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Evolution of Microsatellite Loci in the Adaptive Radiation of Hawaiian Honeycreepers

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Abstract

Previous studies have examined germ-line mutations to infer the processes that generate and maintain variability in microsatellite loci. Few studies, however, have examined patterns to infer processes that act on microsatellite loci over evolutionary time. Here, we examine changes in 8 dinucleotide loci across the adaptive radiation of Hawaiian honeycreepers. The loci were found to be highly variable across the radiation, and we did not detect ascertainment bias with respect to allelic diversity or allele size ranges. In examining patterns at the sequence level, we found that changes in flanking regions, repeat motifs, or repeat interruptions were often shared between closely related species and may be phylogenetically informative. Genetic distance measures based on microsatellites were strongly correlated with those based on mitochondrial DNA (mtDNA) sequences as well as with divergence time up to 3 My. Phylogenetic inferences based on microsatellite genetic distances consistently recovered 2 of the 4 honeycreeper clades observed in a tree based on mtDNA sequences but differed from the mtDNA tree in the relationships among clades. Our results confirm that microsatellite loci may be conserved over evolutionary time, making them useful in population-level studies of species that diverged from the species in which they were characterized as long as 5 Ma. Despite this, we found that their use in phylogenetic inference was limited to closely related honeycreeper species.

Key words: *ascertainment bias, dinucleotide repeats, genetic distance measures, honeycreepers, microsatellites, phylogenetic inference*

Nuclear microsatellite loci have been used extensively in studying within species dynamics but are used less often in interspecific studies. In part, this is because there is not yet consensus on the mechanisms that underlie the high degree of polymorphism seen at many microsatellite loci (Jarne and Lagoda 1996; Goldstein and Pollock 1997; Ellegren 2000; Schlötterer 2000). Although there have been a number of attempts to reconstruct phylogenies between closely related organisms using microsatellite loci (e.g., Roy et al. 1994; Harr et al. 1998; Petren et al. 1999; Muir et al. 2000; Ritz et al. 2000; Uphyrkina et al. 2001; Rout et al. 2008), only a few studies have used data from wild species to investigate changes in microsatellite loci at the sequence level over evolutionary time (Ross et al. 2003; Sokol and Williams 2005; Ochieng et al. 2007).

In contrast, a number of studies have examined germ-line mutations in microsatellite loci. The processes most often inferred are polymerase slippage during DNA replication, which tends to increase variability (Levinson and Gutman 1987; Nishizawa and Nishizawa 2002; Bayliss et al. 2004) and point mutations that break up the tandem repeats, reduce mutation rates, and stabilize allele sizes (Petes et al. 1997; Sainudiin et al. 2004). Mutation rates have been found to be strongly affected by the repeat motif, with dinucleotides having mutation rates 1.5–2.0 times that of tetranucleotides and non–disease-causing trinucleotides having intermediate rates (Chakraborty et al. 1997; Krugylak et al. 1998). Studies have found a positive relationship between microsatellite repeat length and mutation rate (Primmer et al. 1998; Noor et al. 2001; Brohede et al. 2002; Webster et al. 2002) but no detectable effect of size differences between an individual's alleles on mutation rate (Primmer et al. 1998; Ellegren 2000). Although observed mutations have been biased toward expansions rather than

contractions (Primmer et al. 1998; Ellegren 2000), loci with more than 60 repeats have rarely been observed (Goldstein and Pollock 1997). This suggests that there may be an upper limit on repeat size (Garza et al. 1995). Possible evidence for this limit was found by Xu et al. (2000), who demonstrated that the rate of expansion in human tetranucleotide repeat loci was constant, whereas the rate of contraction increased exponentially with allele size, and by Ellegren (2000), who found that long alleles in dinucleotide loci are more likely to decrease in size than short ones. An interesting study by Whittaker et al. (2003) provided further insights. They examined a large number of parent–offspring pairs and found that microsatellite arrays of less than 20 repeats tend to increase, whereas those that contain more than 20 repeats were more likely to contract.

Although studies of germ-line mutations provide insights into the mechanisms that produce and maintain variation at microsatellite loci within species, comparisons between species are needed to investigate the dynamics of these loci over evolutionary timescales. Primmer and Ellegren (1998) investigated changes in 3 microsatellite loci in 39 species spanning the avian phylogeny, demonstrating that the patterns of microsatellite evolution may differ substantially between loci. The first locus they examined contained a perfect $(AG)_n$ repeat, which remained structurally constant over the 15 species in which alleles were sequenced. Those species in which the repeats had been interrupted were monomorphic, having apparently become stabilized by the interruption. Alleles from the second locus, which contained short and interrupted $(GA)_n$ and $(GT)_n$ repeats, were sequenced in 19 species. The microsatellite contained 6 repeat regions as well as a number of subregions defined by interruptions. The complexity of the locus made it difficult to infer the causes of variation, but it was apparent that changes occurred in all regions and that some were likely to have been the result of slippage, whereas others likely represented large deletions. The third locus, with an $(AAAG)_n$ repeat motif, was found to be highly polymorphic in the species in which it was described, but sequencing of alleles revealed that it was highly unstable. The primers did not successfully produce a product in distantly related species, thus alleles from closely related species were sequenced. The repeat region differed within and between species, with some alleles containing more than one motif similar to the original such as $(AG)_n$, $(AAGG)_n$, $(AAAAG)_n$, and $(AAAGAGAG)_n$. The extreme polymorphism that might have made this locus useful in both intra- and interspecific studies was in part due to a structure that seemed to be extremely prone to homoplasy.

Zhu et al. (2000) studied 3 conserved trinucleotide loci in 58 species of the Polistinae, a wasp subfamily that diverged over 140 Ma. In these loci, they found strong conservation of primer sequences and flanking regions, multiple cases of nontriplet insertions or deletions in the repeat region, and numerous imperfections in the repeats that were shared among closely related species. Longer repeats were associated with greater variability within and between species, whether measured as allelic diversity or heterozygosity. One especially interesting finding was that once imperfections occurred in microsatellite arrays, they tended to persist as the elimination of the imperfections was rare. Their results suggest that, to the extent that these imperfections inhibit further slippage, their accumulation may lead to the eventual degradation, and possibly the loss, of microsatellite loci.

