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MITOCHONDRIAL-DNA VARIATION AND EVOLUTIONARY RELATIONSHIPS IN THE AMAKIHI COMPLEX

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ABSTRACT.—An analysis of restriction-site variation in mitochondrial DNA was conducted to examine relationships among five taxa in one group of honeycreepers—the amakihi complex (genus *Hemignathus*). We analyzed 35 ingroup and 3 outgroup samples. Tree topologies, based on both distance and parsimony methods, grouped taxa into two distinct lineages: the *virens-wilsoni* lineage; and the *chloris-stejnegeri-parvus* group. Inter-island sequence divergence (average d_{xy} = 0.0368) is considerably higher than intra-island variation (mean d_x = 0.0035), and is higher than average for avian species. Variability (measured as both nucleotide diversity and maximum divergence between haplotypes) differs among island populations. Molecular evolutionary rates were calibrated on the basis of maximum island age estimates; sequence divergence in this lineage is approximately 2% per million years. The relationships within the *chloris-stejnegeri-parvus* clade generally are consistent with the previously proposed model of double invasion. Genetic distances and the pattern of relationships among amakihi taxa indicate that species status for *H. v. chloris* and *H. v. stejnegeri* may be warranted. *Received 15 July 1992, accepted 18 December 1992.*

THE HAWAIIAN ARCHIPELAGO is widely heralded as a "natural laboratory" for evolutionary studies (Carlquist 1980, Simon 1987). The isolation and ecological diversity of the Hawaiian Islands have served as the backdrop for numerous adaptive radiations. As an example of adaptive radiation of an insular avifauna, the Hawaiian honeycreepers (Drepanidinae) are unsurpassed. This group is renowned for the morphological, ecological, and behavioral diversity that exists among the 23 species described historically (Amadon 1950, Bock 1970, Freed et al. 1987). The recent description of an additional 14 species (James and Olson 1991) has shown that this diversity was once even more extensive.

The drepanidine radiation has been attributed to recurrent double colonization (Amadon 1950, Bock 1970). This process involves colonization of a new island, then differentiation and development of reproductive isolation during a period of allopatry. This is followed by competition and character displacement when secondary contact occurs. Double colonization has been proposed as a general mechanism for speciation of island birds (Lack 1947, Grant 1981). Patterns of colonization consistent with this hypothesis have not been documented for the honeycreepers, in part because phylogenetic relationships of this group have not been fully resolved. In addition to a corroborated phylogenetic hypothesis, an accurate time frame for the diversification of this group needs to be established.

In order to address these issues, we have conducted an analysis of restriction-site variation in one group of closely related honeycreepers of the genus *Hemignathus*, known as the amakihi complex. Speciation via double invasion is thought to have occurred twice within the amakihi complex (Amadon 1950, Bock 1970). The complex includes the following taxa: *Hemignathus sagittirostris*, once found locally on Hawaii, but now extinct; *H. parvus*, a species endemic to Kauai; and *H. virens*, a polytypic species found throughout the main islands. Four subspecies of *H. virens* have been described: *H. v. virens* on Hawaii; *H. v. wilsoni* on Maui, Molokai, and (formerly) Lanai; *H. v. chloris* on Oahu; and *H. v. stejnegeri* on Kauai. Two species of amakihi are found on both Hawaii and Kauai, and it has been proposed that both species pairs have arisen via double colonization from a neighboring island (Amadon 1950, Bock 1970).

