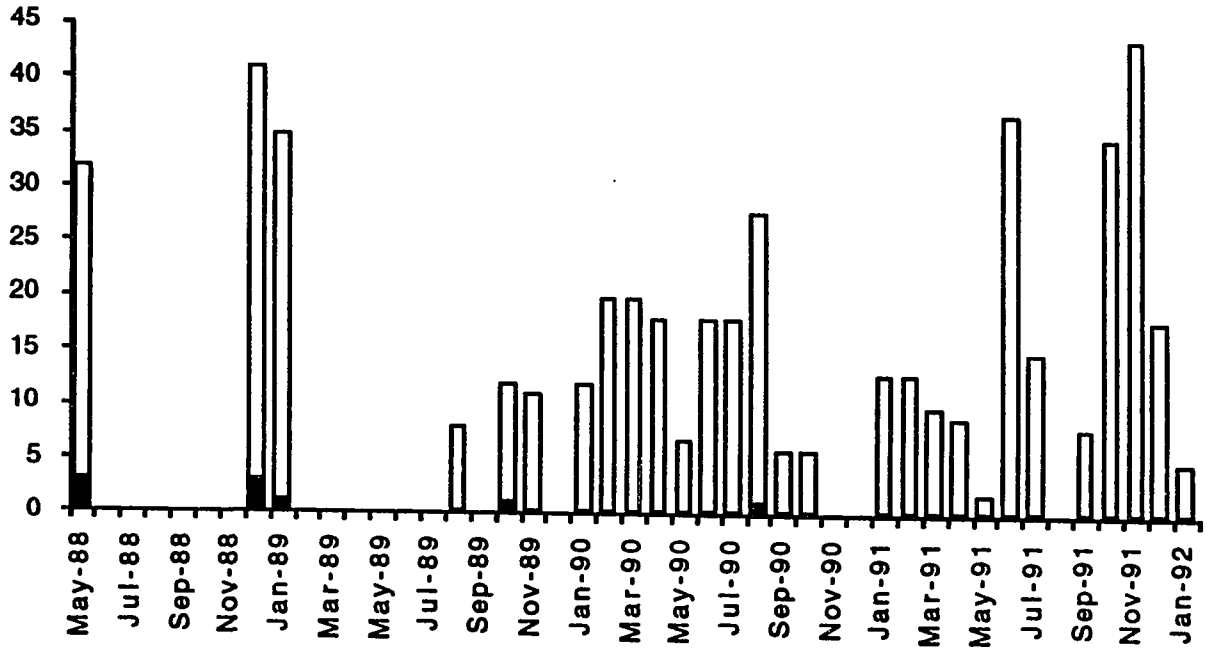

Figure 4.7. Temporal range of tested birds at Pua Akala. Numbers of individuals is given on the Y-axis, the month and year the bird was caught is given on the X-axis. The white bars indicate the numbers of individuals sampled from each time period, the black bars indicate the number of positive individual.

Discussion

I have reported here the successful development of a sensitive and rapid PCR test for avian malaria in bird blood samples. The test is capable of amplifying from as few as 25.42 copies of *Plasmodium* rDNA or the equivalent of 2.12 parasites per reaction under noncompetitive conditions. This is a detection level comparable to that reported for an autoradiographic blot test for the small subunit rRNA from human *Plasmodium* (Waters and McCutchan 1989). The QC-PCR results indicate that the avian malaria test is capable of detecting as few as 8 parasites per 10,000 avian red blood cells under competitive conditions. This is an order of magnitude more sensitive than that reported for DNA blot based tests for human malaria (Barker [1989] reported a detection limit of 1 parasite per 67 human blood cells, or 149 per 10,000). The PCR test covers the DNA equivalent of over 26,000 red blood cells on a typical blood smear. The relative simplicity and high sensitivity of the PCR malaria detection method suggests that it should merit serious consideration as a standard method for detecting low levels of avian malaria infection. Here I consider this test further in relation to the smear test, and discuss the implications of malaria at high elevations in Hawaii.