Primmer and Ellegren's (1998) study spans the entire avian phylogeny, including both passerine and nonpasserine species, which diverged some 50–60 Ma. The study by Zhu et al. (2000) includes Polistinae wasps species that have diverged over the last 140 My. The fact that the loci examined in those studies have been conserved over such long time periods is remarkable, and the results of these studies support the notion that the processes that result in germ-line mutations of microsatellite loci may also operate over evolutionary time. However, the extremely high mutation rates found at the majority of microsatellite loci make them unsuitable for use in studies of long-diverged species. Only 1 of the 6 loci included in those studies contains a CA/GT repeat motif. Dinucleotide loci, with their high mutation rates, are commonly used in studies of species and subspecies that have diverged over much shorter evolutionary timescales (but see Hale et al. 2005).

Here, we investigate the evolutionary dynamics of 8 dinucleotide microsatellite loci in the adaptive radiation of Hawaiian honeycreepers. The Hawaiian Islands are the world's most isolated archipelago; the major islands are arranged in a linear array by age, with the oldest being Kauai (4.7 My) at the northwestern end and the youngest being Hawaii (0.5 My) in the southeast (Figure 1, Clague 1996). The honeycreeper (Drepanidinae) radiation is well known for its morphological, ecological, and behavioral diversity. Because many of the morphological characters that are used for phylogenetic reconstruction may have been modified by selection during an adaptive radiation, relationships between honeycreeper species have also been studied using genetic markers (Tarr and Fleischer 1993; Fleischer et al. 1998, 2001; Fleischer and McIntosh 2001). The results of these studies suggest that the group diverged from a mainland cardueline finch ancestor approximately 5–6 Ma and that the radiation of extant species occurred in the past 3–4 My.

We first examine levels of variability in these loci in 10 honeycreeper species and 1 cardueline finch species. We then investigate the relationship between microsatellite divergence over evolutionary timescales and mitochondrial DNA (mtDNA) divergence and find that these measures are strongly correlated. We compare genetic distances between 3 island-endemic species pairs, whose divergence we assume does not predate the emergence of the younger island (Fleischer et al. 1998; Price and Clague 2002), to island emergence times in order to infer the evolutionary rate of change in our panel of microsatellite loci. Finally, we examine changes at the sequence level over this adaptive radiation using a phylogeny derived from mtDNA sequences. Our results suggest that, over the timescale represented by the honeycreeper radiation, ascertainment bias is not a substantial problem and that genetic distances based on

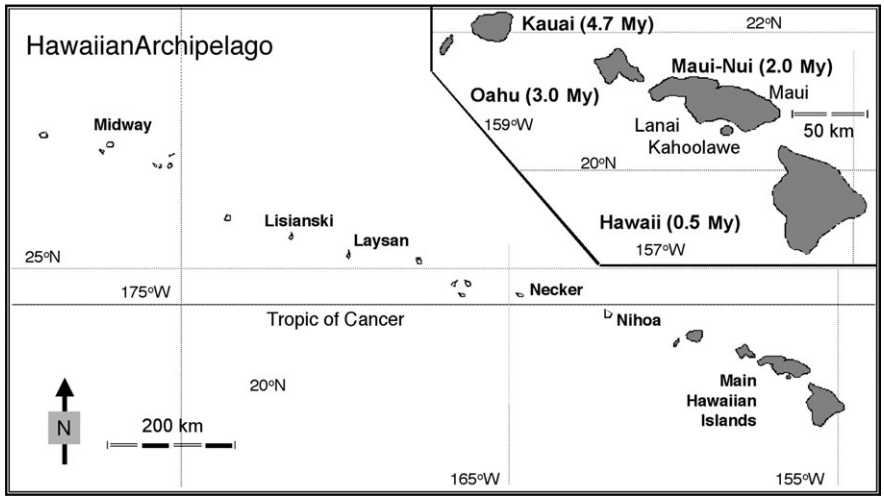

Figure 1. Map of the Hawaiian archipelago, with insert showing the main islands. Island ages shown are from Price and Clague (2002).

microsatellite loci accumulate in a remarkably linear fashion. Phylogenetic inferences using these loci recover several of the major clades but do not agree with inferences based on mtDNA sequences in the relationships among those clades. Although patterns of change may differ considerably between loci, we found that in most loci, there has been strong conservation of flanking region sequences and that imperfections and motif changes may be phylogenetically informative.

Materials and Methods

Sampling of Honeycreeper and Out group Species

Blood samples of honeycreeper and out group species were collected from a number of locations. Samples of Kauai creeper (*Oreomystis bairdi*) were collected at Kokee State Park ($n = 2$) and in 2 areas of the Alakai Wilderness Preserve ($n = 9$). Maui creepers (*Paroreomyza montana*) were sampled at Haleakala National Park ($n = 7$), Hanawa Natural Area Reserve ($n = 4$), Polipoli State Park ($n = 4$), and Waikamoi Preserve ($n = 5$). Palila (*Loxioides bailleui*) samples ($n = 21$) were collected at Pu'u Laa'u on the west slope of Mauna Kea. Laysan finches (*Telespiza cantans*, $n = 21$) were sampled only on Laysan Island. Apapane (*Himatione sanguinea*) were sampled at Haleakala National Park on Maui ($n = 5$), Kokee State Park on Kauai ($n = 6$), and several locations (Mauna Kea State Park, $n = 2$; Volcano, $n = 1$; Kohala mountains, $n = 1$; Nanawale Park, $n = 2$) on the Big Island. Samples of Iiwi (*Vestiaria coccinea*) were collected on the Big Island outside Hilo along Saddle Road ($n = 1$) and Powerline Road ($n = 2$) and at the Mauna Loa Observatory ($n = 1$). On Kauai, Iiwi were sampled at Kokee State Park ($n = 5$), and on Maui ($n = 3$), samples were collected at Haleakala National Park. Oahu amakihi (*Hemignathus flavus*) were sampled at the Makiki Forest Recreation Area ($n = 7$) and Keaiwa State Recreation Area ($n = 6$). Kauai amakihi (*Hemignathus kauaiensis*) were sampled at Kokee State Park ($n = 8$). Maui amakihi (*Hemignathus virens wilsoni*) were sampled at Polipoli State Park ($n = 1$), Haleakala National Park ($n = 9$), Waikamoi Preserve ($n = 2$), and west Maui above Kaanapali ($n = 2$). Hawaii amakihi (*Hemignathus virens virens*) were sampled in the Kohala mountains ($n = 2$), the Waiakea Forest Preserve ($n = 2$), and Mauna Kea State Park ($n = 12$). We used the House finch (*Carpodacus mexicanus*) as a representative of the cardueline finch ancestor of the honeycreepers. Two blood samples were obtained from its introduced range at Pu'u Anahulu on the Big Island of Hawaii and 9 were obtained from across its native range in California.

