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Natural selection of the major histocompatibility complex (*Mhc*) in Hawaiian honeycreepers (Drepanidinae)

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Abstract

The native Hawaiian honeycreepers represent a classic example of adaptive radiation and speciation, but currently face one the highest extinction rates in the world. Although multiple factors have likely influenced the fate of Hawaiian birds, the relatively recent introduction of avian malaria is thought to be a major factor limiting honeycreeper distribution and abundance. We have initiated genetic analyses of class II β chain *Mhc* genes in four species of honeycreepers using methods that eliminate the possibility of sequencing mosaic variants formed by cloning heteroduplexed polymerase chain reaction products. Phylogenetic analyses group the honeycreeper *Mhc* sequences into two distinct clusters. Variation within one cluster is high, with $d_N > d_S$ and levels of diversity similar to other studies of *Mhc* (*B* system) genes in birds. The second cluster is nearly invariant and includes sequences from honeycreepers (Fringillidae), a sparrow (Emberizidae) and a blackbird (Emberizidae). This highly conserved cluster appears reminiscent of the independently segregating *Rfp-Y* system of genes defined in chickens. The notion that balancing selection operates at the *Mhc* in the honeycreepers is supported by transpecies polymorphism and strikingly high d_N/d_S ratios at codons putatively involved in peptide interaction. Mitochondrial DNA control region sequences were invariant in the i'iwi, but were highly variable in the 'amakihi. By contrast, levels of variability of class II β chain *Mhc* sequence codons that are hypothesized to be directly involved in peptide interactions appear comparable between i'iwi and 'amakihi. In the i'iwi, natural selection may have maintained variation within the *Mhc*, even in the face of what appears to a genetic bottleneck.

Keywords: avian *Mhc*, balancing selection, Drepanidinae, Hawaiian honeycreepers, mtDNA, *Plasmodium*

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Introduction

Genes encoded within the major histocompatibility complex (*Mhc*) are thought to undergo positive or balancing selection and a growing body of evidence suggests that this selection is mediated largely through differential recognition by Mhc antigens of peptides from pathogens (Hughes & Nei 1988; Kaufman & Salomonsen 1997; Gilbert *et al*. 1998; Hill & Weatherall 1998; Hughes & Yeager 1998; Hedrick & Kim 2000; Vogel *et al*. 1999). The evidence for balancing selection acting on the *Mhc* is mostly indirect, and includes the relatively high number of alleles and sequence polymorphism, the long-term retention of alleles (i.e. transpecies polymorphism) and relatively high rates of nonsynonymous (d_N) vs. synonymous (d_S) substitution within the peptide-binding region (PBR) (Hedrick 1994, 1999; Hughes & Nei 1988; Hughes & Yeager 1998). Only rarely have cases of epidemic or introduced disease been used to determine the effects of such selection on variation at the *Mhc* (reviewed in: Gilbert *et al*. 1998; Hill & Weatherall 1998; Brown 1999; Vogel *et al*. 1999).

The Hawaiian honeycreeper-malaria disease system provides an exceptional model in which the influences of parasite-driven selection on host genes can be investigated. The Hawaiian honeycreepers are an endemic clade

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of finches renowned for their extensive and rapid adaptive radiation (Freed *et al*. 1987; Tarr & Fleischer 1995; Fleischer *et al*. 1998; Fleischer & McIntosh 2001). The honeycreepers have been severely impacted by the activities of humans: 28 of the 53 known species (34 known from historical specimens, 19 from fossils) are extinct, and 18 of the remaining species are endangered (Jacobi and Atkinson 1995; Jarvi *et al*. 2001). Although direct take, habitat loss and degradation, introduced predators and competitors have all been implicated in the extinction and endangerment of honeycreepers, invasive disease, in particular introduced, mosquito-transmitted avian malaria (*Plasmodium relictum*), is thought to be the most detrimental factor limiting honeycreeper distribution and abundance today (Warner 1968; van Riper *et al*. 1986; Atkinson *et al*. 1995, 2000, 2001; Yorinks & Atkinson 2000; Jarvi *et al*. 2001). While Hawaiian strains of *P. relictum* appear to cause severe disease in Hawaiian honeycreepers relative to introduced bird species (Atkinson *et al*. 1995; van Riper 1991), there is also variation in susceptibility among honeycreeper species (Atkinson *et al*. 1995, 2000, 2001; Yorinks & Atkinson 2000; Jarvi *et al*. 2001). In particular, the 'amakihi (*Hemignathus virens*) has relatively lower mortality rates than its close relative (Fleischer *et al*. 1998, 2001) the i'iwi (*Vestiaria coccinea*) following experimental infections with *P. relictum* (Atkinson *et al*. 1995, 2000, 2001). This dichotomy of malaria resistance in two closely related honeycreeper species sets up an opportunity to begin to define host genetic polymorphisms related to disease resistance and to evaluate the effects of parasite-driven selection on host genes. This study compares levels and patterns of genetic variation of *Mhc* class II β chain peptide-binding region and mitochondrial DNA (mtDNA) control region sequence in four species of Hawaiian honeycreepers.

Materials and methods

Sampling

Whole blood was collected from natural populations of 'amakihi, 'apapane (*Himatione sanguinea*), i'iwi and palila (*Loxiodes bailleui*) from the island of Hawai'i. As part of a study of pathogenicity of avian malaria in honeycreepers (Atkinson *et al*. 1995), juvenile hatch-year i'iwi ($n = 9$) were captured with mist nets on the eastern flank of Mauna Loa volcano in high elevation (1800 m) forests. This elevation is near the upper elevational limits of *Culex quinquefasciatus*, the primary vector of avian malaria in Hawai'i (van Riper *et al*. 1986), and has mean yearly temperatures that are below the lower thermal limits needed to support sporogeny of the parasite in the vector (Benning *et al*. 2002). The 'apapane ($n = 1$) and 'amakihi ($n = 10$) were also involved in pathogenicity studies (Atkinson *et al*. 2000), and were captured in xeric, high-elevation habitat (2000 m) on Mauna Kea volcano. The palila ($n = 8$) were mist-netted in dry woodland forests on Mauna Kea and released (Fleischer *et al*. 1994). Approximately 50–100 μL of blood was collected by jugular or brachial venepuncture. Blood samples were also obtained from one captive bred family of 'amakihi. The dam originated from Pohakuloa, the sire from Mauna Kea, and eight progeny were produced at the Conservation and Research Center, Smithsonian Institution, Front Royal, VA (Scott Derrickson, personal communication). After collection, heparinized samples were mixed with an approximate equal volume of lysis buffer (0.1 M Tris–HCl pH 8.0, 0.1 M sodium EDTA, 2% SDS) and frozen (–50 °C).