The suitability of mitochondrial DNA (mtDNA) for addressing questions of taxonomy and evolutionary relationships among closely

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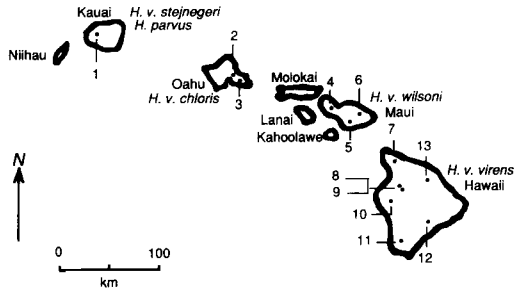

Fig. 1. Collecting localities in the Hawaiian Islands: (1) Pihea trail (n = 7, *H. v. stejnegeri*; n = 5, *H. parvus*); (2) Keaiwa Heiau (n = 2); (3) Makiki (n = 2); (4) West Maui (n = 2); (5) Polipoli (n = 3); (6) Puu Alaea (n = 1); (7) Parker Ranch (n = 2); (8) Puu Laau (n = 2); (9) Puu O Kauha (n = 3); (10) Hualalai (n = 1); (11) Manuka (n = 3); (12) Kulani (n = 1); (13) Puu Kanakaleonui (n = 1).

related organisms has been extensively reviewed (e.g. Avise et al. 1987, Wilson et al. 1985, Shields and Helm-Bychowski 1988, Harrison 1989). Here we report on an analysis of mtDNA variation in five taxa in the amakihi complex. Our goals are to: (1) estimate a phylogeny for the amakihi complex and infer from this the pattern and timing of island colonization; and (2) provide a calibration of nucleotide substitution rates based on estimates of island age. In addition, we reinterpret the classification of *H. virens* in light of the phylogeny and genetic diversity measures.

METHODS

We collected 35 ingroup samples from four of the main Hawaiian Islands. Specific localities and sample sizes are shown in Figure 1. Three individuals of the Laysan Finch (*Telespiza cantans*) were included as an outgroup species; this species was chosen because it represents a lineage that arose earlier than the amakihis in the honeycreeper radiation (Tarr and Fleischer in press). Liver, heart, and breast muscle were removed from specimens, stored in MSB buffer (Lansman et al. 1981), and frozen in liquid nitrogen. Samples were later stored at −90°C for up to two years.

A modification of the sucrose step gradient protocol (Spolsky and Uzzell 1984) was developed and used to prepare mtDNA (Tarr 1991). All mtDNA samples were digested with 15 six-base-recognizing restriction endonucleases (*Bam*HI, *Ban*III, *Bcl*I, *Bgl*I, *Bgl*II, *Bst*BI, *Eco*RI, *Eco*T22I, *Hin*dIII, *Kpn*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I, *Xho*I). For samples without any contaminating nuclear DNA, about 20 ng per sample was digested and end-labelled with ^{32}P and electrophoresed on 1.0 or 1.2% agarose gels at 70 to 90 V for 12 to 18 h. A molecular size standard (lambda DNA cut with *Hin*dIII) was included for determination of fragment sizes. Gels were dried under vacuum and fragments were visualized by autoradiography.

Some tissue samples (n = 11) had been thawed and refrozen prior to mtDNA isolation and yielded mtDNA that was contaminated with nuclear DNA. These samples were digested and electrophoresed as above. The DNA in the gel was depurinated (0.25 N HCl), followed by two denaturing washes (1.5 M NaCl:0.5 N NaOH), and the DNA was transferred in 20× SSC to an MSI Magna NT nylon membrane with a vacuum blotter. The membranes were prehybridized in 4× SSC, 5% sodium pyrophosphate, 1× Denhardt's solution, and 0.5% SDS for 6 h at 65°C. Approximately 100 ng of House Finch (*Carpodacus mexicanus*) mtDNA was labelled with ^{32}PdATP by the random-primed hexamer method to a specific activity of 0.7–2.4 × 10^9 cpm/μg. Hybridizations were carried out in a shaking water bath at 65°C for 18 to 24 h. Filters were washed twice (2× SSC, 0.2% SDS, 0.1× Denhardt's) at room temperature for 15 min, followed by two 20-min washes (2× SSC, 0.1% SDS) at 65°C. One to three individuals were analyzed by both of the above methods for 11 of the 15 enzymes.

Following the approach of Kessler and Avise (1984), a matrix was constructed by scoring the presence or absence of fragments for each individual. Comparisons of fragment sizes were used to infer the minimum number of restriction-site changes that could account for the changes in fragment profiles, and a presence/absence matrix of sites was constructed.

