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Evolution on a volcanic conveyor belt: using phylogeographic reconstructions and K–Ar-based ages of the Hawaiian Islands to estimate molecular evolutionary rates

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Abstract

The Hawaiian Islands form as the Pacific Plate moves over a 'hot spot' in the earth's mantle where magma extrudes through the crust to build huge shield volcanos. The islands subside and erode as the plate carries them to the north-west, eventually to become coral atolls and seamounts. Thus islands are ordered linearly by age, with the oldest islands in the north-west (e.g. Kauai at 5.1 Ma) and the youngest in the south-east (e.g. Hawaii at 0.43 Ma). K–Ar estimates of the date of an island's formation provide a maximum age for the taxa inhabiting the island. These ages can be used to calibrate rates of molecular change under the following assumptions: (i) K–Ar dates are accurate; (ii) tree topologies show that derivation of taxa parallels the timing of island formation; (iii) populations do not colonize long after island emergence; (iv) the coalescent point for sister taxa does not greatly predate the formation of the colonized younger island; (v) saturation effects and (vi) among-lineage rate variation are minimal or correctable; and (vii) unbiased standard errors of distances and regressions can be estimated from multiple pairwise comparisons. We use the approach to obtain overall corrected rate calibrations for: (i) part of the mitochondrial cytochrome *b* gene in Hawaiian drepanidines (0.016 sequence divergence/Myr); (ii) the *Yp1* gene in Hawaiian *Drosophila* (0.019/Myr Kambysellis *et al*. 1995); and (iii) parts of the mitochondrial 12S and 16S rRNA and $tRNA^{val}$ in *Laupala* crickets (0.024–0.102/Myr, Shaw 1996). We discuss the reliability of the estimates given the assumptions (i–vii) above and contrast the results with previous calibrations of *Adh* in Hawaiian *Drosophila* and chloroplast DNA in lobeliods.

Keywords: adaptive radiation, drepanidines, Hawaiian *Drosophila*, Hawaiian Islands, *Laupala*, molecular rate calibration, phylogeography

Introduction

The Hawaiian Islands, in large part because of their unique geological history, provide a premier setting for studies of evolution. First, the Hawaiian archipelago is the most isolated in the world, so few organisms colonize and there may be considerable opportunity for ecological release and adaptation. Second, the islands are arranged in a linear array. Third, this array is ordered sequentially by age (Fig. 1). The youngest subaerial island of Hawaii in the far south-east has been estimated by K–Ar dating at 0.43 Ma, the oldest main island of Kauai at 5.1 Ma, and the north-west or leeward islands range from about 7 to 28 Ma (Clague & Dalyrymple 1987). The Hawaiian native biota contains some of the best examples of adaptive radiation, rapid speciation via founder effects or sexual selection, and major niche shifts (see reviews in Carlquist 1980; Simon 1987; Wagner & Funk 1995). How does this wealth in evolutionary phenomena relate to the geological history of the islands? Thus far, interpretation of observed phylogeographic patterns in the context of geological history has yielded remarkable inference about rates and

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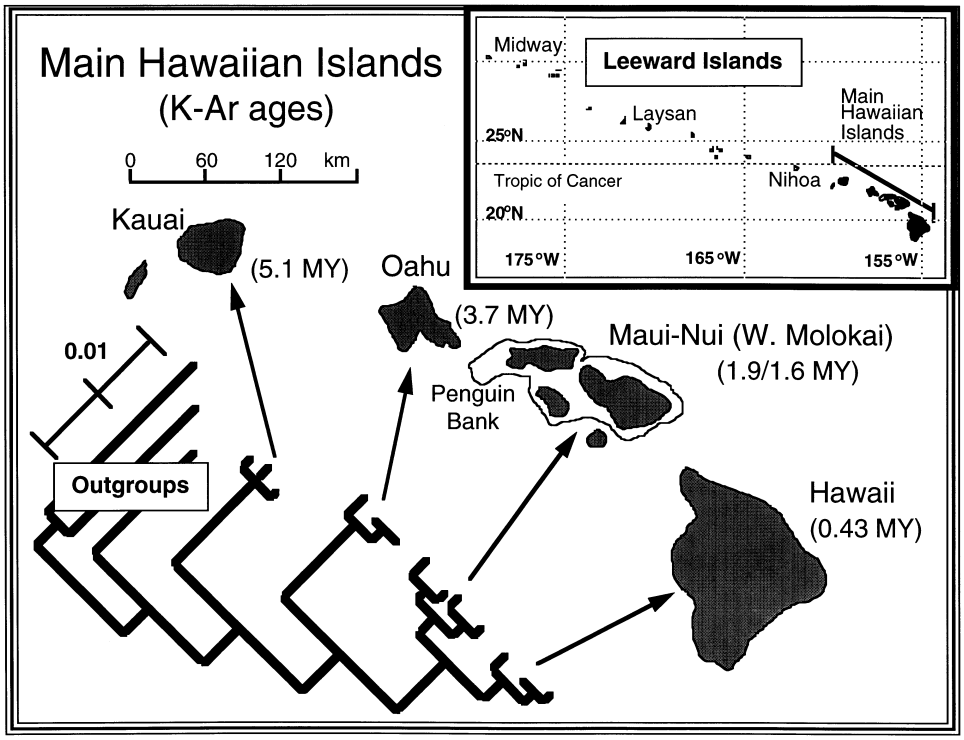

Fig. 1 Map of the main Hawaiian Islands (plus inset map of main and leeward Hawaiian Islands). Ages of the oldest rocks from the main islands based on K-Ar dating are noted (from Carson & Clague 1995). Maui-nui is composed of the islands of Maui, Lanai, Molokai and Kahoolawe, all of which were connected until ≈ 0.3–0.4 Ma (and again at times during Pleistocene periods of low sea level). Oahu and West Molokai were putatively connected via the Penguin Bank for the first ≈ 0.3 Myr after West Molokai formed. Below the map is a neighbour-joining tree (Saitou & Nei 1987) based on Kimura 2-parameter and Γ-corrected distances that shows relationships and relative differentiation among four island populations of amakihi based on 675 bp of cytochrome *b* sequence. Included in the tree are three Kauai amakihi (*Hemignathus kauaiensis*) and 10 common amakihi of three subspecies: *Hemignathus virens chloris*, *H. vs. wilsoni* and *H. vs. virens*, from Oahu, Maui and Hawaii, respectively. Parsimony analyses produce a tree with the same topology as the neighbour-joining tree, as do analyses using sequences of mtDNA ATPase 6/8 (R. C. Fleischer *et al*. unpublished), ND2 and control region (C. Tarr, unpublished). Trees constructed from mtDNA restriction sites show sister taxon status between a clade containing Kauai and Oahu clades, and one containing Maui and Hawaii clades (Tarr & Fleischer 1993). In all of our analyses to date, most for which we have larger samples per island than the case reported here, each island population of amakihi has been found to be monophyletic.

modes of speciation, adaptation and molecular evolution (e.g. Wagner & Funk 1995, and references therein).