DNA isolation

Genomic DNA was extracted from blood samples using standard techniques. Briefly, samples were digested in 1 mL of 50 mM Tris–HCl, 100 mM sodium EDTA, 2% SDS with 0.5 mg/mL proteinase K overnight at 55 °C. DNA extractions were completed with one phenol, two phenol/dimethyl chloride 1 : 1, and one dimethyl chloride/isoamyl alcohol (24 : 1) extraction steps, and dialysed against 10 mM Tris–HCl, pH 8.0 and 1 mM EDTA. DNA concentration was quantified by spectrophotometry (Spectronic Instruments).

Amplification and cloning of partial class II B genes for primer design

Degenerative priming sequences #305 and #306 (Edwards *et al*. 1995a) were initially used for polymerase chain reaction (PCR) amplification from ~ 100 ng total genomic DNA from two individual 'amakihi under conditions described in Edwards *et al*. (1995a). Bands of ~ 180 bp were excised from a 1.5% LMP agarose gel and purified (Glass milk, Geneclean or Qiaquick Gel Extraction Kit, Qiagen Inc.). Purified PCR products were cloned (TA cloning kit, Invitrogen) and sequenced (as described below). Internal primers PBR1.5 (TGAACGGGTGAGGTTCGT) and PBR1.3 (GTACGTGTCCACCGCTGT) were then designed based on the sequences of several clones. The sequences reported in this paper were obtained using these core priming sequences.

PCR + 1 cloning

To specifically avoid the inclusion of mosaic (artefactual) sequences due to the cloning of heteroduplexes, formed when single-stranded DNA from highly similar but distinct alleles re-anneal after the last round of PCR, most of the sequences reported in this study were obtained using either PCR + 1 cloning methods (Borriello & Krauter 1990; L'Abbe *et al*. 1992) or strand separation by SSCP electrophoresis and subsequent re-amplification prior to

Table 1 The origin and number of sequences included in this study. Homoduplexes (HD) were obtained by PCR + 1 cloning methods. Other sequences were obtained by direct cloning of PCR products (unconfirmed homoduplexes, UHD) or by single-stranded gel isolation and direct sequencing of product (SSCP). Species: Hevi (*Hemignathus virens*), Veco (*Vestiaria coccinea*), Hisa (*Himatione sanguinea*) and Loba (*Loxioides bailleui*). Sequences/individual obtained are indicated numerically. Sequences obtained from members of a family are indicated by s (sire), d (dam) or p (progeny) and those obtained from cDNA are indicated by (c)

Sequence	#HD	#UHD	#SSCP	Sequence	#HD	#UHD	#SSCP
Hevi 1	2	0	0	Hevi 16.1p	0	3	0
Hevi 2	2	0	0	Hevi 17.1p	0	2	0
Hevi 2.1	2	0	0	Hevi 17.2p	0	2	0
Hevi 3	2	0	0	Hevi 18.1p	0	1	1
Hevi 4	3	1	0	Hevi 19.1p	0	3	0
Hevi 4.1	1	5	0	Hevi 21.2c	1	0	0
Hevi 5	1	0	0	Hevi 23.6c	1	0	0
Hevi 5.1	0	3	0	Veco 1	2	2	1
Hevi 6	1	0	0	Veco 2	1	1	1
Hevi 6.1	1	0	0	Veco 2.1	1	0	0
Hevi 7	1	2	0	Veco 2.2	1	1	0
Hevi 8	1	0	0	Veco 3	1	0	0
Hevi 9	1	0	0	Veco 4	1	0	0
Hevi 9.1	0	0	1	Veco 4.1	0	0	1
Hevi 10	1	2	0	Veco 4.2	0	0	1
Hevi 11.2 s	0	0	1	Veco 4.3	0	0	1
Hevi 11.3 s	0	0	1	Veco 4.4	0	0	1
Hevi 11.4 s	0	0	1	Veco 5	0	0	1
Hevi 11.6 s	0	2	0	Veco 6	0	0	2
Hevi 12.1d	0	0	2	Veco 7	0	0	2
Hevi 12.3d	0	0	1	Veco 9	0	0	1
Hevi 12.4d	0	6	0	Hisa 1	2	0	0
Hevi 13.1p	0	3	0	Hisa 1.1	2	0	0
Hevi 14.1p	0	2	0	Hisa 1.2	0	2	0
Hevi 15.1p	0	0	1	Loba 1	0	0	8
Hevi 15.2p	0	4	0				

sequencing (Grace *et al*. 1995; Table 1). Some sequences were obtained by direct cloning but were only included in this study if they were verified by replication (Table 1). Replication of a sequence involved duplicate PCR amplification with the same template DNA followed by cloning and sequencing as described. Using methods described by Borriello & Krauter (1990) PCR + 1 amplification of the target DNA was carried out as follows: asymmetric amplification was carried out using primers PBR1.5Bam (GGATCCTGAACGGGTGAGGTTCGT) 2 μL of 2 μM and PBR1.3Sal (GTCGACGTACGTGTCCACCGCTGT) 2 μL of 20 μM in 50 μL reaction volumes following manufacturer's protocols (Perkin-Elmer Cetus) with a 5 min 'hot start' at 94 °C prior to the addition of *Taq* DNA polymerase. The inclusion of *Bam*HI and *Sal*I restriction sites is unrelated to the PCR + 1 protocol, but were found to improve primer annealing. Cycling conditions were 94 °C for 1 min, 52 °C for 2 min and 72 °C for 3 min for 35–40 cycles. Twenty microlitres was subjected to one round of reamplification in a 50 μL total volume using a third primer 1.5Mlu (ACGCGTTGAACGGGTGAGGTTCGT) with similar parameters. Products were gel-purified (1.5% LMP NuSieve, FMC BioProducts; Qiaquick Gel Extraction Kit, Qiagen) and cloned (as described above) in X-L Blue competent cells. Clones were evaluated for the presence of the *Mlu*I site by restriction digest analysis and only clones containing the *Mlu*I site, which served as a marker for homoduplexed DNA, were used for further analysis.

Preparation of cDNA library

To prepare an 'amakihi cDNA library, RNA was isolated with Rna-zol Btm (Cinna Scientific, Inc) from a mid- to late-development embryo collected from the Pohakuloa region on the flank of Mauna Kea volcano, Hawai'i. mRNA was purified (Polytract mRNA Isolation systems, Promega) and a cDNA library constructed with the Zap Express cDNA synthesis kit (Stratagene).