The proportion of shared DNA fragments (F), nucleotide diversity within populations (d_x), and the average number of nucleotide substitutions per site between haplotypes from two populations (d_{xy}) were calculated by the computer program RESTSITE (Miller 1990, Nei and Miller 1990), which uses formulae 5.53, 5.54, and 5.55 from Nei (1987). Similar measures of diversity were calculated for sites (Nei 1987: formulae 5.38 and 5.42). A range of substitution rates was estimated by dividing d_{xy} (uncorrected and corrected values) by the age of the oldest volcanic series for a given island (this assumes an island was colonized soon after emergence and, thus, provides a minimum rate).

The UPGMA option in the RESTSITE program (Miller 1990) was used to cluster the taxa based on distance estimates. The neighbor-joining algorithm (Saitou and Nei 1987) in the analysis package MEGA (Kumar et al. 1993) also was used to construct a tree from the distance matrix. A parsimony analysis was performed by the program PAUP (Swofford 1985) with *T. cantans* as an outgroup to root the tree. A bootstrap was performed on the site matrix (branch-and-bound algorithm with 250 replications).

TABLE 1. Nucleotide diversity in four subspecies of *H. virens*.

Taxon	$d_x \pm$ SE	Maximum d
virens	0.00149 ± 0.00077	0.00460
wilsoni	0.00468 ± 0.00176	0.01255
chloris	0.00197 ± 0.00135	0.00425
stejnegeri	0.00520 ± 0.00299	0.00750

RESULTS

The average size of the mtDNA molecule in *Hemignathus* is approximately 16,712 ± SD of 363 bp. No size variation was detected in this study. The 15 restriction endonucleases produced 115 fragments, 15 of which were unique to *T. cantans*. We identified 18 haplotypes among the 38 individuals; 16 of these were in *H. virens* (there was only one haplotype each in *H. parvus* and *T. cantans*). We estimated 73 sites from the fragment profiles. All enzymes showed restriction-site variation among the taxa, and all except *Sac*II revealed at least one polymorphic restriction site within *H. virens*. The analyses based on sites and fragments did not differ greatly (Tarr 1991), and only the results based on the site data are presented in detail here.

Estimates of intra-island diversity for *H. virens* indicate that variability differs among island populations (Table 1). The greatest degree of differentiation was between *H. v. wilsoni* haplotypes. Two haplotypes from one location (Polipoli, Maui) differed by six restriction sites (d = 0.0122). The maximum sequence divergence between *H. v. stejnegeri* haplotypes also was high, with five restriction-site differences. Haplotypes of *H. v. virens* and *H. v. chloris* differed by no more than two restriction sites, and the maximum sequence divergence was less than 0.5% (Table 1).

The average sequence divergence among island populations of *H. virens*, based on analysis of sites, was 0.0370 ± 0.012. *Hemignathus v. virens* and *H. v. wilsoni* were the most closely related taxa, and the highest divergence was between *H. v. chloris* and *H. v. wilsoni* (Table 2).

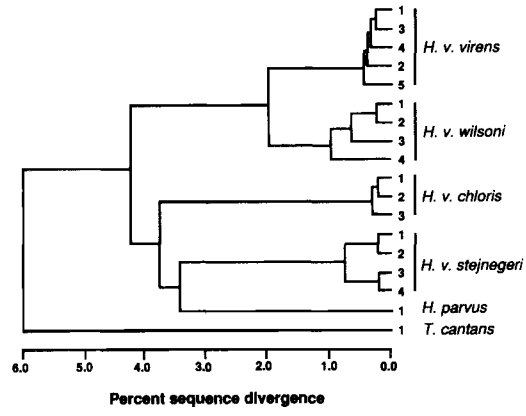

Fig. 2. UPGMA phenogram produced by analysis of restriction-site distances.

The UPGMA analysis shows island populations as distinct clusters (Fig. 2). Two major lineages are evident: the *virens-wilsoni* group; and the *parvus-stejnegeri-chloris* group. Within the latter lineage, *H. v. parvus* and *H. v. stejnegeri* form a cluster.