Whole genomic DNA was extracted from blood or tissue samples using either a standard proteinase K-phenol/chloroform/isoamyl alcohol extraction method (Sambrook et al. 1989) or the DNeasy Tissue Extraction Kit (Qiagen, Valencia, CA). Although it would have been desirable to include other honeycreeper species in this study, it was not possible to obtain a sufficient number of samples given that many of the taxa are either extinct or critically endangered.

Microsatellite Amplification and Analysis

All samples were scored for 8 microsatellite loci developed for the Laysan finch, using amplification conditions as previously described (Tarr et al. 1998). Forward primers were fluorescently labeled and genotyping was done using GeneScan in an ABI377 DNA Sequencer (Applied Biosystems, Norwalk, CT). Allele sizes were analyzed in Genotyper 2.5. Genotype data were analyzed in GenePop 3.2a (Raymond and Rousset 1995) for evidence of linkage disequilibrium between loci or for significant deviations from Hardy–Weinberg expectations, and results were evaluated after a table-wide Bonferroni correction. Values of allelic diversity and observed heterozygosity were

determined in GenePop, and the average range of repeats was computed for each species by subtracting the smallest allele from the largest allele at each locus and averaging these values over all loci.

If ascertainment bias (Ellegren et al. 1995) were present in our data set, we would predict that the average number of alleles and the range of allele sizes would decline with increasing genetic distance from the species in which the loci were developed. As allelic diversity measures are strongly affected by unequal sample sizes (Leberg 2002), we used rarefaction in the computer program HP-RARE (Kalinowski 2005) to compute the allelic diversity for each species based in the smallest sample size in our data set ($n = 8$). These were tested for correlations with mtDNA-based genetic distances from the Laysan finch (K81uf + G, where $G = 0.1607$, based on the Akaike information criterion in MODELTEST [Posada and Crandall 1998; Fleischer RC, unpublished data]), the species in which these loci were characterized. Mitochondrial distances were based on 2241 bp of sequences from cytochrome *b* (675 bp), adenosine triphosphate synthase subunits 6 and 8 (858 bp), nicotinamide adenine dinucleotide dehydrogenase subunit 2 (457 bp), and the 5′ end of the control region (251 bp). Analyses and rate calibrations based on these mtDNA sequences for the same taxa are discussed in Fleischer RC, 1998, unpublished data. Analyses were performed without the values for the Laysan finch, a species that underwent a severe bottleneck approximately 80 years ago (Olson 1996) and thus is unlikely to have regained equilibrium levels of genetic diversity.

To investigate the divergence of microsatellite loci over evolutionary time, we used 5 methods of calculating genetic distances between species: 2 that focus on the sum of the frequencies of alleles shared between taxa (DC, Cavalli-Sforza and Edwards 1967; DAS, Jin and Chakraborty 1994a, 1994b) and are thus independent of mutation models; 1 that is expected to increase linearly over time under the infinite alleles model (DS, Nei 1987); 1 that was developed for microsatellites under the stepwise mutation model (R_{st}, Slatkin 1995); and 1 traditional method that has not been optimized for microsatellite loci (F_{st}). The measures DC, DAS, DS, and F_{st} have been evaluated in both simulation studies (Takezaki and Nei 1996) and empirical studies (Bowcock et al. 1994; Manica et al. 2005; Takezaki and Nei 2008) and found to be superior to other measures in recovering correct tree topologies. R_{st} was included for comparison of these measures with one based on the stepwise mutation model. Values for DC, DAS, and DS were determined in Populations 1.2.19 (Langella 2000). Values for R_{st} (Rho_{st} as defined in Rousset 1996 and estimated in Michalakis and Excoffier 1996) and F_{st} (as estimated in Weir and Cockerham 1984) were determined in GenePop 3.2a (Raymond and Rousset 1995). We used a Mantel test in XLSTAT2008 to test for correlations between these distances and genetic distance based on mtDNA sequences between species.

We then focused on 3 species pairs whose divergence time is based on the age of maximal shield building of the main Hawaiian islands, using the estimated dates in Price and Clague (2002): Oahu at 3.0 My, Maui at 2.0 My, and Hawaii at 0.5 My. The comparisons involve 3 species pairs: Kauai and Maui creepers (3.0 My), Oahu and Maui amakihi (2.0 My), and Maui and Hawaii amakihi (0.5 My). Genetic distances based on microsatellite loci were tested for correlations with divergence time.

Using the genetic distance matrices obtained for each method, phylogenetic trees based on microsatellite data were constructed using the neighbor joining algorithm in Populations 1.2.19 (Langella 2000). The resulting phylograms were visualized in TreeView (Page 1996).

To investigate patterns of microsatellite evolution at the DNA sequence level in the honeycreeper radiation, we cloned and sequenced one individual that was homozygous for the most common (modal) allele of each locus in each species using a Topo TA Cloning Kit (Invitrogen, Carlsbad, CA). In doing this, we assumed that the pattern of repeats and the flanking sequence in the modal allele were representative of the locus in that species. To avoid analyzing polymerase chain reaction artifacts (stutter bands or fragments that incorporated polymerase errors), at least 2 clones were selected and sequenced using the M13 forward and reverse primers. Only clones whose sequenced size matched the expected allele size were used in the analysis. Sequences were aligned and analyzed in Sequencher 4.1, and patterns of changes in both the repeat and the flanking regions were examined.