Single-stranded polymorphism assays (SSCP)

PCR products of the approximate expected size produced using primer set PBR1.5Bam/PBR1.3Sal were gel purified as before, and quantified visually in a 1.5% agarose gel. SSCP conditions and sequence isolation were carried out as described in Grace *et al*. (1995), and following

manufacturer's protocol (Novex™, San D iego, CA) Briefly, ~ 50–100 ng DNA (5 μL direct PCR product or 10 μL purified product) was added to load dye containing 95% formamide, 20 μM EDTA, 10 μM NaOH and 0.1% dye (bromophenol blue and xylene cyanol, w/v) up to a total volume of 20 μL. The sample was then fully denatured by boiling for 4 min with immediate quenching on ice until loaded into a Novex™ acrylamide gel. Generally, 4–20% Novex™ gradient gels were used at a constant running temperature of 15 °C for 2 h at 200 V. Gels were stained with Syber® Green II (FMC Bioproducts). A 1 mL aerosol-resistant pipette tip was used to core out a sample from the band of interest. PCR re-amplification with cycling parameters of 94 °C for 30 s, 50 °C for 1 min, 72 °C for 1 min. The products were then purified as described above and sequenced.

Nucleotide sequence determination

All nucleotide sequences were determined by the PCR dye-terminator method on a ABI 373 automated sequencer using plasmid-derived primers for cloned sequences and primers PBR1.5 and PBR1.3 for SSCP-derived sequences. Sequences were aligned, compared and corrected using SEQUENCHER (Gene Codes Corp.) or SEQ-ED (version 3.0.1, Applied Biosystems). Sequences were translated and checked for stop codons or other evidence that they might be pseudogenes.

Measures of diversity

Aligned and corrected DNA sequences were analysed using MEGA 2.0 (Kumar *et al*. 2000) and ARLEQUIN 2.000 (Schneider *et al*. 2000). Measures of genetic diversity were calculated from various groupings of the sequences, and compared with variation in other avian taxa, including Darwin's finches (Geospizinae) exon 2 sequences (GenBank Accession nos Z74411–Z74470; Vincek *et al*. 1997) and others (*Acrocephalus arundinaceus* U24405–8, *Agelaius phoeniceus* U24412–3, U24415–9, U24427, *Aphelocoma coerulescens* U24399–401, U24420–26, *Carpodacus mexicanus* U24409–11, U23968–9, U23976; Edwards *et al*. 1995b). We divided the Hawaiian drepanidine sequences by species and use the following acronyms: 'amakihi, Hevi; i'iwi, Veco; 'apapane, Hisa; and palila, Loba. Captive-bred 'amakihi were used for phylogenetic analyses but removed from estimates of variation.

Analyses were made on all 45 PBR codons, and on the 13 hypothesized (Brown *et al*. 1993) peptide-binding codons (PBCs) and 32 nonpeptide-binding codons (non-PBCs) separately. In addition, based on tree topology (see below), honeycreeper sequences fell into two very differentiated clusters. These clusters also appear to differ in degree of within-cluster diversity, so analyses were also made on each cluster separately. Darwin's finch sequences also showed two clusters, one of which appeared to have low diversity. We analysed these sequences separately as well. We estimated d_S and d_N using the method described by Kumar *et al*. (2000) in the computer program MEGA, which corrects for multiple substitutions at a site, transition/transversion bias, and degeneracy. We also calculated Kimura 2-parameter (K2P) distances on all substitutions.

Phylogenetic relationships

We assessed the relationships of *Mhc* DNA sequences by constructing a neighbour joining tree (Saitou & Nei 1987) from K2P corrected distance matrices using PAUP*. In addition to the drepanidine sequences obtained in this study, we included 60 Darwin's finch sequences (Vincek *et al*. 1997), one sparrow sequence Pasa39.43 (AF420007, Freeman-Gallant *et al*. 2002), one blackbird sequence Agph117.5 (U24418) and two galliform sequences used to root the tree (*Phasianus cochicus*, X75404 and *Gallus domesticus* BLBIIB, X07447).

Mitochondrial control region sequence determination

We amplified portions of the mitochondrial control region (CR) for both i'iwi and 'amakihi using primers from Tarr (1995). We compared sequences of 11 i'iwi samples from the islands of Kaua'i ($n = 4$), Maui ($n = 3$) and Hawai'i ($n = 4$) with sequences of 11 'amakihi samples from Kaua'i ($n = 1$), Maui ($n = 4$) and Hawai'i ($n = 6$). Two sections of the CR were amplified: (i) part of the 5′ or left domain using primers LGL4 (5′-CAATTGTATTCGCCCACCTC-3′) or LGL2 and H417 (Tarr 1995); and (ii) part of the 3′ or right domain using primers LCR1 and H1248 (Tarr 1995). Amplified products were cleaned with Qiagen kits and sequenced using either manual or automated methods (see above). Sequences were aligned in SEQUENCHER AND exported for analyses of variation within taxa and populations. Means and SEs of Tamura–Nei and γ-corrected distances within each taxon and population were calculated in MEGA (Kumar *et al*. 2000).

Results

PCR amplification, and cloning of partial Mhc class II β genes

A total of 51 cloned *Mhc* or SSCP products are included in this study, originating from 38 different individuals representing four species of Hawaiian honeycreepers.

The sequence length we determined was 135 bp, of which 58 nucleotide sites were variable in the honeycreepers. An alignment of the 45 unique inferred amino acid sequences