While the Hawaiian Islands are primarily depauparate in taxonomic lineages relative to less isolated islands and continents, they have become secondarily enriched by the formation of many endemic species within the few lineages that did colonize (Simon 1987; Carson & Clague 1995). The best studied endemic lineages of organisms in the islands have been insects, birds and plants, in particular *Drosophila* (Carson & Kaneshiro 1976; Kaneshiro 1988; DeSalle & Hunt 1987; DeSalle 1995; Kambysellis *et al*. 1995), drepanidines (cardueline finches often called honeycreepers; Amadon 1950; Raikow 1977; James & Olson 1991; Tarr & Fleischer 1995), lobelias (Givnish *et al*. 1995; Lammers 1995) and silverswords (Carr 1987; Baldwin & Robichaux 1995).

More than 900 endemic species of *Drosophila* are thought to occur in the islands (not including an unknown number that probably existed prior to human colonization and associated habitat changes). Molecular data suggest that the Hawaiian lineage(s) separated from mainland *Drosophila* 10–32 Ma, long before the formation of the main islands that exist today (Beverley & Wilson 1984; Thomas & Hunt 1991; DeSalle 1992; Russo *et al*. 1995). Givnish *et al*. (1995) also provide molecular evidence supporting the origin of bird-pollinated lobelias (*Cyanea*) prior to the formation of the current main islands. The avifauna of the islands, while not as speciose as the invertebrate fauna and the flora, offer similar patterns of adaptive radiation and niche shifts. The drepanidines, in particular, have speciated to a great extent (33 historical and > 17 subfossil species; James & Olson 1991; H. James, personal communication) and have become highly differentiated phenotypically from putative mainland relatives. Biochemical and molecular

data suggest that they did not radiate prior to the formation of the current main islands (Johnson *et al*. 1989; Tarr & Fleischer 1993, 1995; Fleischer *et al*. unpublished; see below).

While these examples showcase the potential for major adaptive radiations in the Hawaiian Islands, many endemic lineages have not radiated significantly, and some not at all. Is the variance in the amount of speciation and phenotypic differentiation related to the length of time that a lineage has been evolving in the islands (e.g. Simon 1987; Carson & Clague 1995)? Or are there other factors that have promoted stasis in some lineages and change in others, regardless of time? Although there are invaluable Holocene fossil records for birds and pollen (Olson & James 1991; James & Olson 1991; Selling 1948), the record for the late Pleistocene is limited (although one excellent fauna dates to > 0.12 Ma, James 1987). In addition, there is almost no fossil record earlier than this: the extreme subsidence and erosion that occurs as islands age (see below) has probably erased it. Thus the age of separation from mainland ancestors or the age of a radiation within the islands can only be inferred from the amount of molecular divergence among the taxa under study and the rate of molecular evolution. It is preferable in these cases to use internal or 'local' molecular rate calibrations (Hillis *et al*. 1996), and the geological history of the Hawaiian Islands offers unique opportunities for making such calibrations (e.g. Bishop & Hunt 1988; Tarr & Fleischer 1993; Givnish *et al*. 1995).

Here we expand on approaches developed in these earlier studies in which phylogeographic reconstructions and geological history were used to infer rates of molecular evolution. We begin with a brief overview of the geological history of the Hawaiian archipelago. We then outline the regression approach for calibrating 'local' molecular rates: K–Ar dates of island age are used to estimate various benchmarks of population or species formation. Appropriately corrected, among-taxon genetic distances are then regressed against the appropriate ages to estimate the divergence rate (i.e. the slope). The procedure involves assumptions or caveats, and we identify and evaluate seven categories of these below. We apply the method to estimate overall divergence rates for: (i) part of the mtDNA cytochrome *b* gene in the Hawaiian drepanidines; (ii) sequences of yolk protein gene 1 (*Yp1*) in the β lineage of the *planitibia* subgroup of Hawaiian *Drosophila* (Kambysellis *et al*. 1995); and (iii) mtDNA rRNA and tRNA sequences in Hawaiian *Laupala* crickets (Shaw 1996). We compare the results of rate estimates from other studies of Hawaiian organisms, including *Adh* in *Drosophila* and chloroplast DNA in lobelias and discuss the overall reliability of the approach.

The conveyor belt: geological history of the Islands

The Hawaiian Islands arise as the Pacific tectonic plate moves slowly north-west (≈ 8 cm/year) over a plume of magma or 'hot spot' that spikes through the plate and pours out onto the ocean floor (for informative reviews of Hawaiian volcanology and geology see Clague & Dalrymple 1987; Walker 1990; Carson & Clague 1995). The magma plume spreads and is carried along below the plate, so lava extrudes at more than a single point over more than 100 km linear range (thus more than a single volcano can be active at the same time). Hawaiian lavas generally flow at a relatively constant rate. Lava is slowly layered to build a rounded shield on the ocean floor which usually extends above the ocean's surface (becomes 'subaerial') to great heights. The extant volcano of Mauna Loa ('long mountain' in Hawaiian), for example, rises to 4169 m above sea level and is the most massive single mountain on earth.

When the plate moves an island off the hotspot two major types of events cause the islands to decrease in size and change in character. First, the bulging of the crust over the hotspot diminishes as the crust cools. This, combined with the great weight of the volcano above the crust, causes a fairly rapid subsidence of the crust and a decrease in island elevation. Initially, the rate is very rapid (> 3 mm/y during the first ≈ 0.3 Myr, Clague & Dalrymple 1987), but then subsidence slows as the island moves further away from the hotspot (to an average over the entire chain of 0.04 mm/y, Walker 1990). Second, slow erosive processes – such as wind, rain and ocean waves – and abrupt, massive landslides, begin to whittle away at the islands (Carson & Clague 1995). The islands first decrease to smaller subaerial volcanic masses (i.e. reduced areas and elevations, such as Niihau or Nihoa). Next, the volcanic portions continue to subside under the sea, and only coral and sand atolls remain above the surface (e.g. Pearl and Hermes Reef or Laysan Island). Finally, the volcanoes sink further, forming the undersea mounts or guyots that extend to the north of Kure Atoll.