Results

No instances of linkage disequilibrium were detected between loci in any species. The average number of alleles per locus varied from 4.5 ± 2.0 (standard deviation [SD]) in the Laysan finch to 12.0 ± 3.0 (SD) in the Maui creeper (Table 1). When these were estimated for a sample size of 8 using rarefaction, the range became 3.0 (Kauai amakihi) to 5.5 (Maui creeper, Table 1). Heterozygosity averaged 0.607 (range, 0.409–0.771). Significant deviations from Hardy–Weinberg equilibrium were observed for locus 5A1B in Hawaii amakihi ($P = 0.0007$) and Maui amakihi ($P = 0.0000$), for locus 12B5E in Hawaii amakihi ($P = 0.0004$), and for loci 11B4E ($P = 0.0003$) and 11A4D ($P = 0.0008$) in Laysan finch. Further tests in GenePop showed that all these deviations were due to heterozygote deficiencies. As no population-level inferences were to be made in this study, individuals were chosen from several populations to obtain a broadly representative sample of the range of alleles present in each species. Thus, the deviations are not surprising and we do not believe they bias our results.

For the loci used in this study, neither the average number of alleles per locus ($R^2 = 0.0001$, $P = 0.975$) nor the average range of repeats ($R^2 = 0.0007$, $P = 0.940$) was significantly correlated with mtDNA genetic distance from the Laysan finch. Thus, we did not detect ascertainment bias in our panel of loci using what we believe would be highly sensitive measures in a study of closely related species.

Table 1. Allelic diversity (A), heterozygosity (Ho), and range of alleles for the species used in this study

Species	Number of samples	3A2C		11B1C		5A5A		4A4E		11B4E		12B5E		5A1B		11A4D		Average A, uncorrected	Average A, corrected	Average, Ho	Range of alleles (bp), average (range)
		A	Ho	A	Ho	A	Ho	A	Ho	A	Ho	A	Ho	A	Ho	A	Ho				
House finch	11	12	0.818	4	0.273	0	0.000	0	0.000	11	0.727	11	0.727	6	0.727	1	0.000	5.9 (4.9)	3.6	0.409	16.8 (6–36)
Kaui creeper	11	6	0.636	5	0.818	6	0.875	7	0.727	2	0.100	13	0.909	9	1.000	8	0.818	7.0 (3.2)	4.5	0.736	18.5 (2–36)
Maui creeper	20	13	0.700	9	0.800	13	0.900	14	0.667	7	0.500	16	0.850	10	0.750	14	1.000	12.0 (3.0)	5.5	0.771	28.8 (10–42)
Palila	21	6	0.810	8	0.810	7	0.650	7	0.952	1	0.000	7	0.667	7	0.762	6	0.667	6.1 (2.2)	3.7	0.665	15.0 (0–30)
Laysan finch	21	2	0.400	6	0.500	3	0.400	7	0.500	7	***0.550***	3	0.650	3	0.263	5	***0.368***	4.5 (2.0)	3.1	0.454	10.2 (2–30)
Apapane	17	14	0.706	10	0.917	15	1.000	7	0.410	5	0.706	12	1.000	11	***0.333***	10	0.667	10.5 (3.3)	5.5	0.717	31.2 (12–48)
Iiwi	12	5	0.622	8	0.589	6	0.822	5	0.933	5	0.756	5	0.356	9	0.589	8	0.689	6.6 (1.8)	4.1	0.669	23.5 (12–46)
Oahu amakihi	13	5	0.538	10	0.768	9	0.615	11	0.615	5	0.615	10	0.615	12	0.769	2	0.385	8.0 (3.6)	4.5	0.615	27.8 (2–48)
Kauai amakihi	8	2	0.625	2	0.375	6	0.750	4	0.375	2	0.000	7	0.438	4	0.500	2	0.375	7.3 (2.0)	3.0	0.430	8.5 (2–18)
Maui amakihi	14	11	0.857	7	0.714	6	0.714	6	0.357	4	0.563	12	0.714	8	***0.286***	2	0.285	7.8 (4.0)	4.6	0.561	20.0 (6–42)
Hawaii amakihi	16	12	0.938	11	0.812	9	0.500	9	0.563	8	0.750	11	***0.625***	6	***0.375***	3	0.625	8.5 (2.8)	4.8	0.649	20.5 (4–32)

Significant departures from values predicted under Hardy–Weinberg equilibrium are shown in bold italics. Values of the average number of alleles are presented before and after rarefaction was used to estimate the allelic diversity for each species based in the smallest sample size in our data set ($n = 8$).

Microsatellite-based genetic distances were found to be strongly correlated with mtDNA-based distance, regardless of the method used for computing microsatellite distances (Table 2, Figure 2). When we narrowed our analysis to the 3 species pairs whose divergence times were based on island ages, we also found strong correlations (Table 3, Figure 3). Because the methods of estimating genetic divergence based on microsatellite loci involve different sorts of measures, the resulting rates are not directly comparable.

The phylogeny based on mtDNA sequences (Figure 4A) shows strong bootstrap support for most of the traditional clades (i.e., the amakihi species and subspecies, the finch bills including the Laysan finch and Palila, the creepers, and the nectarivores including Apapane and Iiwi). Phylogenetic reconstructions based on microsatellite loci using DAS, DC, *F*st, and *R*st grouped the creepers into a single clade and the amakihi lineages into another clade but did not place the finch bills or the nectarivores into monophyletic clades (Figure 4C–F). The DS tree recovered the amakihi clade and the finch bill clade but not the creeper or nectarivore clades (Figure 4B). None of the microsatellite trees agreed with the mtDNA tree in the relationships among these clades, and none reflect the divergence patterns among amakihi species seen in the mtDNA tree.

Microsatellite allelic variation at the sequence level is summarized in Table 4. At many loci, conservation of modal allele size can be seen among closely related species, especially those of the amakihi complex. In most cases, this reflects a pattern of conservation of flanking sequence as well as of repeat length and composition. We found length polymorphism in the flanking region in only 2 loci. In the locus 3A2C, a $(TG)_3$ repeat in the 5′-flanking region was identical in all species but the House finch, in which it was found to be a $(TG)_4$. In the locus 11A4D, the 5′-flanking region contained a string of T's and an inserted sequence TGTT in 7 of the taxa.

Locus 11A4D was found to be monomorphic in the House finch, with only 7 repeats, but polymorphic with between 9 and 22 repeats in all other species. Following the divergence pattern seen in the mtDNA phylogeny, we traced the number of repeats in the modal alleles through the phylogeny (Figure 5). The large decrease in repeat number in the amakihi lineages was accompanied by a large decrease in the number of alleles.