The PCR test and the smear test have different types of sensitivity limitations. The PCR test represents a random

sample of bird and *Plasmodium* DNA in solution. The physical property of being in solution, where diffusion of small molecules is caused by Brownian motion, increases the likelihood that a sample of the solution would contain DNA from both organisms. The limitation of the PCR test is the bias of the polymerase toward amplifying smaller fragments during initial stages of the reaction (Saiki 1989). As parasitemia (or level of parasites in the blood) becomes low, the potential for this bias increases. Because the sensitivity of the PCR test is a stochastic function of initial conditions, detection of low levels of parasitemia may require replicate runs of the same sample. Chance initial conditions in one of the runs may give the rare *Plasmodium* fragment an early expression.

The smear test, in contrast, has the potential to examine each of the 40,000,000 cells on a typical slide, but there is no physical basis to expect that the localized sample of 10,000 to 25,000 cells actually observed represents a random sample. Uneven distribution of parasite-infected cells on the slide (Garnham 1966), especially under conditions of low parsitemia (Barker et al. 1989), and perhaps due to the stacking and clumping of *Plasmodium* infected misshapen cells, may influence the probability of detecting a parasite. In addition, observer fatigue and motivation may result in missing a rare parasite in a field. A study that compared reproducibility of smear diagnosis

found that significant differences in detection can result upon microscopic reexamination of the same slides (Barker et al. 1989).

This analysis can only begin to address why the PCR test, with an average sensitivity of 31 parasites per 10,000 red blood cells, detected *Plasmodium* when a smear test that examined approximately 25,000 red blood cells did not. The estimates of sensitivity of the PCR test are based on estimates of genome size of both *Plasmodium* and birds. There is no reported information of the error that surrounds the estimate of genome size. In addition, passerine birds may have smaller genome size than that reported for chickens. Even slight revision of genome size upward for *Plasmodium* and downward for birds would increase the estimated sensitity of the PCR test.

The apparent difference in sensitivity between the tests can be useful. The PCR test can in principle be used in conjuction with the smear test to distinguish tolerance and resistance to malaria in birds. The birds in which these tests were compared fell into 3 classes; smear positive and PCR positive, smear negative and PCR positive, and both smear and PCR negative. The smear negative/PCR positive category reflects low levels of parasitemia. While such low levels could indicate either tolerance or resistance, the proportion of birds in a challenge experiment that harbor few or virtually no parasites can help identify resistance and the

threshold between tolerance and resistance. Another promising feature of the PCR test is that it can identify specimens for which additional detailed screening of smears could be worthwhile. Information about the stages of infection require direct inspection of morphology.

The presence of malaria at elevations of 1900-2000 m, and in an endangered species, indicates that the endangered species whose core area of distribution is at such elevations are at risk of exposure to the disease. This is higher than the 1500 m elevation line that has been the basis of reserve design for endangered forest birds based on mosquitos and disease (Scott et al. 1985, 1986, 1988). Two issues are generated by the presence of positive Apapanes, Common Amakihis, and an Omao at upper elevations with prevalences that are close to those observed by van Riper et al. (1986) for these species below 1500 m, also on Hawaii, and by the positive native birds on Maui. First, where did the positive individuals get the infection? Second, is there a chance that vectors exist at upper elevation or can persist there long enough to cause an epizootic?

A case can be made that the Common Amakihis and the Omao on Hawaii contracted the disease on the Pua Akala study site. One Amakihi had been initially captured in the same area slightly more than a year before the blood sample was taken. Observations of these birds indicate that they are resident in the study area for the entire year. They are territorial

during the mating and breeding season and join interspecific flocks of insectivorous birds within their home range during the non-breeding months. Studies of banded Common Amakihi in other study areas indicate that these birds are generally resident throughout the year (Baldwin 1953, van Riper 1987). The Omao is also found on the site throughout the year and is known to be highly sedentary elsewhere on the island (Ralph and Fancy 1994).