The bootstrap consensus tree of 76 steps is shown in Figure 3. The neighbor-joining algorithm produced a topology identical to the parsimony tree (Fig. 4). Most features of the topology are concordant with the phenogram in Figure 2. For example, all three analyses partition the amakihis into two distinct lineages; and each island population is a monophyletic group. However, the position of *H. parvus* differs in the analyses: *H. v. chloris* and *H. parvus* are sister taxa in the cladogram and neighbor-joining tree; and *H. v. stejnegeri* is the sister taxon to *H. parvus* in the UPGMA phenogram. Also,

TABLE 2. Average sequence divergence (d_{xy}) between taxa (above diagonal) with jackknifed standard errors (below diagonal).

	Taxon					
Taxon	1	2	3	4	5	6
1 *H. v. virens*	—	0.0205	0.0475	0.0373	0.0448	0.0473
2 *H. v. wilsoni*	0.0055	—	0.0551	0.0440	0.0476	0.0609
3 *H. v. chloris*	0.0088	0.0094	—	0.0374	0.0416	0.0851
4 *H. v. stejnegeri*	0.0092	0.0074	0.0107	—	0.0357	0.0626
5 *H. parvus*	0.0077	0.0090	0.0139	0.0121	—	0.0573
6 *T. cantans*	0.0113	0.0135	0.0192	0.0137	0.0132	—

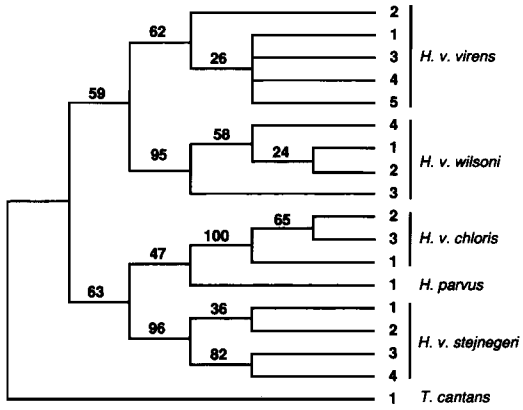

Fig. 3. Bootstrap consensus tree produced by parsimony analysis of presence/absence matrix of sites. Numbers along branches denote percentages of trees in which a particular node appears.

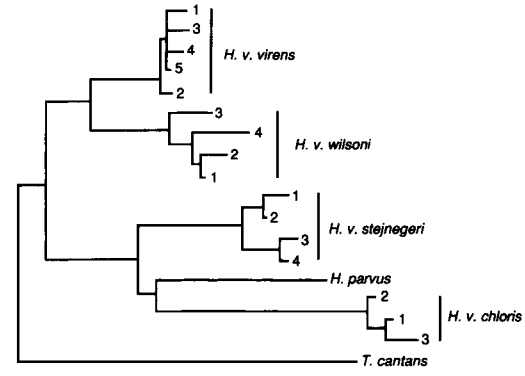

Fig. 4. Distance-parsimony tree constructed by neighbor-joining algorithm.

relationships among haplotypes within islands are not consistently resolved.

Discussion

mtDNA diversity in amakihi populations.—Diversity measures indicate average to high levels of intra-island variation in populations of *H. virens*. The nucleotide diversity in the Hawaii and Oahu populations, although low compared to Kauai and Maui, are similar to values reported for other avian taxa (Avise and Zink 1988, Seutin et al. 1993). In contrast, nucleotide diversity is high in the Maui and Kauai populations, and in some cases the pairwise divergence between individuals is much higher than values normally reported for birds collected from the same site, and even exceed the divergence between some congeneric species of birds (e.g. Avise and Zink 1988:tables 4 and 5). The percent sequence divergence between two individuals from Polipoli (1.22%) is as high as the value reported between two subspecies of *Platycercus elegans;* Ovenden et al. (1987) reported a maximum nucleotide divergence of 1.17% between individuals of *P. e. nigrescens* from the northeastern coast of Australia and *P. e. adelaidae* from the south-central coast of Australia. One other example of co-occurring divergent lineages within a population of birds has been reported; a study of mtDNA polymorphism in populations of *Parus caeruleus* in France (Taberlet et al. 1992) revealed two maternal lineages that differed by 1.23%. The phylogeographic pattern of *P. caeruleus* was attributed to either recent admixture of two previously isolated populations, or introgression through hybridization with *P. cyanus*.