Discussion

The microsatellite loci used for this study were developed in the Laysan finch, a species that underwent a severe bottleneck in the early 1900s after introduced rabbits decimated the vegetation on Laysan Island. When rabbits were removed in 1923, scientists estimated that only about 100 finches remained (Olson 1996). Thus, it is not surprising that the Laysan finch has fewer alleles and lower overall heterozygosity than many of the species in this study. The surprising finding is that these loci are highly variable across a large sampling of the Hawaiian honeycreepers. It is

Table 2. Correlations between microsatellite-based genetic distance values and mtDNA-based genetic distance (K81uf + G, where $G = 0.1607$)

	DC	DAS	F_{st}	R_{st}	DS
R^2	0.372	0.517	0.314	0.291	0.543
P	<0.0001	<0.0001	<0.0001	2.9×10^{-4}	<0.0001

Microsatellite-based distances are DAS = Jin and Chakraborty's (1994a, 1994b) allele sharing distance, F_{st} = as estimated in Weir and Cockerham (1984), R_{st} = Rho_{st} (Rousset 1996, Michalakis and Excoffier 1996), DC = Cavalli-Sforza and Edwards (1967) chord distance, and DS = Nei's (1987) standard distance. Correlation coefficients and probability values were computed using a Mantel test in XLSTAT2008.

interesting to speculate that by isolating long and polymorphic microsatellites in a severely bottlenecked honeycreeper species, Tarr et al. (1998) characterized loci that may be among the most variable in the radiation as a whole.

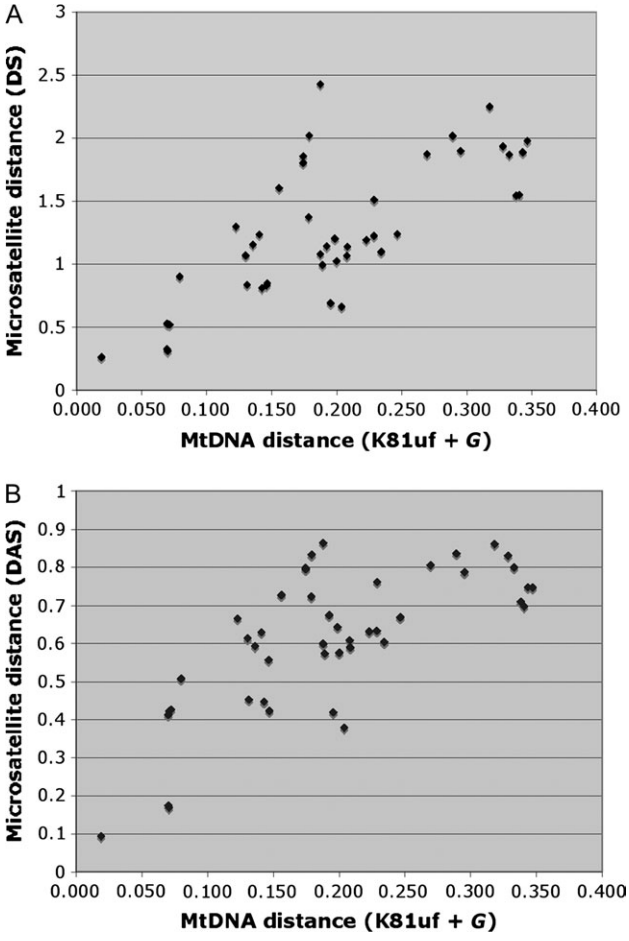

Figure 2. The relationship between genetic distances derived from microsatellite loci and the genetic distance based on mtDNA sequences (K81uf + G, where $G = 0.1607$): (**A**) Nei's (1987) standard distance, DS, and (**B**) Jin and Chakraborty's (1994a, 1994b) allele sharing distance, DAS.

The lack of ascertainment bias with respect to allelic diversity and allele size ranges is also surprising but is consistent with the results of Zhu et al. (2000), who found little or no evidence for a correlation between overall repeat region length and a measure of genetic distance from the species in which the loci were cloned. Their study did, however, find a negative correlation between the number of perfect repeats and genetic distance from the original species. We did not examine this in our study as 3 of the 8 were characterized in the Laysan finch as imperfect or compound microsatellites, and an additional locus was found to have an imperfect motif in the modal Laysan finch allele.

Largely due to allele size constraints and homoplasy, it has been proposed that genetic distance measures for microsatellite loci will lose their linear relationship with time after several thousand generations (Nauta and Weissing 1996; Feldman et al. 1997). Using simulated data, Goldstein et al. (1995) found that DAS and DS approached an asymptote within 2500 generations. Simulations by Takezaki and Nei (1996) found that DS increases linearly with time under the infinite alleles model of microsatellite evolution but that the rate of increase quickly diminishes in DC under both the infinite alleles model and the stepwise mutation model.

In the alleles we sequenced, we found no instances of more than 22 repeats and multiple instances in which the longest microsatellite alleles contained imperfect motifs, both of which may be the result of length size constraints. We also found evidence for allele size homoplasy at every locus (Table 4). We might expect distance measures that are based on the frequencies of shared alleles to be more sensitive to these factors and to lose their linear relationship with time. However, the 2 distance measures that are based on the sum of the frequencies of shared alleles (DC and DAS) were strongly correlated with mtDNA sequence divergence as well as having the most linear relationships with divergence time for the 3 species pairs. F_{st}, R_{st}, and DS were also strongly correlated with mtDNA sequence divergence but were less strongly correlated with divergence time for the same species pairs.