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Hevi9         KRFIYNREQY VHFDSDLGHF VGDTPYGEEV ARYWNSDPEW MEHRR
Hisa1         .......... .......... .......... .......... .....
Hevi8         .......... .......... .......... .......... .....
Hevi7         .......... .......... .......... .......... .....
Hevi6.1       .......... .......... .......... .......... .....
Veco4.3       .......... .......... .......... .......... .....
Hevi10        .......... .......... .......... .......... .....
Hevi4.1       .......... .......... .......... .......... .....
Hevi11.2s     .......... .......... .......... .......... .....
Hevi11.6s     .......... .......... .......... .......... .....
Hevi12.4d     .......... .......... .......... .......... .....
Hevi16.1p     .......... .......... .......... .......... .....
Hevi17.2p     .......... .......... .......... .......... .....
Loba1         .K........ .......... .......... .......... .....
Veco2.2       .......... .......... .......... .......... ....Q
Hevi2.1       .......... ........R. .......... .T........ .....
Hevi19.1p     .......... .......... .......... .T........ .....
Pasa39.43     .......... ......V... .......... .......... .....
Agph117.5     .......... ......V... .......... ..H....... .....
Veco9         E......... .......... .......... .M........ .....
Hevi5.1       V.Y....... AM....V..Y ..F.....MN .KH.....AL L.Y..
Hisa1.2       DTY....... AM....V..Y ..FNAF..MN .KH....... ..YKP
Veco2.1       DTY......F MM....V..Y ..F.A...MN .KRL....AI L.DK.
Veco7         E.Y....... AM....V..Y ..F.A...MN .KS.....AI L.YK.
Veco6         E.H......F .M....V..Y ..F.AF..MN .KRL...... ..YK.
Hevi2         E.Q....... AM....V..Y ..F.....MN .KR....... ..N..
Hevi3         E.K......F MM....V..Y ..FNAF..MN .KS.....AI L.N..
HevicDNA21.2  E.Q....DE. AM....V..Y ..F.....MN .KR....... ..N..
Veco4.1       E.Y....... .M....V..Y ..F.AF..MN .KS.....AK L.YK.
Veco4.2       E.H......F MM....V... ..FNAF..MN .KRL....AK L.YK.
Veco5         E.Y....... AM....V... ..FNAF..MN .KS.....AI L.YK.
Veco1         E.Y....... AM....V..Y ..F.AF..MN .KS.....AK L.YK.
Hevi5         D.Y......F AMY...V... ..FNAF..MN .KR.....AI L.N..
Hevi6         DTY....... AM....V..Y ..SNAF..MN .KH....... ..YK.
Hevi4         E.K......F MM....V..Y ..FNAF..MN .KR....... ..N..
Veco4         DTY......F MM....V..Y ..F.A...MN .KRL....AI L.GK.
Hevi1         E.Q....L.D .M....V..Y ..F.....MN .KR.....AK L.N..
Veco2         E.Y....... AM....V..Y ..F.AF..MN .KS.....AK L.YK.
Veco3         DTY....... AM....V..Y ..FNAF..MN .KH....... ..YK.
HevicDNA23.6  E.Q....... AMV...V..Y ..F.....MN .KR....... ..N..
Hisa1.1       E.Y......L AI....V..Y ..F.....MN .KH.....AL L.DS.
Hevi9.1       E.Q....L.D .M....V..Y ..F.....MN .KR.....AK L.N..
Veco4.4       E.Q....L.D .M....V..Y ..F.....MI .KR.....AK L.NT.
Hevi11.3s     E.H....... MM.......Y ..F.....MN .KS....... ..N..
Hevi11.4s     E.H....... .K....V..Y ..F.....MI .KN....... ..N..
Hevi12.1d     D.Y......F AM....V... ..F..F..MN .KR.....AK L.N.L
Hevi12.3d     E.Y......F .K....V... ..F..F..MN .KR.....AK L.N..
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Fig. 1 Predicted amino acid sequences of the 45 distinct nucleotide sequences originating from honeycreepers. Honeycreeper sequences defined by species use the following acronyms: 'amakihi, Hevi; i'iwi, Veco; 'apapane, Hisa; and palila, Loba. Included are highly conserved sequences from a Savannah sparrow (Pasa39.43) and a red-winged blackbird (Agph117.5).

from honeycreepers, which varied at 27 sites (60%) in the drepanidinae, is shown in Fig. 1 (MEGA 2.0). Also included are one sequence each from a Savannah sparrow Pasa39.43 (Freeman-Gallant *et al*. 2002) and a red-winged blackbird Agph117.5 (Edwards *et al*. 1995b). All honeycreeper *Mhc* sequences from this study are deposited in GenBank (AY583059–AY583109).

Phylogenetic relationships

The result of the phylogenetic analyses is shown in Fig. 2. The honeycreeper sequences clustered into two distinct groups (clusters 1 and 2). Cluster 1 comprised a collection of 28 diverse sequences (average $d = 0.101$; Table 3). The cluster was nested within a larger grouping that included the Darwin's finch sequences. The sequences from each drepanid species did not cluster together, but instead were interdigitated on the phylogeny. In contrast, the 15 sequences from putative nonrelatives in cluster 2, although highly divergent from the other *Mhc* sequences, show a high degree of similarity to each other (average $d = 0.014$). Also, there is extensive allele sharing among the drepanid species (see Fig. 2). The sparrow and red-winged blackbird sequences also fall within the low-variability cluster 2. The distinctness and limited variability of sequences in cluster 2 suggest that they may represent alleles of a distinct locus, whereas the remaining sequences comprise several groupings, possibly originating from several other loci.

A neighbour-joining (NJ) tree was constructed from the predicted amino acid sequences of honeycreeper cluster 2 (Fig. 2 insert). The high degree of conservation among sequences in honeycreeper cluster 2 is particularly apparent at the amino acid level. Of the 25 sequences (23 honeycreeper sequences) in cluster 2, 11 sequences originating from 10 apparently unrelated individuals of three species are identical at the amino acid level (Figs 1, and 2). The sequence from a fourth honeycreeper species (Loba1) appears highly conserved in 8 individual palila (data not shown), and differs from the remaining highly conserved 11 sequences by only a single nonsynonymous nucleotide substitution. This highly conserved sequence was also detected in a Savannah sparrow Pasa39.43 and a red-winged blackbird Agph117.5.

The Darwin's finch sequences also form two major clusters, likely consisting of multiple loci. Just as seen for honeycreepers, the Darwin's finch sequences fell into a highly variable grouping (clusters/groups 1, 2, 3; average $d = 0.116$), as well as a low-variability cluster that is distinct from the rest (cluster/group 4; average $d = 0.025$). [The data from Darwin's finches are presented here in a consolidated form; see Vincek *et al*. (1997) for individual sequence relationships.] For each taxonomic group, the low-variability cluster assumes a basal position in the phylogeny of alleles.