Since the mitochondrial genome is haploid and maternally inherited, its effective population size is about four times smaller than that for nuclear genes (Nei 1987). Thus, mtDNA variation can be lost more rapidly through population reduction than nuclear-DNA variation (Wilson et al. 1985). The model of Avise et al. (1984) predicts that, unless the number of generations is small or N_e is very large, the probability of maintaining more than one lineage is very low. However, the probability of survival of two or more mtDNA lineages is increased if a population is subdivided (Avise et al. 1984). Although mtDNA diversity will decay within each subpopulation, at least one mtDNA lineage per deme will be maintained. Occasional migration between demes could introduce moderately divergent mtDNA lineages into a population.

Levels of variability indicate that long-term effective population sizes have varied among amakihi populations; the factors causing such variation are unknown. However, population subdivision may play a role in maintaining diversity in at least one deme. The islands of Maui, Molokai, and Lanai were joined to form the Maui Nui complex during the Wisconsin glacial period, and may have been contiguous during previous glacial periods (Stearns 1966, Macdonald et al. 1983). Secondary contact between previously isolated demes could occur during lower stands of the sea and, thereby, enhance genetic diversity. The monophyly of island populations

and the presence of intermediate haplotypes makes subspecies hybridization an unlikely source of divergent haplotypes in *H. v. wilsoni.*

Rate of mtDNA evolution and island colonization.—Some authors have suggested the average rate of substitution over the mitochondrial genome may be decelerated in birds compared to other vertebrates (Kessler and Avise 1985, Ovenden et al. 1987; but see Shields and Wilson 1987). Because the ages of the Hawaiian Islands impose maximum ages on the populations, the minimum substitution rate of the mtDNA genome can be estimated. We use only the two youngest islands because the time of divergence of the amakihis from other drepanidines is not known; the time may be well accommodated within the ages of Kauai and Oahu.

Assuming a substitution rate of 2% per million years (Shields and Wilson 1987), the Hawaii subspecies (*H. v. virens*) and the Maui subspecies (*H. v. wilsoni*) diverged approximately 1,000,000 years ago. If the most recent age estimate for Hawaii (400,000 years; McDougall and Swanson 1972) is accurate, the divergence event would predate the formation of Hawaii by about 600,000 years. The branch leading to the Maui and Hawaii populations diverged from the Kauai-Oahu lineage approximately 2.2 mybp. This would predate the formation of Maui (1.6 mybp; Naughton et al. 1980) by about 600,000 years. Assuming Hawaii and Maui were colonized in the order of formation, we used the ages of the islands to calibrate a substitution rate of 2.7 and 5.0% sequence divergence per million years for the Maui and Hawaii lineages, respectively. However, because Maui and Molokai have been joined in the past, the age of Molokai (1.84 my; Naughton et al. 1980) may be more appropriate for calibrating a substitution rate for *H. v. wilsoni*; this yields a rate of substitution of 2.4% per million years.