Thus, the Hawaiian Islands exist on a sort of geological conveyor belt, being built up while over the hotspot, then subsiding and eroding to atolls and seamounts. Lineages that 'ride' an island must continue the colonization process or face extinction when the island that they are located on either diminishes in size or submerges. One can only wonder at the types of organisms and radiations that were not able to continue down the chain because of limited or lost dispersal capabilities. Each new island is devoid of taxa at first, and the subsequent filling of niches presumably results in new selective regimes for taxa and the formation of novel community structures. In comparison to less

dynamic island systems, this necessary continuous colonization of new islands by lineages greatly increases the likelihood of divergence from ancestral populations, and consequently should increase the rate of speciation and adaptive change. The formation of new islands does appear to have greatly affected the rate and pattern of radiation in the Hawaiian honeycreepers (Fleischer *et al*., unpublished).

The eight main or high islands (Fig. 1) really comprise four island groups: (i) Kauai/Niihau; (ii) Oahu; (iii) Molokai/Lanai/Maui/Kahoolawe ('Maui Nui'); and (iv) Hawaii. There is no evidence that Kauai or Hawaii were ever connected by land bridges to Oahu or Maui, respectively. However, some reconstructions of historical geography indicate that the Penguin Bank was a land bridge that connected Oahu and Molokai for perhaps as long as 0.33 Myr after the West Molokai volcano became subaerial (Carson & Clague 1995). These dates are based on a maximal estimated elevation of the Penguin Bank of 1000 m above sea level (Table 2.1 in Carson & Clague 1995), and a minimal subsidence rate of 0.003 m/y (Clague & Dalrymple 1987). If this is correct, then the date of separation of populations currently residing on Oahu and Maui-Nui would not be about 1.9 Myr (the age of west Molokai), but rather about 1.6 Myr. Maui and Molokai apparently remained joined in Maui-Nui until about 0.3–0.4 Ma, and there is evidence for the conjoining of a volcano called Mahukona with the Kohala volcano of Hawaii which would increase the date of origin of 'Hawaii' from 0.43 to about 0.50 Myr (Carson & Clague 1995).

Rationale and methods for rate calibrations based on island age

The geological evidence for the plate tectonic events that formed the Hawaiian Islands is extensive, and one of the key pieces of evidence is the K–Ar dating of the oldest exposed rocks on each island (Fig. 1; Clague & Dalyrymple 1987). The estimated island ages can also provide valuable information for evolutionary inference. For example, the geological age of an island establishes a maximum age for a population of organisms living on the island. These ages, in concert with a set of assumptions or caveats (1 to 7 outlined below), can be used to calibrate minimum rate, 'local' molecular clocks (e.g. Bishop & Hunt 1988; Rowan & Hunt 1991; Tarr & Fleischer 1993; Givnish *et al*. 1995; see Lynch & Jarrell 1993 and Hillis *et al*. 1996 for general problems with rate calibrations).

In such a calibration we assume that the K–Ar age of the younger island represents an approximate date for a split between the 'offspring' population on the younger island and the 'parental' population of the older island (given a topology described in assumption 2 below). We estimate the divergence between parental and offspring populations on adjacent islands. Divergence can be estimated by the absolute number of all substitutions (or only synonymous or nonsynonymous changes or transversions, depending on the level of saturation and proportion of sites free to vary), or as a distance (which can be corrected with an appropriate model for multiple hits, transition bias or rate variation among sites). In our approach, mean parent–offspring distance is regressed against the age of formation of the offspring's island (or the age of an appropriate vicariance event). The slope of the regression line is the rate of sequence divergence, the intercept should not be significantly different from zero, and the between-matrix correlation coefficient (e.g. Mantel matrix *r*, Rohlf 1990) and associated significance level represents the fit of the data to the regression line. Regressions with low values of *r* or high standard errors have poor predictive power (Hillis *et al*. 1996). In this approach we make a number of assumptions that need to be addressed. We list and discuss their implications here:

Assumption 1: the K–Ar dates of earliest subaerial lavas and island ages are correctly estimated

The literature contains numerous examples using K–Ar methods to calculate ages of lavas in the Hawaiian Islands (see Clague & Dalyrymple 1987; Carson & Clague 1995 for a summary of literature). The K–Ar dates are highly repeatable, with low standard errors and an established temporal calibration. However, it is not critical that K–Ar dates be calibrated accurately with time, as absolute time is not as important to answering some of our questions as is time relative to the formation of the main islands.

Assumption 2: topology of the phylogeny for a lineage implies successive colonization of islands in parallel to island age and each island's taxon is monophyletic

In support of this, many estimated phylogenies of Hawaiian organisms have yielded topologies with serial branching of parent and offspring clades, in which the order of the clades sequentially arises in parallel to the linear physical (and thus temporal) arrangement of the Hawaiian Islands (e.g. Fig. 1; Rowan & Hunt 1991; Tarr & Fleischer 1993; Kambysellis *et al*. 1995, and several examples within Wagner & Funk 1995). Taxa on each island should also be (and often are) monophyletic: hybridization or gene flow subsequent to founding of an island population could severely bias a calibration. A serial area cladogram (Fig. 1) is most parsimoniously interpreted as having arisen via successive colonization of the islands by the lineage (i.e. Kauai to Oahu, Oahu to

Molokai, etc.), but it does not necessarily imply colonization at or even near the time of island formation (see Assumption 3).

Assumption 3: each island population was formed at the time the island became subaerial

In some cases, islands may have been colonized sequentially after most or even all of the islands had formed, thus overestimating the age of each population. However, the error in the calibration will only occur in one direction, i.e. the estimated rate of sequence evolution will be a minimum one and time frames predicted from the calibration will be maximum ones. This assumption can be violated in some cases and still yield valuable evolutionary information (e.g. about maximum ages of taxa, or whether substitution rates for certain taxa may be decelerated relative to others). For volant species, such as many bird species, one might expect that islands would be colonized fairly rapidly after their formation. This also implies that there may be continuing gene flow (in the absence of reproductive isolation), which might counter Assumption 2.

Assumption 4: DNA sequence variation within the parental population today is similar to what it was at the time of population splitting, and considerably less than that between island populations

Calibration also requires that the coalescent point of DNA sequence variants of the parental and offspring populations did not greatly predate the formation of the new island. This 'lineage sorting' source of error is usually small relative to the distances between differentiated taxa (e.g. Bishop & Hunt 1988; Tarr & Fleischer 1993; Moore 1995). With the assumption that within-island variation in the ancestral population today is similar to variation at the time of offspring population formation, the between-population distance can be at least partly corrected by subtracting the mean within-island variation (Wilson *et al*. 1985; Nei 1987; Tarr & Fleischer 1993). Only variation in the ancestral or parental population is taken into account when population separation is the result of a founder event and the direction of colonization is known. Thus, we correct the inter-island distance (d_{xy}) by subtracting the mean within-population distance of the parental population (d_x, e.g. $d_A = d_{xy} - d_x$).