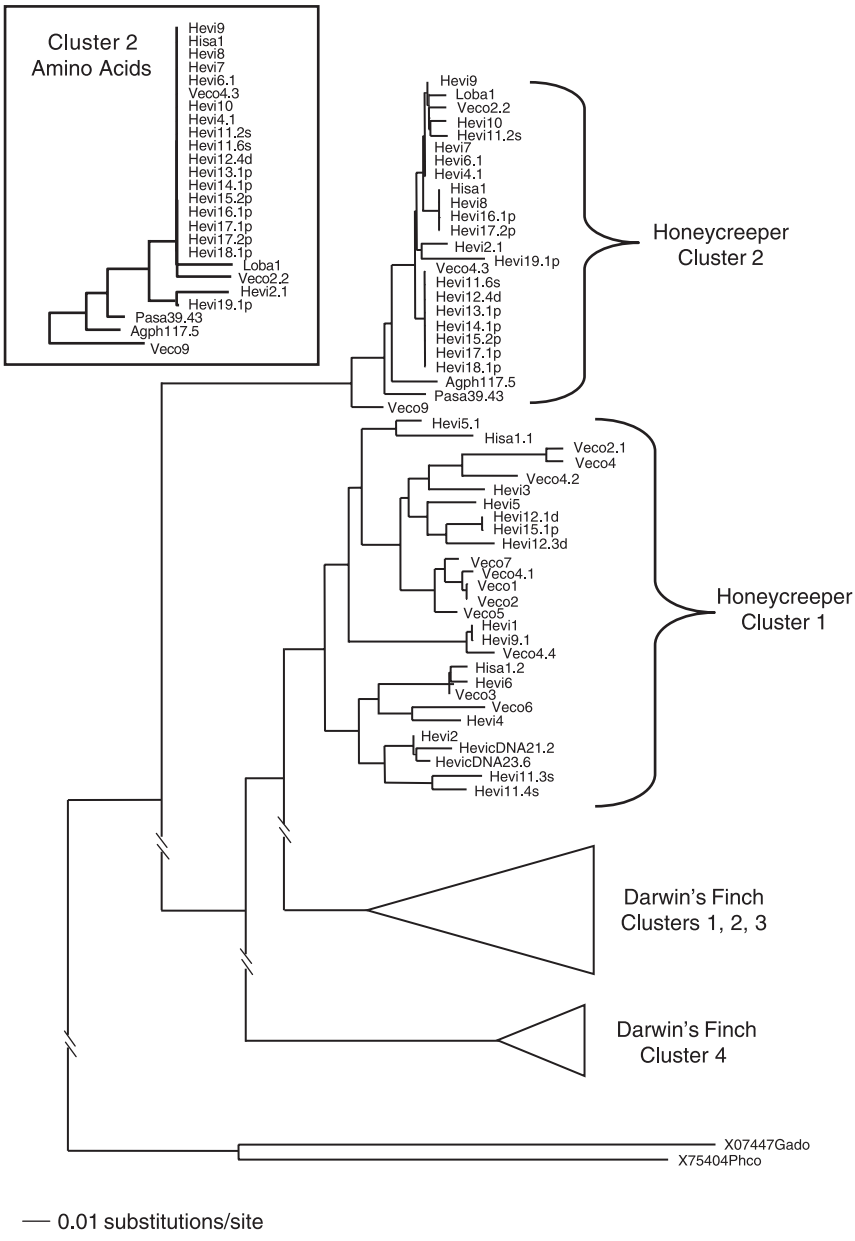

Fig. 2 A neighbour-joining tree (Kimura 2-parameter corrected distances) generated using PAUP. Included are 51 drepanidinae sequences ('amakihi, Hevi; i'iwi, Veco; 'apapane, Hisa; and palila, Loba) 60 Darwin's finch sequences (Z74411–Z74470), one sparrow sequence (AF42007), one blackbird sequence (U24418) and two galliform sequences (pheasant X75404 and chicken X07447). The data from Darwin's finches are presented here in a consolidated, truncated form; see Vincek *et al*. (1997) for individual sequence relationships.

Measures of diversity

Calculations of d_N and d_S are shown in Table 2 for different groupings of the Hawaiian honeycreeper sequences and for Darwin's finch exon 2 sequences and others. The mean values of d_N and d_S across all 42 honeycreeper sequences, and the ratio of the two (1.96), are typical of other studies of *Mhc* sequences in birds. This ratio suggests that most of the changes have been in nonsynonymous sites, and provides evidence that the variability in this region is maintained by positive Darwinian or balancing selection. The 45 codon sequence was divided into the 13 codons hypothesized to be involved in peptide binding (peptide-binding codons or PBCs) and 32 which are presumably not directly involved (non-PBCs), based on the 3D structure of the human class II antigen (Brown *et al*. 1993). The finding that the 13 PBC sites in all honeycreepers and in i'iwi and 'amakihi separately (Table 2) have a significantly higher d_N value than the 32 non-PBC sites ($Z = 4.0$, $P < 0.001$), but not d_S ($Z = 0.3$, $P > 0.50$), provides further support for positive selection on this region. The d_N/d_S ratios at the 13 PBC sites in honeycreepers are more than twice that of the Darwin's finches and others, but the ratios appear comparable at the other 32 non-PBC. This increase in d_N/ds ratio at the 13 PBC sites appears to be due to a decrease in d_S, while d_N values appear comparable. In all comparisons, variation

Table 2 Estimates of d_N and d_S (SE) in all 45 PBR (peptide-binding region) codons, and also partitioned into the 32 (hypothesized) non-PBCs (peptide-binding codons) and the 13 (hypothesized) PBCs (peptide-binding codons) in presumably unrelated individuals (Kumar distance estimates). Comparisons are made within groupings of Drepanidinae (Honeycreepers) and Geospizinae (Darwin's finches) sequences then selected subsets within by species. Other comparisons are within species