The nectarivorous Apapane, which becomes rare at Pua Akala following breeding when fewer blossoms are available, and which is known to move considerably in response to floral blooms (Warner 1968, Baldwin 1953, MacMillan and Carpenter 1980), could have contracted the disease off the study site. It could still contribute to the dynamics at Pua Akala because its movement onto the study site may create or augment a local reservoir of disease available to a vector. The long patent period of this species (van Riper et al. 1986) contributes to this effect.

The detection of malaria in the high elevation population at Hanawi on Maui suggests that malaria may be more widespread in remaining populations of Hawaiian birds than previously thought. Capture rates of juveniles and adults at Hanawi support the hypothesis that the birds contracted the disease at Hanawi. Juvenile Red-Billed Leiothrix, Maui Creeper, and Common Amakihi were caught in July but only adults were present in February. This

indicates that these birds are residents who breed at the Hanawi site and probably contracted the disease at the site. Crested honeycreepers are there in equal numbers of adults and juveniles at both times of the year but they are known to move to lower elevations (Scott et al. 1986) where they could have contracted the disease. Hanawi is at 2000 m elevation, above the normal range of mosquitos (van Riper et al. 1986). However, it is possible that mosquitoes infrequently appear at Hanawi, and the presence of malaria in either the Apapane or the Red-Billed Leiothrix (*Leiothrix lutea*) must be considered a potential reservoir for transmission to the sedentary or geographically restricted species.

Although *Culex* mosquitos are known from elevations as high or higher than Pua Akala or Hanawi, they are much rarer than at elevations below 1650 (Goff and van Riper 1980, van Riper et al. 1986). Nevertheless, the temporal clumping of infected birds, particularly those that were resident, suggests that mosquitos may have been present on the study site, at least temporarily. They are known to breed throughout the year at elevations of 1500m in mesic habitat on Mauna Loa (Goff and van Riper 1980). It is thus possible that conditions were right for infected mosquitos downslope on Mauna Kea or Haleakala to appear at Pua Akala or Hanawi, and to persist long enough to infect some native birds. Vortices of warm air might carry the insects upslope (Scott et al. 1986). They were not collected in Malaise or light

traps at Pua Akala when many of the bird samples were collected (Peck 1993), but insect sampling did not begin until after the possible epizootic. No mosquito sampling occurred at Hanawi. There is also the possibility that a different and less efficient vector exists in Hawaiian forests.

Summary

The combination of a sensitive PCR test for avian malaria and the discovery of malaria in the habitats of the last remaining populations of endangered forest birds suggests a strong role for molecular ecology in conservation. An integrated approach of smear and PCR diagnosis, challenge experiments, and knowledge of host genotypes has the potential to identify lineages that tolerate or resist *Plasmodium* infection. This information could affect the choice of specific birds for captive breeding programs and translocation experiments. A conservation strategy which takes advantage of natural genetic variability for parasite tolerance or resistance has the highest probability of maintaining viable populations (Freed and Cann 1989).

Chapter 5

Conclusions and Areas for Future Work

As with most research, the results generated in these studies raise new and intriguing questions for further work. Here I will discuss some of these.

Further Work on Phylogenetics

The origin of the Drepanidinae within the Carduelinae may become known when a large scale phylogenetic study of the Carduelines using the cyt b gene is completed. The data generated here regrading the *Paroreomyza* question should be combined with the cardueline phylogenetic data to find the closest outgroups of the Drepanidinae. This information could be used to infer a geographic origin for the Hawaiian honeycreepers and answer this old question (Wilson and Evans 1890-99, Amadon 1950, Johnson et al. 1989).

A more complete phylogeny of the honeycreepers that examines the systematic positions of the rare and extinct taxa is necessary to better understand the evolution of this group. This should include the psittirostrine taxa and all of the creeper-like birds in *Paroreomyza*.