Assumption 5: DNA sequences are not saturated, or saturation can be corrected with the appropriate model of sequence evolution

Most saturation effects arise in mitochondrial DNA and other DNA sequences following a relatively long period of time. Such effects do not normally become a problem for rate calibrations until uncorrected sequence divergences are greater than 10% (Nei 1987; Meyer 1994; Moore & DeFilippis 1997). Because the oldest island we use for rate calibrations is only 3.7-Myr old (Oahu), it is unlikely that divergences for most mtDNA or nuclear sequences will exceed the saturation threshold. However, applying our rate calibrations to significantly more divergent sequences, within other carduelines or passerines, for example, would require detection and correction of saturation effects. Problems of compositional biases can also affect divergence estimates and can be corrected with maximum likelihood or distance models, or with a log-Det transform (Swofford *et al*. 1996). A general problem with applying corrections is that each additional parameter increases the random error of the distance estimate (Swofford *et al*. 1996). However, in the rate calibrations reported here, distance corrections and associated errors should be minimal because of the recent age (< 5 Myr) of most Hawaiian lineages. The added error may also be offset somewhat by the reduced standard error of regression resulting from the greater precision of the corrected estimates.

Assumption 6: relative rates tests should show no significant among-lineage variation in rate for the taxa used for calibration, or heterogenous taxa should be removed

Significant variation in rates among lineages could bias calibrations and subsequent estimates of absolute divergence times. Extreme variation in rates has been shown to be related to variation in body size and its correlates (e.g. metabolic rate, generation time; Wu & Li 1985; Martin & Palumbi 1993; Cantatore *et al*. 1994; Martin 1995). Application to species of similar size and metabolism should often control for this source of error. Rate variation may also be related to population size fluctuations under the assumptions of the nearly neutral model (e.g. Ohta 1976, 1993; DeSalle & Templeton 1988). Rates may also be biased by selection on the gene being studied, and differences among lineages in the sites that are free to vary (Palumbi 1989). Use of more than one unlinked marker (e.g. Ruvolo 1996) may help to detect such cases of heterogeneity. In spite of these possible biases, rates can be tested for heterogeneity by several statistical methods (Wu & Li 1985; Wilson *et al*. 1987; Li & Bosquet 1992) and via comparison of log-likelihood values from maximum likelihood trees constructed with and without a molecular clock constraint (Hasegawa *et al*. 1985; Felsenstein 1993). Trees that show rate variation can then be 'linearized' (Takezaki *et al*. 1995) and only the reduced set of sequences subsequently used for calibration or prediction.

Assumption 7: calculations of standard errors and significance levels will be biased by non-independence among elements in matrices of pairwise comparisons

Calculation of the standard error of the average among-clade genetic distance using more than one pairwise comparison can be biased by non-independence among the comparisons. Unbiased standard errors of divergence can be estimated between individuals in different clades using the method of Steel *et al*. (1996) (see also Li & Bosquet 1992). The significance of regressions of pairwise genetic distance against time can also be biased by non-independence among the taxa used to calculate the genetic distances (Lynch & Jarrell 1993; Hillis *et al*. 1996). Standard errors of regression can be corrected for non-independence and heteroscedasticity using generalized least-squares regression (Lynch & Jarrell 1993). The significance of time–distance relationships can also be assessed using the Mantel and its associated permutation test (Rohlf 1990; Wray *et al*. 1996).

Rate calibration in the Hawaiian honeycreepers

Here we present an example of a 'local' rate calibration for part of the cytochrome *b* gene (*Cytb*) in Hawaiian drepanidines. Elsewhere (Fleischer *et al*., unpublished), we use this and other rate calibrations to estimate the timing of the drepanidine radiation. Here we use three calibration points: two from the amakihi lineage and one from the creeper lineage. We sequenced 675 bp of *Cytb* for 13 individuals of four amakihi taxa (*Loxops stejnegeri* from Kauai, and *Loxops virens* from Oahu, Maui and Hawaii; see Fig. 1) and an additional 14 drepanidine species in 10 genera (see Fig. 2a).

We amplified *Cytb* from either genomic DNA (three taxa only: Hawaii creeper, *Loxops mana*; Maui parrotbill, *Pseudonestor xanthophrys*; and akiapolaau, *Hemignathus wilsoni*) or purified mitochondrial DNA (all other taxa in Figs 1 and 2a; see Tarr & Fleischer 1993, 1995 for details). We used primers L14841 (modified from Kocher *et al*. 1989), 5'-AACATCTCAGCATGATGAAA-'3; H15149 (modified from Kocher *et al*. 1989), 5´-CAGAATGATATTTGTCCTCA-3´; BS2H, 5´-GAATCTACTACGGCTCATAC-3´ (designed from our drepanidine sequences); B7, 5´-CTAGTAGAATGAGCCTGAGG-3´ (designed from our drepanidine sequences); H15573 (modified from S. V. Edwards, unpublished, by Taberlet *et al*. 1992): 5´-AATAGGAAGTATCATTCGGG-3´; and L15162 (modified from S. Pääbo, unpublished, by Taberlet *et al*. 1992): 5-´AGCTTCTACCATGAGGACAAATATC-3´.

Amplification reaction components were 10 mM Tris buffer (pH 8.3), 50 mM KCl, 0.2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 U ampli*Taq*® DNA polymerase (Cetus), and 1 μM of each of a pair of primers in a 50 μL total volume.