	45 PBR codons				32 non-PBCs			13 PBCs		
Comparisons	*n*	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S	d_N	d_S	d_N/d_S
Honeycreepers	42	0.174 (0.037)	0.089 (0.044)	1.96	0.121 (0.038)	0.092 (0.053)	1.32	0.341 (0.103)	0.076* (0.095)	4.49
'Amakihi	24	0.163 (0.035)	0.092 (0.045)	1.77	0.117 (0.036)	0.099 (0.052)	1.18	0.301 (0.110)	0.074 (0.094)	4.06
I'iwi	14	0.162 (0.035)	0.063 (0.032)	2.57	0.101 (0.033)	0.040 (0.025)	2.23	0.388 (0.102)	0.068 (0.072)	5.71
Cluster 1 only										
Honeycreepers	28	0.113 (0.026)	0.040* (0.020)	2.83	0.069 (0.027)	0.044 (0.025)	1.57	0.238 (0.067)	0.027* (0.017)	8.81
'Amakihi	14	0.107 (0.028)	0.048 (0.024)	2.23	0.076 (0.027)	0.061 (0.032)	1.25	0.192 (0.070)	0.020 (0.018)	9.60
I'iwi	11	0.089 (0.022)	0.022* (0.014)	4.05	0.049 (0.021)	0.022 (0.016)	2.23	0.207 (0.063)	0.024* (0.021)	8.63
Cluster 2-All	15	0.008 (0.003)	0.028 (0.014)	0.29	0.009 (0.004)	0.037 (0.021)	0.24	0.005 (0.005)	0.000 (0.000)	0.00
Darwin's finch	60	0.156 (0.032)	0.061* (0.031)	2.56	0.107 (0.034)	0.075 (0.042)	1.43	0.298 (0.081)	0.151 (0.082)	1.97
G. fortis	2	0.171 (0.035)	0.097* (0.038)	1.76	0.107 (0.037)	0.085 (0.048)	1.26	0.336 (0.099)	0.175(0.107)	1.92
G. magnirostris	10	0.159 (0.033)	0.091 (0.035)	1.74	0.103 (0.034)	0.069 (0.041)	1.49	0.304 (0.073)	0.175 (0.102)	1.73
*Clusters 1, 2, 3**	52	0.126 (0.028)	0.065 (0.030)	1.94	0.084 (0.029)	0.069 (0.038)	1.22	0.239 (0.062)	0.077 (0.061)	3.10
*Cluster 4**	8	0.020 (0.010)	0.036 (0.020)	0.56	0.016 (0.009)	0.031 (0.022)	0.52	0.030 (0.024)	0.063 (0.059)	0.48
A. arundinaceus	4	0.257 (0.058)	0.135 (0.059)	1.90	0.192 (0.054)	0.124 (0.062)	1.55	0.430 (0.193)	0.186 (0.145)	2.31
A. phoeniceus	8	0.225 (0.050)	0.201 (0.070)	1.12	0.151 (0.048)	0.190 (0.075)	0.79	0.459 (0.153)	0.359 (0.135)	1.28
A. coerulescens	10	0.134 (0.033)	0.117 (0.047)	1.15	0.094 (0.031)	0.117 (0.055)	0.80	0.246 (0.088)	0.140 (0.090)	1.76
C. mexicanus	6	0.211 (0.049)	0.100 (0.042)	2.11	0.165 (0.051)	0.103 (0.047)	1.60	0.354 (0.124)	0.127 (0.099)	2.79

*Test of the hypothesis that $d_N = d_S$ $P < 0.001$. Clusters 1, 2 and 3 differ from cluster 4 for all 45, and for 32 and 13 codons separately.

as measured by d_S and d_N is lower in cluster 2 than in cluster 1, and the rate of nonsynonymous substitutions is lower than that of synonymous substitutions (d_N/d_S = 0.29). A similar result is seen with the sequences from the Darwin's finches. Both d_S and d_N are much lower in cluster 4 than clusters 1, 2, 3 and the rate of non-synonymous substitution is lower in cluster 4 ($d_N/d_S = 0.56$) relative to clusters 1, 2, 3 ($d_N/d_S = 1.94$). Corrected sequence divergences (Table 3) were notably higher at all 13 PBC sites, when compared with the 32 non-PBC sites in all comparisons, except in honeycreeper cluster 2 and in cluster 4 of Darwin's finches.

Mitochondrial DNA variation in i'iwi and 'amakihi

The total length of the CR sequenced was 330 bp (75 sites from the 5'-end and 255 sites from the 3'-end). Previous analyses of mtDNA restriction fragment length polymorphism (RFLP) and cytochrome *b* sequence variation revealed high within and among population variation among 'amakihi (Table 4; Tarr & Fleischer 1993, 1995; Fleischer *et al*. 1998). By contrast, no mtDNA sequence variation was detected by RFLP analyses (Tarr & Fleischer 1995) of i'iwi samples, including birds from Kaua'i, Maui and Hawai'i islands. CR region sequences revealed similar results: we found no variation among 11 i'iwi CR sequences from three islands, whereas we found significantly higher variation ($P < 0.05$) among the 11 'amakihi CR sequences (Table 4). All CR sequences from this study are deposited in GenBank (AY582899–AY582920).

Discussion

A key finding in this study is the extensive variation at the hypothesized PBCs in a species that otherwise shows very low levels of genetic diversity. The i'iwi, a species for which other genetic markers show extremely low variation, has roughly equal levels of nonsynonymous *Mhc* variation in comparison with the 'amakihi at the hypothesized 13 PBC sites. We also find that among the class II β chain antigen-binding region sequences, there are distinct clusters in both the honeycreepers and Darwin's finches that show overall low variation and d_N/d_S ratios that are atypical for this *Mhc* region. This contrasts with the other sequences presented here, which collectively show a high rate of nonsynonymous substitution, and the high levels of diversity that are typical for avian *Mhc* sequences. We first discuss the advantages of our methodological approach, which eliminates sequencing artefacts that would otherwise complicate analysis and interpretation of *Mhc* variation. We then detail functional explanations for the patterns of variability that we see in the honeycreeper sequences.

Table 3 Estimates of mean genetic distance (*d*) based on nucleotide divergence (standard error) in presumably unrelated individuals (Kimura 2-parameter distance). Comparisons are made within groupings of Drepanidinae (Honeycreepers) and Geospizinae (Darwin's finches) sequences then selected subsets within by species

	N	d45 PBR codons	d 32 non-PBCs	d 13 PBCs
All honeycreepers	42	0.160 (0.024)	0.122 (0.025)	0.273 (0.069)
Amakihi	24	0.151 (0.024)	0.120 (0.024)	0.250 (0.061)
I'iwi	14	0.144 (0.022)	0.105 (0.022)	0.260 (0.061)
Cluster 1	28	0.101 (0.017)	0.069 (0.017)	0.187 (0.047)
Amakihi	15	0.100 (0.018)	0.078 (0.018)	0.154 (0.044)
I'iwi	11	0.078 (0.015)	0.047 (0.015)	0.166 (0.042)
Cluster 2	15	0.014 (0.005)	0.018 (0.006)	0.004 (0.004)
All Darwin's finches	60	0.145 (0.022)	0.104 (0.022)	0.263 (0.060)
G. fortis	20	0.161 (0.024)	0.110 (0.024)	0.300 (0.075)
G. magnirostris	10	0.150 (0.021)	0.104 (0.023)	0.217 (0.070)
Clusters 1, 2, 3	52	0.116 (0.020)	0.085 (0.021)	0.202 (0.049)
Cluster 4	8	0.025 (0.007)	0.021 (0.007)	0.034 (0.019)
A. arundinaceus	8	0.243 (0.035)	0.188 (0.035)	0.411 (0.101)
A. phoeniceus	4	0.222 (0.030)	0.167 (0.031)	0.382 (0.087)
A. coerulescens	10	0.135 (0.020)	0.106 (0.025)	0.217 (0.053)
C. mexicanus	6	0.191 (0.029)	0.167 (0.032)	0.289 (0.071)