Examining patterns of diversity for locus 11A4D, we can see stages in the "life cycle" of a microsatellite locus (Zhu et al. 2000; Sokol and Williams 2005). Figure 5 illustrates changes in repeat number and the number of alleles along the divergences of species as inferred from the mtDNA phylogeny. In the House finch, this locus was monomorphic, having only 7 repeats. It expanded in the species of the honeycreeper radiation, with both the Maui creeper and the Iiwi having 22 perfect repeats. In the Laysan finch, however, the modal allele contains mutations that break up the perfect array. The number of repeats dropped dramatically in the amakihis, as did the number of alleles and (in all but the Hawaii amakihi) the heterozygosity. This decreased variability is likely the result of the decreased mutation rates that would be predicted for shorter alleles. In Hawaii amakihi, the modal allele is unchanged, but a third allele, one repeat longer, was found at low frequency. If the

Table 3. Genetic distance values used in the tests for correlations between microsatellite-based genetic distances and divergence time for 3 species pairs

Taxon split	Time (My)	DC	DAS	F_{st}	R_{st}	DS
Kauai creeper–Maui creeper	3.0	0.587	0.423	0.086	0.123	0.725
Oahu amakihi–Maui amakihi	2.0	0.539	0.183	0.044	0.032	0.339
Maui amakihi–Hawaii amakihi	0.5	0.488	0.028	0.028	0.011	0.302
	R^2	0.991	0.944	0.870	0.803	0.719
	Rate	0.039	0.154	0.022	0.019	0.157

Estimates of divergence time are based on maximal shield building of the main Hawaiian Islands of Oahu, Maui-Nui, and Hawaii (Fleischer et al. 1998; Price and Clague 2002).

locus does not suffer mutations that interrupt its perfect repeat motif, over time we might expect that mutation will replenish allelic diversity.

Simulation results have indicated that as many as 50–100 loci may be needed to recover a well-supported phylogeny for intraspecific studies, whereas 300 or more polymorphic loci would be needed for one that included different species (Takezaki and Nei 1996). Empirical studies, however, have indicated that fewer loci may actually be needed to obtain phylogenetic reconstructions that are as well supported as those developed using more traditional methods. Petren et al. (1999) analyzed 16 microsatellite loci to infer phylogenetic relationships between 13 of 14 species of Darwin's finches, an adaptive radiation that may have occurred over as long as 3 My (Grant 1994). Previous studies in this group using mtDNA and nuclear DNA sequences had failed to resolve relationships between the more closely related species. The work of Petren et al. (1999) supported the monophyly of the group and agreed with other studies based on morphology, plumage, and song.

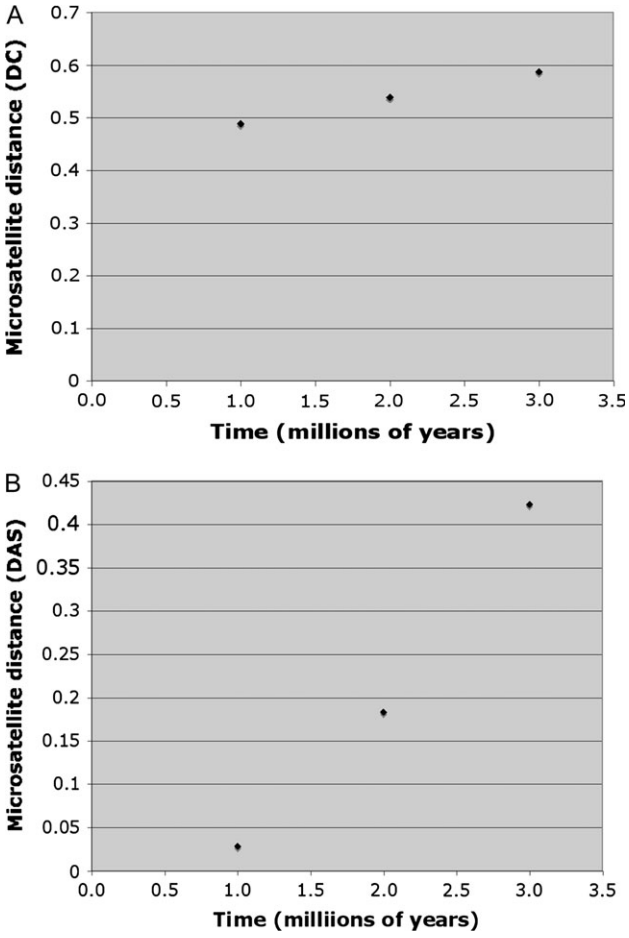

Figure 3. The relationship between genetic distances derived from microsatellite loci and the divergence time of 3 species pairs based on island age: (**A**) Cavalli-Sforza and Edwards (1967) chord distance, DC, and (**B**) Jin and Chakraborty's (1994a, 1994b) allele sharing distance, DAS.

Schlötterer (2001) cites several other such examples. On the whole, it appears that some taxonomic groups may require fewer loci for accurate phylogenetic reconstruction than previously thought (Schlötterer 2001; Koskinen et al. 2004). For many wild species, the availability of microsatellites may be similar to the number of loci used in our study. When analyzing only allele sizes, our results indicate that 8 loci may be sufficient to resolve relationships between very closely related species or subspecies. For more distantly related species, however, they confirm previous suggestions that more loci will be needed (but see Ochieng et al. 2007).

At the sequence level, changes in flanking sequences, repeat motif, or patterns of interrupted repeats may provide data that will help to resolve phylogenetic relationships (Makova et al. 2000). Caution is needed, however, as flanking regions up to 50 bp on either side of microsatellites have been found to have both increased mutation rates and nonrandom evolutionary patterns (Vowles and Amos 2004). Results of that study suggest that loci with 10 or more repeats are not as strongly affected and will be less likely to confound phylogenetic analyses. In our study, the node that joins the amakihi group to the finch bills in our mtDNA tree is not supported by strong patterns of microsatellite allele sharing, and this group does not appear in most trees based on allele lengths. At the sequence level, however, we found that the Laysan finch, Kauai amakihi, and Maui amakihi share a unique repeat motif at the locus 5A1B. Thus, sequence data from this locus provides support for a close genetic relationship between the 2 clades that could not be detected when scoring microsatellite alleles based on size polymorphism.