The above calibration does not account for polymorphism at the time of population splitting. Haplotypes could diverge in the ancestral population prior to inter-island dispersal, thereby leading to an overestimate of the genetic distance between the two populations. Such an overestimate could be extreme in the case of *H. v. wilsoni* and *H. v. virens*, because divergent lineages presently exist on Maui, and may also have existed in the past. We maintain that one population was derived from the other (on basis of close genetic relationship between *H. v. virens* and *H. v. wilsoni*); the direction of colonization was most likely from Maui to Hawaii (if Maui were colonized from Hawaii, then a rate of 10% divergence per million years would be required to account for the distance between the *virens-wilsoni* and *chloris-stejnegeri* lineages). To provide a maximum correction for estimating the net nucleotide substitutions per site (d_A; Nei 1987), we subtract the maximum divergence between haplotypes on Maui (0.012) from d_{xy} (0.020), which yields a d_A of 0.008, and a substitution rate of approximately 2% per million years. As the potassium-argon ages for Hawaii are equivocal (e.g. McDougall 1969, Dalrymple 1971), the calibration for *H. v. virens* is not as reliable as that for *H. v. wilsoni.* We have made conservative assumptions to provide a minimum rate in our calibrations, and we conclude that the rate of molecular evolution in this group of birds is not decelerated relative to other vertebrates. We also note that some rate heterogeneity exists among lineages of *H. virens* (Fig. 4), and the substitution rate may be accelerated in the populations on the two older islands.

The phylogeny does not provide further insights into patterns of colonization (beyond those inferred from comparisons of genetic distance and island age). We cannot be certain whether the Kauai or Oahu population is ancestral, and the position of *H. parvus* has proven difficult to resolve (see also Johnson et al. 1989). The topology derived from the parsimony and neighbor-joining analyses is consistent with secondary contact of taxa on Kauai, as suggested by Amadon (1950) and Bock (1970); a neighbor-joining analysis, which includes other drepanidine taxa, supports the double-invasion hypothesis as well (Tarr and Fleischer in press). However, the UPGMA tree does not support the double-invasion hypothesis, but instead is consistent with a sympatric origin for *H. parvus* and *H. v. stejnegeri*; a parsimony analysis (Tarr and Fleischer in press) is inconsistent with inclusion of *H. parvus* in the amakihi complex. Future studies which include *H. v. sagittirostris* and other drepanidine taxa will provide a more rigorous test of the double invasion hypothesis.

Taxonomic considerations.—The taxonomic status of *H. v. stejnegeri* has been debated, primarily because of its morphological divergence from other populations of *H. virens.* The mtDNA differentiation of the Kauai Amakihi reported here is consistent with previous investigations. Morphological (Amadon 1950, Bock 1970, Pratt 1979),

osteological (James and Olson 1991), ecological and behavioral (Pratt 1979) differentiation, as well as nuclear differentiation (Johnson et al. 1989), all indicate that the divergence of *H. v. stejnegeri* is sufficient to consider it a separate species. The nomenclature for the Kauai Amakihi remains problematic, as the epithet *H. stejnegeri* is preoccupied (Olson and James 1988); Pratt (1989) has suggested the name *H. kauaiensis*. However, James and Olson (1991) have retained the amakihis in the genus *Loxops*, with the Kauai Amakihi designated as *Loxops stejnegeri*. A molecular-phylogenetic analysis of the Hawaiian honeycreepers will provide an independent assessment of the relationship of the amakihis to other drepanidines (Fleischer, McIntosh, and Tarr pers. comm.).

Surprisingly, our data also suggest the divergence of *H. v. chloris* may be sufficient to consider it a separate species. The sequence divergence between the Hawaii-Maui clade and the Kauai-Oahu clade (4.4%) is similar to the average distance among taxa in five avian genera (Avise and Zink 1988). The divergence between the Kauai and Oahu amakihi (3.4%) also is similar to levels of interspecific differentiation in other avian taxa.

Some authors caution against delineating species limits on the basis of a gene tree (e.g. Quinn et al. 1991). Examination of nuclear genes can reveal different patterns of divergence, and there is no absolute value of divergence above which two taxa should be considered separate species. *Hemignathus v. chloris* shows a slight to moderate divergence in nuclear genes (Johnson et al. 1989) and morphological differentiation appears slight. However, if the phylogeny presented here accurately reflects the pattern of descent among the amakihis, then *H. virens* as it is currently defined is polyphyletic. The case for separating *H. v. chloris* is not as strong as that for *H. v. stejnegeri*, but these data are suggestive, and further analysis seems warranted.

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