Table 4 Summary of genetic comparisons between I'iwi and Amakihi (SE)

	I'iwi	Amakihi
*d*mtDNA-RFLP*	0.000	0.021 (0.006)
d mtDNA-CR		
Hawaii	0.000	0.018 (0.007)
Maui	0.000	0.008 (0.006)
Kauai	0.000	Not determined
d RFLP MhcII†	0.120	0.380
Cluster 1		
*d Mhc*II (32)	0.047 (0.015)	0.078 (0.018)
*d Mhc*II (13)	0.166 (0.042)	0.154 (0.044)
d_N (32)	0.049 (0.021)	0.076 (0.027)
d_S (32)	0.022 (0.016)	0.061 (0.032)
d_N (13)	0.207 (0.063)	0.192 (0.070)
d_S (13)	0.024 (0.021)	0.020 (0.018)
d_N/d_S (32)	2.23	1.25
d_N/d_S (13)	8.625	9.600

*Tarr & Fleischer (1993), (1995); Fleischer *et al*. (1998).
†Jarvi *et al*. (2001).

Sequence reliability

Standard PCR amplification protocols and subsequent cloning in *Escherichia coli* have been used routinely to obtain sequence data from avian *Mhc* genes (Edwards *et al*. 1995a,b; Vincek *et al*. 1997; Sato *et al*. 2000; Freeman-Gallant *et al*. 2002). A number of studies document the formation of recombinant variants using standard protocols in, especially, multigene families like the *Mhc* (Nagamine *et al*. 1989; Borriello & Krauter 1990; Jansen & Ledley 1990; Triggs-Raine & Gravel 1990; L'Abbe *et al*. 1992; Tombline *et al*. 1996; Zhao *et al*. 1998; Zylstra *et al*. 1998; Longeri *et al*. 2002). Some of the technical variables shown to influence the formation of recombinant artefacts are the type of polymerase, number of PCR cycles (Zylstra *et al*. 1998), *in vitro* recombination events (Jansen & Ledley 1990; Meyerhans *et al*. 1990; Zhao *et al*. 1998), and formation of mosaics by cloning of heteroduplexes (Nagamine & Chan Kand Lau 1989; Borriello & Krauter 1990; Jansen & Ledley 1990; Triggs-Raine & Gravel 1990; L'Abbe *et al*. 1992; Longeri *et al*. 2002). *In vitro* recombination is thought to account for only 1–5% of variant (artefactual) sequences and can generally be easily detected (Jansen & Ledley 1990). *In vitro* recombination events result from template switching when incompletely amplified sequences of one allele pair with the template of another allele. This amplified chimera then contains sequence information from two distinct alleles. Estimates of heteroduplex formation, however, have been documented as high as 38–50%; (Jansen & Ledley 1990; L'Abbe *et al*. 1992). Heteroduplexes are formed during the final cycle of PCR and are hybrids of full-length complementary sequences originating from distinct alleles (Borriello & Krauter 1990). When cloning occurs in bacterial systems that are not mismatch repair deficient, mismatches can be repaired producing mosaic sequences that may or may not be authentic. We used methods of sequence isolation for *Mhc* class II antigen-binding region that are designed to avoid sequencing mosaics, formed when heteroduplexes are cloned. Accurate distinction between authentic and mosaic sequences that originate from individuals of wild populations is unlikely, but mosaics can be detected under controlled experimental conditions or in families in which the parental allele sequences are known and thus provide a basis for

comparison. A controlled study of exon 2 sequences from a bovine *Mhc* class II *DRB3* gene from heterozygous animals (Longeri *et al*. 2002) compared recombinant variants with parental alleles and documented that most of the recombinant variants were not produced by *in vitro* recombination, but resulted from the mismatch repair of heteroduplex PCR products during cloning in *E. coli*. Here, the use of PCR + 1 cloning methods or single-stranded sequence capture eliminates the potential for sequencing spurious, mosaic artefacts.

Phylogenetic analyses

A NJ analysis (Fig. 2) separated the honeycreeper sequences into two main clusters. An interesting finding is the high degree of sequence conservation in honeycreeper cluster 2, which includes a sparrow and a blackbird sequence. Taken together, the phylogenetic distinctiveness and the evidence for purifying selection (low divergence and $d_N/d_S < 0.29$) suggest that these sequences represent a separate locus in the *Mhc* family with an undetermined role in antigen presentation. It is possible that these sequences represent a DM-like locus. DM facilitates the loading of peptides by class II molecules (Kropshoffer *et al*. 1997), and consists of an α and β chain homologous to the corresponding chains of the class II heterodimer (Hughes *et al*. 1994; Freemont *et al*. 1998). Alternatively, the high degree of conservation of sequences within cluster 2 appears reminiscent of the *Rfp-Y* genes in chickens (Guillemot *et al*. 1988; Miller *et al*. 1994, 1996). The molecularly defined *Rfp-Y* system is an independently segregating cluster of *Mhc* class I and II genes, from the serologically and molecularly defined *B* complex (*Mhc*). The class II β genes of the *Rfp-Y* system in chickens belong to the B-LBIII family of genes, while the class II β genes of the *B* system, belong to the B-LBII family of genes (Zoorob *et al*. 1993). The class II genes of these families differ tremendously in levels of polymorphism, transcription and time of divergence. The *B* system genes (B-LBII) appear to be more polymorphic, are predominantly transcribed, and have diverged much more recently than those of the *Rfp-Y* system, B-LBIII (Zoorob *et al*. 1993). However, at least one class I *Rfp-Y* gene has been shown to be transcribed and is polymorphic (Afanassief *et al*. 2001). A similar organization is observed among class II genes of the *Mhc* of the ring-necked pheasant (*Phasianus colchicus*) (Wittzell *et al*. 1994, 1995; Jarvi *et al*. 1996), as well as independent segregation of at least two systems of class I genes (Jarvi *et al*. 1996). Independent segregation of *Mhc* RFLP bands has been reported in warblers (Westerdahl *et al*. 2000), cranes (Jarvi *et al*. 1999 and unpublished data) and sparrows using clone Pasa39.43 (Freeman-Gallant *et al*. 2002). Sparrow clone Pasa39.43 clusters within honeycreeper cluster 2 (Fig. 2), which as with Darwin finch cluster 2, has a basal location to the remaining passerine sequences. Attempts were made to determine linkage relationships between clusters 1 and 2 in the single honeycreeper family available for testing, but both the dam and sire of the family appear to be homozygous at this locus, so independent segregation of alleles among the progeny could not be evaluated. The high degree of sequence conservation and phylogenetic distinctiveness of cluster 2, which includes a passerine sequence known to segregate independently of other *Mhc* loci (Pasa39.43), suggests that cluster 2 may eventually be found to represent an independently segregating locus that is the counterpart to the *Rfp-Y* system of chickens.