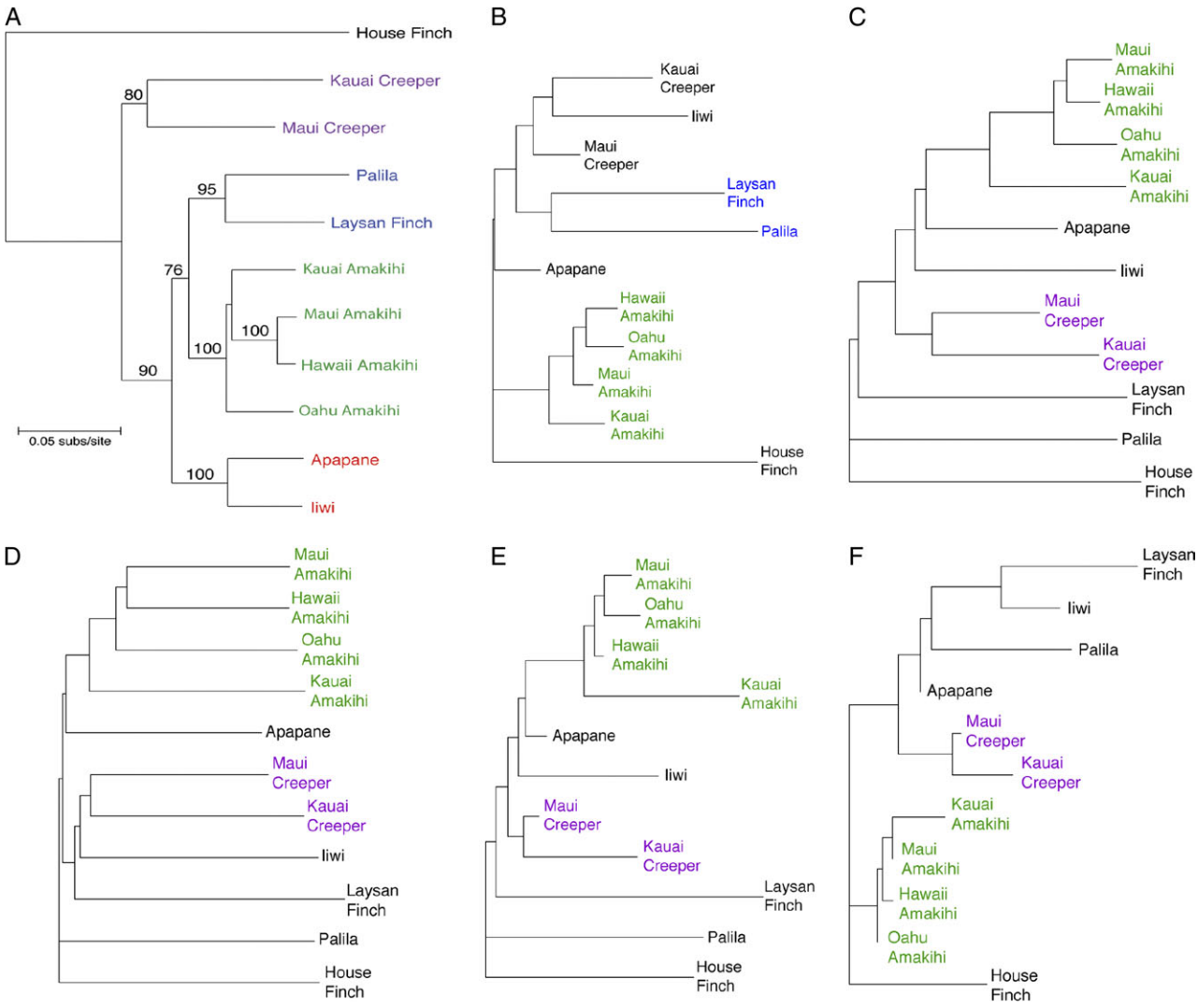

Figure 4. Phylogenetic inferences using (**A**) mtDNA sequences (K81uf + *G*, where $G = 0.1607$), bootstrap values from 500 replicates are shown above the nodes; (**B**) DS (Nei 1987); (**C**) DAS (Jin and Chakraborty 1994a, 1994b); (**D**) DC (Cavalli-Sforza and Edwards 1967); (**E**) F_{st} (Weir and Cockerham 1984); and (**F**) R_{st} (Slatkin 1995, as defined in Rousset 1996 and estimated in Michalakis and Excoffier 1996).

However, our results also demonstrate that sequence-level variation at one or a small number of microsatellite loci is not likely to be sufficient to resolve difficult phylogenetic relationships. Tarr and Fleischer (1993) were unable to determine whether the Oahu amakihi was derived with respect to the Kauai amakihi, or vice versa, based on mtDNA restriction site variation. Our mtDNA tree is based on over 2 kb of sequences from multiple genes, yet there is no strong bootstrap support for the relationship between these species. This ambiguity was echoed in conflicting results between microsatellite loci. At locus 3A2C, Oahu, Maui, and Hawaii amakihi share a unique mutation in the flanking region as well as identical repeat motifs. At locus 4A4E, Kauai, Maui, and Hawaii amakihi share repeat patterns. At locus 5A1B, Kauai and Maui amakihi have modal alleles that are identical in size and interrupted repeat motif, whereas Oahu and Hawaii amakihi have uninterrupted repeats. At locus 11B1C, Oahu, Maui and Hawaii amakihi share modal alleles that are identical in both flanking region and the complex repeat motif. Thus, neither mtDNA sequences nor the sequences of 8 nuclear microsatellite loci have been sufficient to conclusively resolve the relationship between these taxa.

Our results support the notion that the processes that generate germ-line mutations in microsatellite loci also appear to operate over evolutionary time. Within the bounds of probable length constraints, the loci we surveyed were highly variable within the honeycreeper radiation, and

Table 4. Values of the most common (modal) allele and patterns of change in the flank and repeat regions of the microsatellite loci used in this study