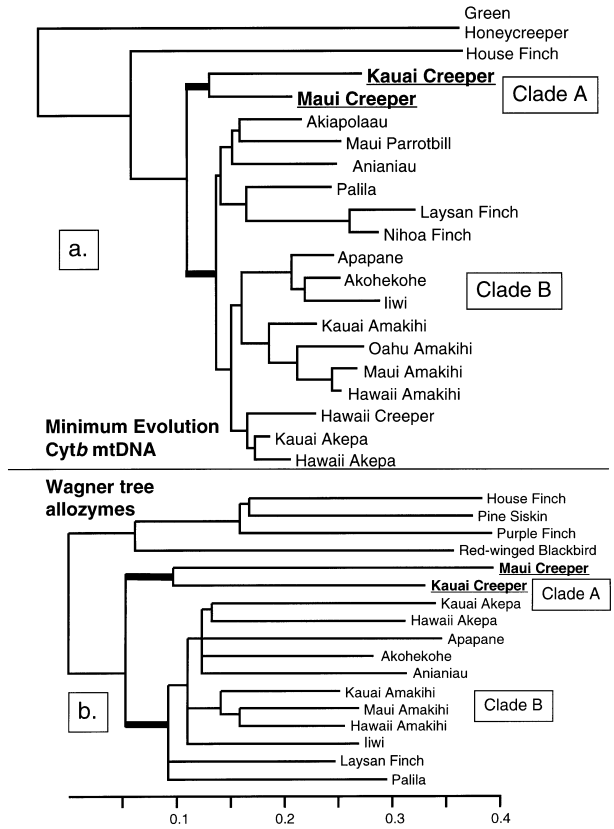

Fig. 2 a. Phylogeny of drepanidines based on 675 bp of the mitochondrial cytochrome *b* gene. A minimum evolution criterion was used to find the optimal tree via branch swapping on a neighbour-joining tree constructed from Kimura 2-parameter and Γ-corrected distances in PAUP* (Swofford 1997). This tree is very similar in topology to the majority consensus of nine trees produced by a heuristic search in a cladistic parsimony analysis in PAUP*. b. Phylogeny of drepanidines constructed from 20 variable allozyme loci (R. C. Fleischer, unpublished) using the distance Wagner procedure and Roger's distances in BIOSYS-1 (Swofford & Selander 1981). Note here, and in trees in Johnson *et al*. (1989), the basal split into 'Clade A,' the Kauai and Maui creepers and 'Clade B,' all the other drepanidine lineages for which sequence data are available. Outgroup taxa included are: green honeycreeper (*Chlorophanes spiza*), house finch (*Carpodacus mexicanus*), purple finch (*Carpodacus purpureus*), pine siskin (*Carduelis pinus*), red-winged blackbird (*Agelaius phoeniceus*). Drepanidine ingroups are: Kauai creeper, Maui creeper, akiapolaau (*Hemignathus wilsoni*), Maui parrotbill (*Pseudonestor xanthophrys*), anianiau (*Loxops parva*), palila (*Loxioides bailleui*), Laysan finch (*Telespiza cantans*), Nihoa finch (*Telespiza ultima*), apapane (*Himatione sanguinea*), akohekohe (*Palmeria dolei*), iiwi (*Vestiaria coccinea*), Kauai amakihi, Oahu amakihi, Maui amakihi, Hawaii amakihi, Hawaii creeper, Kauai akepa (*Loxops caeruleirostris*), and Hawaii akepa (*Loxops coccineus*).

Reactions were repeated for 35 cycles at 94 °C for 1 min (denaturing), 50 °C for 1 min (primer annealing), and 72 °C for 2 min (polymerization). Products were concentrated, electrophoresed in 2% agarose gels, and the excised product bands were isolated from the agarose.

The double-stranded products were sequenced according to standard double-stranded protocols using Sequenase, [^{35}S]-dATP and one of the above primers. Some sequences were also analysed on an ABI 373 automated sequencer via PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing Kit (ABI). To ensure accuracy, taxa were sequenced in both directions at least twice. Sequences were aligned in MacVector.

We constructed trees using character-based (maximum parsimony) and distance-based (minimum evolution and neighbour-joining) approaches, and used the program PAUP* (Swofford 1997) for both approaches and MEGA (Kumar *et al*. 1993) only for the latter. Amakihi phylogenies obtained from both approaches are nearly identical (only apical rearrangements within islands) and, as required by Assumption 2, their topologies indicate that the Maui subspecies was derived from the Oahu subspecies, and the Hawaii subspecies from the Maui subspecies (Fig. 1; Tarr & Fleischer 1993). In addition, we found no haplotypes shared among island populations, and that inter-island divergences are larger than intra-island ones. There were no significant differences in relative branch lengths among the Oahu, Maui and Hawaii lineages based on the maximum likelihood method of Hasegawa *et al*. (1985: $G = 1.92$, 8 d.f., $P > 0.9$). Thus these two nodes provide two calibration points.

Our third calibration point is the divergence between the Maui (*Paroreomyza montana*) and Kauai (*Oreomystis bairdii*) creepers. Maui and Oahu creepers (*Paroreomyza maculata*) are very similar morphologically, and are thought to be close sister taxa (H. James, personal communication), but we had no DNA sample from the Oahu taxon to test this hypothesis. A phylogeny based on osteological characters (H. James, personal communication) places the *Paroreomyza* as a sister clade to *Oreomystis*. This placement is also well supported by phylogenies reconstructed from *Cytb* (Fig. 2a, clade A; see below) and ATPase6/8 sequences (Fleischer *et al*., unpublished), our allozyme results (Fig. 2b, clade A) and those of Johnson *et al*. (1989), but not by restriction fragment data (Tarr & Fleischer 1995). We note that the Hawaii creeper (*Loxops mana*, formerly *Oreomystis mana*) falls within the clade containing the akepas and amakihis based on mtDNA and morphology (Fig. 2a; R. C. Fleischer *et al*., unpublished; James & Olson 1991), and is therefore not useful for our rate calibration. Thus, we assume that the Oahu/Maui creeper clade is sister to the Kauai one, and the divergence between them occurred after the formation of Oahu 3.7 Ma.

A matrix of Kimura 2-parameter (K2-P, Kimura 1980) and Γ-corrected (Uzzell & Corbin 1971) pairwise distances was constructed using PAUP* (Swofford 1997; Swofford *et al*. 1996). The value of α for the latter correction (0.254) was estimated by the Sullivan *et al*. (1995) method in PAUP* using the shortest drepanidine trees obtained in a heuristic search in a maximum parsimony analysis. For the multiple pairwise comparisons of amakihis we used the program 'CIProgPPC 1.01b' (C. McIntosh, unpublished) to generate unbiased standard errors on Jukes–Cantor (J–C) corrected distances following the method of Steel *et al*. (1996). These standard errors are included in Fig. 3: the ratio between J–C and K2-P/Γ-corrected values for our most distant ingroup comparison is only about 0.75. Thus the unbiased standard errors for K2-P/Γ-corrected distances would not be more than about 1.3 times larger than the J–C values. For the single pairwise comparison of Maui and Kauai creepers we used MEGA (Kumar *et al*. 1993) to estimate a standard error for the corrected distance (Fig. 3). Only for the two amakihi comparisons could we correct for potential divergence of ancestral alleles prior to population separation (see Assumption 4; Wilson *et al*. 1985; Nei 1987; Bishop & Hunt 1988; Tarr & Fleischer 1993).