A phylogenetic examination of the extinct honeycreepers is also necessary to fully appreciate the extent of the radiation of this group. This could be accomplished by taking advantage of the extensive museum collections of the honeycreepers (Banko 1979) and the fossil and subfossil bones of extinct honeycreeper genera and species (Olson and James 1982, James and Olson 1991).

It would also be of great interest to include the Poouli in these analyses. This genus may not be a honeycreeper (Pratt 1992b) but if DNA was extracted from museum skins it could be sequenced and added to the large cardueline data set. If the honeycreepers group monophyletically in this larger set of carduelines it may be obvious if the Poouli belongs with them.

Phylogenetic relationships of the *Hemignathus* genus may be resolvable using the cyt b gene. *Hemignathus* is the most speciose genus of the extant Hawaiian honeycreepers and *Hemignathus virens* is highly diverse at the population level. A more complete phylogeny of the creepers is also called for. It would be highly informative to look at *Paroreomyza* from Oahu, Lanai, Molokai. It would also be interesting to look at more *Oreomystis* from Kauai and other *Oreomystis* from different Hawaii populations.

Other endemic Hawaiian passerines should also be examined including the old world monarchine flycathers of the genus *Chasiempsis*, the native thrushes (*Myadestes*) and the

melaphagid honeyeaters (*Moho*). A comparative study of these taxa with the drepanids could reveal the dates of origin of these groups and if they were possible competitors with honeycreepers.

Further Work on Populations Genetics

It could be highly informative to look at other species groups that vary morphologically by island and see if the phylogenetic pattern agrees with the Amakihi. These studies would examine the interface between species and populations for honeycreepers found on several islands: Hawaii, Maui, Oahu and Kauai. Good target taxa for these studies would be the Akepa (*Loxops*) and all the members of the genus *Hemignathus*.

A somewhat surprising result of these studies was the finding that two of the endangered species at Pua Akala (the Akepa and Hawaii Creeper) had mitochondrially diverse populations. This is a situation that should be explored considering that captive breeding efforts for the honeycreepers are underway at several zoos in North America. A more extensive analysis of mitochondrial diversity should emaphasize more individuals from the Pua Akala site in order to define the number of individuals that must be sampled in order to have a high level of confidence that most of the rare genotypes are detected. Populations in locations around

Pua Akala, in more disturbed habitat, should also be compared. This type of analysis might reveal disjunctions between once continuous populations or detect subpopulations, undergoing inbreeding, now cut off from a larger metapopulation.

An analysis of nuclear genome diversity is needed especially for understanding the fine scale genetic relationships required for breeding efforts. These studies could examine diversity of anonymous random amplified polymorphic DNAs (RAPDs), microsatellites, and DNA fingerprints. Information about the nuclear genome is also necessary to identify markers that are under selection and possibly linked to malaria resistance and tolerance.

Further Work on Malaria

A more extensive screening study could indentify significant species reservoirs of avian malaria and geographic disease hotspots. Screening birds with low levels of infection may help to track malaria in the latency period. Screening studies of mosquitoes could identify specific malaria carrying vectors and help to model the demographics of disease movements.

The results of the sensitivity experiments on the malaria test indicate it is more sensitve than standard smear based methods. A combined smear and PCR study has the

potential to reveal the quantitative differences between tolerance and resistance.

The rDNA test allows for the genetic identification of parasite populations. A sequence study of positives from Hawaii (Hakalau and Volcanoes) and Maui may reveal the extent of *Plasmodium* genetic diversity in Hawaiian birds. Molecular phylogenetic information on the diversity of *Plasmodium* in Hawaiian birds could determine if the introduction of malaria was a singular event or one that recurs.

A major advance in the study of the malaria-honeycreeper system would be one that links malaria challenge studies to honeycreeper genetic variability studies. The goal of this research would be to directly identify the markers that are under selection in the birds and which lineages have these markers. Individuals of these lineages would then be suitable candidates for breeding stock. This type of project would link basic information from molecular genetic, population diversity and conservation studies. Perhaps future developments in DNA sequencing collection and analysis will make this a realistic dissertation topic for future students.

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