	3A2C			4A4E			5A1B			5A5A		
	Modal allele	Flank	Repeat region	Modal allele	Flank	Repeat region	Modal allele	Flank	Repeat region	Modal allele	Flank	Repeat region
House finch	104	A*	$(GT)_{11}$	**	n/a	n/a	95	A	$(GT)_{20}$	**	n/a	n/a
Kauai creeper	108	B	$(GT)_{14}$	91	A	$(GT)_2GC(GT)_{11}AT(GT)_2$	85	B	$(GT)_{15}$	100	A	$(GT)_{14}GA$
Maui creeper	104	B	$(GT)_{12}$	93	A	$(GT)_2(GC)_4(GT)_9AT(GT)_2$	85	B	$CT(GT)_{14}$	102	A	$(GT)_{16}$
Palila	106	B	$(GT)_{13}$	105	A	$(GT)_2GC(GT)_{21}$	93	B	$(GT)_{19}$	116	A	$(GT)_{13}TT(GT)_9$
Laysan finch	102	B	$(GT)_{11}$	103	A	$(GT)_2(GC)_3(GT)_1GC(GT)_{12}$ $AT(GT)_3$	89	B	$(GT)_{14}AT(GT)_2$	104	A	$(GT)_{17}$
Apapane	104	B	$(GT)_{12}$	109	A	$(GT)_2GC(GT)_{20}AT(GT)_2$	91	B	$(GT)_{18}$	114	A	$(GT)_{22}$
Iiwi	96	B	$(GT)_8$	89	A	$(GT)_2GC(GT)_{13}$	81	B	$(GT)_{12}CA$	108	A	$(GT)_{19}$
Oahu amakihi	108	C	$(GT)_6TT(GT)_7$	95	A	$(GT)_2GC(GT)_{16}$	91	B	$(GT)_{18}$	112	A	$(GT)_{20}GC$
Kauai amakihi	106	B	$(GT)_7TT(GT)_5$	95	A	$(GT)_2GC(GT)_{13}AT(GT)_2$	85	B	$(GT)_{12}AT(GT)_2$	112	A	$(GT)_{21}$
Maui amakihi	108	C	$(GT)_6TT(GT)_7$	95	A	$(GT)_2GC(GT)_{13}AT(GT)_2$	85	B	$(GT)_{12}AT(GT)_2$	116	A	$(GT)_{22}GC$
Hawaii amakihi	108	C	$(GT)_6TT(GT)_7$	93	A	$(GT)_2GC(GT)_{12}AT(GT)_2$	95	B	$(GT)_{20}$	114	A	$(GT)_{22}$
	11A4D			11B1C			11B4E			12B5E		
	Modal allele	Flank	Repeat region	Modal allele	Flank	Repeat region	Modal allele	Flank	Repeat region	Modal allele	Flank	Repeat region
House finch	61	A*	$(GT)_7$	102	A	$(GT)_4CTGTGTGC$	98	A	$(GT)_{18}CT$	116	A	$(GT)_{12}GC$
Kauai creeper	73	B*	$(GT)_{12}$	114	B	$(GT)_{12}(GC)_2$	88	B	$(GT)_{14}$	114	B	$(GT)_6GTACGTAT$ $(GT)_9(GC)_2$
Maui creeper	93	B*	$(GT)_{22}$	110	B	$(GT)_2GA(GT)_5(GTGC)_2$	90	B	$(GT)_{15}$	120	C	$(GT)_4(GTAT)_2$ $(GT)_{14}(GC)_2$
Palila	77	C*	$(GT)_{15}$	132	B	$(GT)_{13}(GTGC)_5$	84	B	$(GT)_{12}$	122	C	$(GT)_6(GTAT)_3$ $(GT)_9(GC)_4$
Laysan finch	97	D	$(GT)_{18}(TTGT)_2$	132	B	$(GT)_{15}(GTGC)_4$	90	B	$(GT)_{15}$	134	C	$(GT)_6GCAT$ $(GT)_{21}(GC)_2$
Apapane	83	D	$(GT)_{15}$	128	B	$(GT)_{15}(GTGC)_3$	90	B	$(GT)_{15}$	108	C	$(GT)_2GCGTGTAT$ $(GT)_{10}(GC)_2$
Iiwi	97	D	$(GT)_{22}$	128	B	$(GT)_{15}(GTGC)_3$	90	B	$(GT)_{15}$	114	C	$(GT)_4GTAT$ $(GT)_{13}(GC)_2$
Oahu amakihi	71	D	$(GT)_9$	118	B	$(GT)_{12}(GC)_2GTGC$	90	B	$(GT)_{15}$	100	C	$(GT)_{12}(GC)_2$
Kauai amakihi	71	D	$(GT)_9$	128	C	$(GT)_2GG(GT)_8$ $GG(GT)_5(GTGC)_2$	90	B	$(GT)_{15}$	108	C	$(GT)_{16}(GC)_2$
Maui amakihi	71	D	$(GT)_9$	118	B	$(GT)_{12}(GC)_2GTGC$	90	B	$(GT)_{15}$	104	C	$(GT)_{13}(GC)_3$
Hawaii amakihi	71	D	$(GT)_9$	118	B	$(GT)_{12}(GC)_2GTGC$	90	B	$(GT)_{15}$	104	C	$(GT)_{13}(GC)_3$

Flanking regions were classified based on point mutations, only those shown with an asterisk contain insertions or deletions. Double asterisk denotes those species for which a locus did not amplify. Sequences correspond to GenBank accession FJ596990-FJ597067. n/a, not applicable.

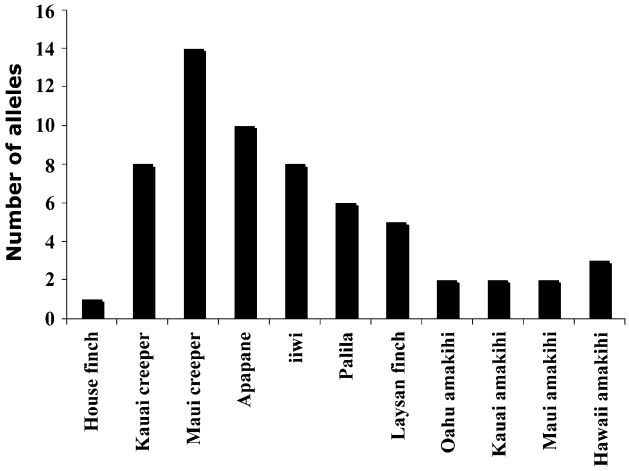

Figure 5. A single allele was found at locus 11A4D in the House finch. Within the honeycreeper radiation, allele number appears to have increased until there was a major decrease in repeat size accompanied by a decrease in allele number in the amakihi lineages.

ascertainment bias was not evident. Although genetic distance measures based on microsatellite loci had a strongly linear relationship with divergence time up to 4.7 My, phylogenetic trees based on microsatellite genetic distances recovered only some of the major clades resolved using mtDNA sequences and differed in the relationships among those clades. In examining the patterns of variability between species, we found that changes in flanking sequences, repeat motifs, or patterns of interrupted repeats may be phylogenetically informative. Thus, although loci may be useful in population-level studies of species that diverged from the species in which they were characterized as long as 5 Ma, we caution that their use in phylogenetic inference should be limited to closely related species.

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