Evidence for balancing selection

We provide evidence for balancing selection on the *Mhc* of honeycreepers with the finding of an overall d_N/d_S ratio of 1.96 and that the 13 PBC sites have a significantly higher d_N value than the 32 non-PBC sites. The d_N/d_S ratios at the 13 PBC sites in honeycreepers are notably higher than those of the Darwin's finches and others, but the ratios appear comparable at the 32 other sites. This increase in d_N/d_S ratio at the 13 PBC sites appears to be due to a decrease in d_S, whereas d_N values appear comparable. If d_S represents the substitution rate (Hughes & Yeager 1998), then the relatively low d_S in honeycreepers (0.027–0.044) is accompanied by a relatively high d_N value (0.238), as compared with other finches, and is reflected in the d_N/d_S ratios. This suggests high intensity of selection at critical regions within the PBR in the honeycreepers. Because distinct sets of primers are used in all these studies it could be that the difference in d_N/d_S ratios observed is due to the detection of different loci. However, none of the class II PBR region primers (ours and others) included in this study appear to be locus-specific thus, presumably, all d_N/d_S ratios have been calculated across multiple loci.

The existence of identical *Mhc* alleles among species has been reported in many mammalian (Klein *et al*. 1993) and some avian species (Vincek *et al*. 1997). Alleles shared between species are presumed to have arisen by either convergent evolution, interbreeding of species or, as is more likely the case at least in the honeycreepers, may have been present in the ancestral species and were retained, possibly by selection, throughout the process of speciation (transpecies hypothesis, Klein *et al*. 1993). Genes that display long-term balanced polymorphism (e.g. *Mhc* genes) often have coalescence times that predate speciation events (Takahata & Nei 1990; Takahata 1990). The interdigitation of *Mhc* sequences from different honeycreeper species may provide additional evidence for balancing selection (long-term retention of alleles) on the *Mhc*. However, it is clear that the honeycreepers have undergone a rapid radiation (Freed *et al*. 1987; Tarr & Fleischer 1995; Fleischer *et al*. 1998, 2001),

so it is unclear how nuclear sequences that evolve neutrally and under purifying selection would assort phylogenetically.

Species comparison

Studies involving experimental infection with malaria have provided evidence that susceptibility to malaria varies among species of honeycreepers. 'Amakihi have substantially lower mortality (65%) than do i'iwi (90–100%) under comparable experimental conditions (Atkinson *et al*. 1995, 2000; Yorinks & Atkinson 2000). This difference in susceptibility appears to translate to wild populations as well as wild 'amakihi typically have prevalences of infection with chronic malaria that are up to 5–10 times higher than wild i'iwi, suggesting that significantly more 'amakihi are able to survive the initial acute phase of the disease under natural conditions (CT Atkinson, unpublished). While 'amakihi are generalists, quite sedentary and are currently abundant throughout the main Hawaiian islands, i'iwi are considered specialists, are a mobile species and although historically abundant, are currently limited to forests above 1500 m on Hawai'i, Maui and Kaua'i (Fancy & Ralph 1998). The leading hypothesis for restriction of this species to higher elevations is high susceptibility to malaria (Atkinson *et al*. 1995).

In a comparison based on RFLP analyses (Jarvi *et al*. 2001) i'iwi appear significantly less variable ($P < 0.001$) at class II loci than 'amakihi (Table 4). In the current study, we found no difference in *Mhc* sequence variability between i'iwi and 'amakihi at the 13 PBC sites, d_S and d_N values and genetic distances did not differ overall. However, at the 32 non-PBC sites diversity in i'iwi appears consistently lower than that observed in 'amakihi (summarized in Tables 2, 3). Thus, the RFLP/Southern blot analyses of class II genes may reflect the low variability in i'iwi at non-PBC or other adjacent or linked sites, but cannot resolve the higher level of variability at the 13 PBC sites. Additional studies of nuclear genes of individuals subjected to experimental infection with malaria and from natural populations are currently underway. These studies will allow us to directly relate particular *Mhc* variants with resistance to malaria.

Studies of mtDNA, as assessed by RFLP analyses of whole mtDNA, cytochrome *b* sequences, and CR region sequences, show no variation among i'iwi, but show substantial variation within and among populations of 'amakihi (Table 4; Tarr & Fleischer 1993, 1995; Fleischer *et al*. 1998). This suggests that i'iwi have had a considerably more recent coalescence time for their mtDNA sequences than 'amakihi. The extremely low variation in i'iwi mtDNA may have been caused by a genetic bottleneck or a selective sweep. A bottleneck is further supported by the finding of low allozyme variation in i'iwi relative to 'amakihi (Johnson *et al*. 1989): it would be unlikely that a selective sweep at one locus would impact variation at many unlinked loci. This reduction in variation likely also involved a recent spread among the islands, as i'iwi on all islands, including distantly located ones (e.g. Kaua'i and Hawai'i), have identical haplotypes. Such low genetic variation suggests that the event happened relatively recently — thousands or tens of thousands of years ago, but not much earlier. We do not think the putative bottleneck occurred more recently than thousands of years ago because nuclear variation in the i'iwi as assessed by AFLP (SI Jarvi *et al*. unpublished) and microsatellite data (L Eggert *et al*. unpublished) appears comparable with that found in the 'amakihi. In other words, there may have been time since a major bottleneck for the rapidly mutating microsatellites and some other nuclear loci to recover variation, while loci with lower mutation rates (i.e. mtDNA) might not have had time to accumulate mutations. In the case of microsatellites, assuming a mutation rate of 10^{-4}, a simple equilibrium level for the heterozygosity we observed would be ~ 10 000 years. Because transmission of the parasite causing the current malaria epidemic could not occur until the arrival of its vector *Culex quinquefasciatus* in 1826 (Hardy 1960) it is unlikely related to this historic bottleneck. One interesting implication of this putative historical bottleneck is that the level of variation in the *Mhc* might also expected to be low, as there is no evidence of a higher mutation rate for *Mhc* genes. The finding of similar *Mhc* variation at the 13 PBC sites suggests that selection may have maintained variation in i'iwi, even in the face of what appears to be a major genetic bottleneck.

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