The K2-P/Γ-corrected *Cytb* distances for the two amakihi and the one creeper calibration point were then regressed against the oldest K–Ar estimated ages of subaerial rock of the younger island for each comparison (Fig. 3). The dates used are from Table 1 of Carson & Clague (1995): for Oahu, 3.7 Myr; for Maui-nui (i.e. West Molokai, with and without correction for the subsidence of the Penguin Bank separating it from Oahu), 1.9 and 1.6 Myr, respectively; and for Hawaii (Kohala range), 0.43 Myr.

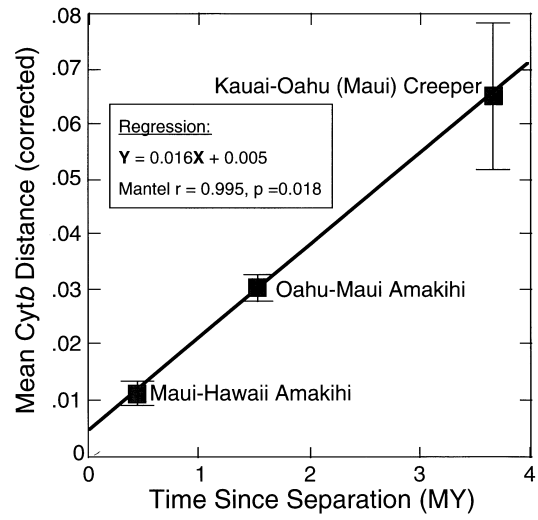

Fig. 3 Rate calibration for drepanidine mitochondrial cytochrome *b*. The two amakihi points are the mean of all pairwise Kimura 2-parameter and Γ-corrected distances, with intrapopulation variation from the parental population subtracted and unbiased standard error bars (Steel *et al*. 1996). The standard error bar for the single Maui and Kauai creeper pairwise comparison was calculated in MEGA (Kumar *et al*. 1993). The regression was significant based on 1000 permutations in a Mantel test (Rohlf 1990).

Because of non-independence in comparisons involving Maui amakihi we calculated the Mantel matrix *r* and used a permutation test (1000 replications) to assess significance of the relationship between time since separation (Myr) and genetic distance (Rohlf 1990). The relationship is significant for both Maui-nui ages (for 1.6 Myr: Mantel matrix $r = 0.995$, $P = 0.018$; for 1.9 Myr: Mantel matrix $r = 0.999$, $P = 0.015$; the regression for both was $Y = 0.016X + 0.005$). Thus we find an overall minimum sequence divergence rate for this region of *Cytb* to be 0.016/Myr (0.008 substitutions per site/Myr). If we use J–C-corrected values (without a Γ-correction) we obtain a slightly slower rate (e.g. for 1.9 Myr: Mantel matrix $r = 0.999$, $P = 0.027$; regression of $Y = 0.014X - 0.000$). Previously we (Tarr & Fleischer 1993) calculated substitution rates of 0.020–0.024/Myr for the entire mtDNA molecule using RFLP data, but we might expect the more conservative *Cytb* to be slower than the average for mtDNA. Both RFLP and cytochrome *b* rate estimates are similar to previous ones for small homeothermic vertebrates, and suggest that mtDNA substitution rates are not decelerated in birds, at least at these relatively low levels of divergence.

Our drepanidine trees (Fig. 2a,b), and those of Johnson *et al*. (1989) show a basal split between the 'true' creepers (clade A) and all other drepanidines (clade B). We have applied our rate calibrations to this basal split and estimated that this point occurred about 4–5 Ma (R. C. Fleischer *et al*., unpublished). Sibley & Ahlquist (1982) applied a rate to DNA hybridization divergences and suggested that drepanidines branched from the carduelines 15–20 Ma. This date is possibly inflated because they used cardueline outgroups that may not be closely related to the drepanidines (Groth 1994). Application of rate estimates from other molecular data sets also differ from these dates: allozymes (Johnson *et al*. 1989) suggest a cardueline–drepanidine split of 7.6 Ma and a radiation in 5.6 Myr, while our mtDNA restriction fragment data suggest that the split occurred only 3.5 Ma (Tarr & Fleischer 1993, 1995). These *Cytb*-based dates are also generally matched by ages inferred from internal rate calibrations for other genes (R. C. Fleischer *et al*., unpublished).

A number of other lineages of Hawaiian passerine taxa may be suitable for rate calibrations, including drepanidine akialoa and nukupuu, *Myadestes* thrushes and *Moho* honeyeaters. Because most of these taxa exist today only as museum specimens, mtDNA analyses will not be as straightforward as for the amakihi and Kauai/Maui creeper calibration points. Such comparisons, however, are important in evaluations of the among-taxon component of rate variation. We expect, however, that the rates may be similar, as rates for organisms of similar body size and metabolic rate tend not to vary greatly (Martin & Palumbi 1993) and these Hawaiian passerines are well within an order of magnitude in body size.

Rate calibration for *Yp1* in Hawaiian *Drosophila*

Using the same approach as above we calculated rates of sequence divergence for yolk protein 1 for the β lineage of the *planitibia* subgroup of the picture-winged Hawaiian *Drosophila* (*Yp1*, Kambysellis *et al*. 1995). The phylogeny from *Yp1* sequences (Fig. 4) is largely similar to ones generated from allozyme and chromosome data, *Adh* and mtDNA restriction site and *Adh* sequence data (see below; compare Kambysellis *et al*. 1995 to DeSalle & Giddings 1986; Bishop & Hunt 1988; Rowan & Hunt 1991), and the 'cascade' of clades in parallel to island order supports Assumption 2 (Kambysellis *et al*. 1995). Missing or misplaced taxa could, of course, invalidate this assumption.

In the *Yp1* tree we assume that the node (M–H, Fig. 4) between the two Hawaii and the two Maui-nui taxa occurred at the time of formation of Hawaii, thus less than 0.43 Ma (there is no way to rule out that each Hawaii taxon resulted from an independent colonization by a Maui form, but this would be a less parsimonious assumption). Similarly, we assume that the O–M node (Fig. 4) represents the split between *D. hemipeza* (Oahu) and the remainder of the β lineage. Bishop & Hunt (1988) and Rowan & Hunt (1991) argued that previous mtDNA and chromosomal data indicate that *D. differens* and *D. planitibia* arose from a single colonization of Maui; thus they consider the O–M split to have occurred after 1.3 Myr (as opposed to 1.6–1.9 Ma on Molokai). Maui and Molokai split apart about 0.3–0.4 Ma (Carson & Clague 1995), thus we use 0.35 Myr for the *D. differens*/*D. planitibia* (Mo–Ma) node. The K–O node between

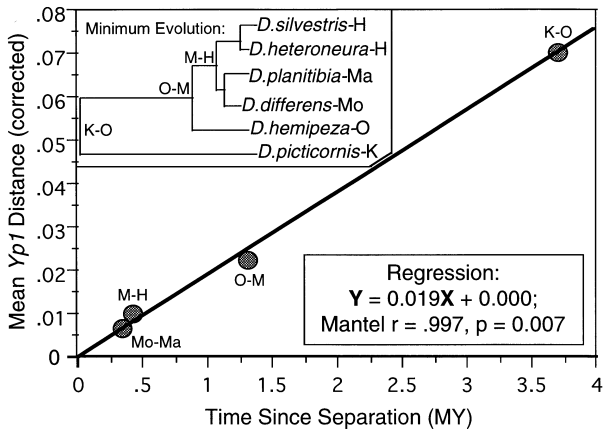

Fig. 4 Rate calibration for the *Yp1* gene (Kambysellis *et al*. 1995) for Hawaiian *Drosophila*. Distances are corrected by the Kimura 2-parameter method and with a Γ correction (Swofford 1997). M, Maui-nui (see Fig. 1); Mo, Molokai; Ma, Maui; H, Hawaii; O, Oahu; and K is Kauai. The regression was significant based on 1000 permutations in a Mantel test.

D. picticornis (Kauai) and *D. hemipeza* is assumed to have occurred after the formation of the Waianae range on Oahu 3.7 Ma.

We obtained the *Yp1* sequences from Kambysellis *et al*. (1995) from GenBank, including *D. hemipeza* (accession number U51800) and all the other taxa in Bishop & Hunt (1988; *D. silvestris*, U52056; *D. heteroneura*, U51801; *D. planitibia*, U52011; *D. differens*, U51582; and *D. picticornis*, U52010). Pairwise distances among the sequences were corrected by the K2-P method (Kimura 1980) and a Γ-correction in PAUP* ($\alpha = 1.29$ by the Sullivan *et al*. (1995) method; Swofford 1997). We could not correct for intraspecific variation (Assumption 4) because there was only one sequence per species. We found a significant relationship (Mantel matrix $r = 0.997$, $P = 0.007$ based on 1000 permutations) between the K2-P/Γ-corrected pairwise distances and the geologically based estimates of the times since their separation (Fig. 4). The overall rate of sequence divergence was estimated as 0.019/Myr (0.008 substitutions per site/Myr). If we instead use a date for the origin of Maui-nui as the West Molokai age corrected by the persistence time of the Penguin Bank (e.g. 1.6 Myr) it does not greatly alter either the rate estimate or its significance level (Mantel matrix $r = 0.987$, $P = 0.007$).

An interesting finding in the *Yp1* parsimony tree is the placement of *D. neopicta* within the '*cyrtoloma*' (or α) clade of the *planitibia* subgroup rather than as an outgroup to both it and the β clade (Ho *et al*. 1996). DeSalle & Templeton (1988) used *D. neopicta* as the outgroup to test whether the putatively bottlenecked β clade had longer branch lengths than the α clade, as predicted by the nearly neutral model (Ohta 1976). If *D. neopicta* is actually part of the α clade, then the 'shorter' branch lengths of the α lineage may not have resulted from repeated population bottlenecks, but rather are an artefact of the closer relationship between the α lineage and the putative outgroup. In fact, a neighbour-joining tree constructed using the mtDNA data (Table 3 in DeSalle & Templeton 1988) in MEGA (Kumar *et al*. 1993) places *D. neopicta* basal only to the α lineage. The use of additional outgroups might help to resolve this inconsistency.

Rate calibration for mtDNA in *Laupala* crickets

Shaw (1996) analysed sequence variation in portions of mtDNA 12S rRNA, 16S rRNA and the entire $tRNA^{val}$ among 17 species of Hawaiian *Laupala* crickets plus two outgroup taxa. Shaw's cladistic parsimony and maximum likelihood analyses supported a topology that mostly meets Assumption 2. First, a 'Hawaii' clade (seven species in clade 4 of Fig. 3 in Shaw 1996) appears to be derived from a Maui clade (three species in clade 3). Clade 4 does contain two Maui taxa, but these probably originated on Hawaii and represent a back-colonization from Hawaii to Maui. Second, the Maui clade appears to be derived from a clade containing both Kauai and Oahu taxa (four species in clade 2). Because the Kauai taxon (*L. kokeensis*) is basal to the Oahu taxa in clade 2, it is unlikely that the 'paraphyly' is due to a back-colonization of Kauai from Oahu. Shaw (1996) suggests that the three Oahu taxa in the tree used in that study may have resulted from a relatively recent colonization of Oahu from Kauai. Thus only two comparisons appear useful for rate calibrations: Maui vs. the Kauai-Oahu clade, and Hawaii vs. Maui.

We calculated J–C-corrected pairwise distances and the means, standard errors and 95% confidence limits of these distances among groups of taxa (as above; Steel *et al*. 1996). Additional corrections did not appear to be necessary (e.g. Table 2 of Shaw 1996). A maximum likelihood relative rate test (Hasegawa *et al*. 1985) revealed significant differences in branch lengths across 10 taxa sampled from among the four island groupings ($G = 15.45$, $P = 0.05$), as did independent pairwise comparisons of branch lengths of Hawaii vs. Oahu taxa (all $P < 0.05$). This results from considerably long branch lengths in the Hawaii clade relative to intermediate and small branch lengths in the Maui and Oahu/Kauai clades, respectively. This finding rejects Assumption 6; thus a rate calibration using linear regression would not be valid.

Nonetheless, we do calculate rates for each point separately. For the Hawaii–Maui calibration point we used two extremes that probably represent the upper and lower bounds of the rates. First, as in the drepanidine and *Drosophila* calibrations above, we used the node between the Maui and Hawaii clades as the maximum depth for the Hawaii clade. The unbiased mean distance across this node (0.044) divided by 0.43 Myr results in a mean between-lineage divergence rate of 0.102/Myr (95% confidence range is 0.068–0.142/Myr). Second, for a minimum rate we used the mean maximum depth within the Hawaii clade itself, as measured by pairwise J–C distances through the clade's basal node. The mean unbiased distance (0.0184) results in a mean sequence divergence rate of 0.044/Myr (95% confidence range is 0.023–0.064/Myr). The latter estimate requires the assumption that the basal-splitting major lineages within the Hawaii clade do not each represent independent colonizations of Hawaii from Maui (i.e. of lineages that became extinct, or were missed during sampling on Maui). The rates estimated from this comparison are far higher than expected for mtDNA and especially for rRNA and tRNA genes. The second calibration point involves comparison of the Maui and Kauai-Oahu clades. Assuming colonization of Maui-Nui 1.6 Ma results in a mean divergence rate of 0.024/Myr (95% confidence range is 0.015–0.034/Myr). This value is closer to, but still considerably higher than, other reported rates of

sequence evolution for this mtDNA region (e.g. Lynch & Jarrell 1993; Caccone *et al*. 1994; Cooper & Penny 1997).

Comparisons with rate calibrations for other Hawaiian taxa

The ages of the Hawaiian Islands have previously been used to estimate rates of molecular evolution within the dipterid genus *Drosophila* and the lobelioid genus *Cyanea*. Initial attempts for *Drosophila* were based on distances from analyses of allozyme variants (Carson 1976) and DNA–DNA hybridization (Hunt *et al*. 1981), but more recent analyses using restriction sites and DNA sequences of the alcohol dehydrogenase gene (*Adh*, Bishop & Hunt 1988; and Rowan & Hunt 1991, respectively) have provided substantially greater resolution. The rate estimates for *Cyanea* are based on restriction-site variation in chloroplast DNA (Givnish *et al*. 1995).

Bishop & Hunt (1988) were the first to use a regression approach in their analysis of *Adh* restriction-site divergence among members of the β lineage of the *planitibia* subgroup. They calculated pairwise distances and obtained a topology similar to that in Fig. 4 (but not including *D. hemipeza*). This topology is also supported by chromosome and allozyme data (Carson & Kaneshiro 1976), DNA hybridization (Hunt & Carson 1983), and *Yp1* sequences (Kambysellis *et al*. 1995). It is equivocally supported by *Adh* sequence data (Rowan & Hunt 1991; in their tree, *D. affinidisjuncta* positions between *D. picticornis* and the β lineage), and not entirely supported by mtDNA restriction-site data (DeSalle & Giddings 1986). In the latter tree, *D. differens* branches before a *D. planitibia*/*D. silvestris*/*D. heteroneura* clade. Nevertheless, either tree supports Assumption 2.

Bishop and Hunt (1988) corrected distances for intraspecific variation (Assumption 4) and regressed four points against younger island age. Their regression was significant ($P < 0.005$), and provided a sequence divergence rate of 0.012/Myr (or 0.006 substitutions per site/Myr). Rowan & Hunt (1991) followed a similar approach with *Adh* sequence data, but did not use a *D. planitibia*/*D. differens* point (and ignored *D. affinidisjuncta*). As in Bishop and Hunt, they used the age of West Maui (1.3 Myr) for the divergence of the *D. differens*/*D. planitibia* clade. They used two regressions, one dating the *D. picticornis* node at 3.7 Myr (Oahu) and one at 5.1 Myr (Kauai), and found rates of 0.023/Myr and 0.014/Myr for total nucleotides, respectively. The latter is close to what was found for the restriction-site data, but that regression used the 3.7 Myr date.

The only other Hawaiian rate calibration we know of is that of *Cyanea* (Givnish *et al*. 1995). This speciose lobeliod genus has undergone an extensive adaptive radiation, with variation in growth form and leaf and floral morphology. Givnish *et al*. (1995) used restriction-site analysis (nine enzymes) of chloroplast DNA to assess the phylogeny of 24 of the 55 species. Their single most parsimonious tree, including outgroups and the apparently congeneric *Rollandia*, was based on 78 informative sites of 150 total variable sites. From a phylogeographic analysis they were able to identify seven cases where lineages appeared to colonize younger islands. They averaged the number of site changes within cases involving colonizations of Oahu, 'Maui-Nui,' and Hawaii, respectively, and regressed these means against the dates of island formation (as in our Assumption 3). The regression was not significant ($P = 0.27$; $r^2 = 0.83$), but the regression coefficient revealed a rate of 1.52 site changes per Myr. Because they found a mean of 26.4 site changes along branches leading to the 'common ancestor' of the group, they inferred that the radiation began as much as 17 Ma. The poor fit of their regression may be caused by the lack of correction for saturation at the higher level of divergence, resulting also in a reduced rate. Regression with an exponent of the number of changes results in a better fit ($r^2 = 1.00$), and in a considerably faster overall rate.

Conclusions

Above, we describe a procedure that uses molecular phylogeographic reconstructions for lineages of Hawaiian organisms in combination with the geological history of the islands to infer rates of molecular evolution. We discuss seven assumptions or caveats, many of which should be met to justify using the approach. Assumption 2, for example is critical: tree topologies must show that monophyletic taxa arise in parallel to the temporal ordering of island formation. Assumption 3, that populations should be established very near to the time of island formation, is important for accuracy in estimating rates. For some assumptions, problems need only to be detected and then can often be easily corrected. The rate calibrations that we present here for the drepanidines and *Drosophila* are surprisingly well supported (Figs 3 and 4), even when we relax some assumptions about the timing of population founding. Less consistent calibrations for other Hawaiian taxa (e.g. for the extremely high rates and high variability among lineages found in *Laupala* crickets) suggest that one or more assumptions may have been compromised. In these cases the calibrations may be incorrectly estimated, or they may be correct and the rates simply vary for some biologically valid reason. In spite of the seemingly vast array of assumptions and caveats required in implementing the approach, the results we report here, along with those of a few previous studies, suggest that the procedure can be useful for estimating rates of molecular evolution. We will be interested in seeing how well future phylogeographic rate analyses support this conclusion.

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Note added in proof

A recent study (DeSalle and Bower 1997, Systematic Biology **46**, 751–764) of part of the β lineage of the *planitibia* subgroup of *Drosophila* summarizes support for the tree topology shown in Fig. 4. One new mtDNA analysis (COII) favours a 'differens (planitibia (silvestris, heteroneura))' phylogeny, while one new nuclear gene (*vg*) favours a (d,p)(s,h) phylogeny. A second nuclear gene (*ACHE*) provides an unresolved topology. Thus two mitochondrial phylogenies (i.e. RFLP and COII) favour d(p(s,h)), while six studies based on nuclear markers (i.e. allozymes, chromosomes, DNA–DNA hybridization, *Adh, YP1,* and *vg*) favour (d,p)(s,h). This nuclear vs. mtDNA difference suggests reticulation and/or lineage sorting, and slightly complicates our use of these taxa for estimation of rates. If we used the mtDNA-based topology rather than the *Yp1*-based one, this would remove the 'Mo–Ma' comparison from our regression (Fig. 4). It will be interesting to determine whether additional mtDNA and nuclear markers support this incongruency.

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