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Adaptive Radiation of Hawaiian Honeycreepers

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Report

Multilocus Resolution of Phylogeny and Timescale in the Extant Adaptive Radiation of Hawaiian Honeycreepers

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Summary

Evolutionary theory has gained tremendous insight from studies of adaptive radiations. High rates of speciation, morphological divergence, and hybridization, combined with low sequence variability, however, have prevented phylogenetic reconstruction for many radiations. The Hawaiian honeycreepers are an exceptional adaptive radiation, with high phenotypic diversity and speciation that occurred within the geologically constrained setting of the Hawaiian Islands. Here we analyze a new data set of 13 nuclear loci and pyrosequencing of mitochondrial genomes that resolves the Hawaiian honeycreeper phylogeny. We show that they are a sister taxon to Eurasian rosefinches (*Carpodacus*) and probably came to Hawaii from Asia. We use island ages to calibrate DNA substitution rates, which vary substantially among gene regions, and calculate divergence times, showing that the radiation began roughly when the oldest of the current large Hawaiian Islands (Kauai and Niihau) formed, ~5.7 million years ago (mya). We show that most of the lineages that gave rise to distinctive morphologies diverged after Oahu emerged (4.0–3.7 mya) but before the formation of Maui and adjacent islands (2.4–1.9 mya). Thus, the formation of Oahu, and subsequent cycles of colonization and speciation between Kauai and Oahu, played key roles in generating the morphological diversity of the extant honeycreepers.

Results

More than 3,700 km from any major landmass, the Hawaiian Islands form the most remote archipelago in the world. Consequently, evolution on the Hawaiian archipelago has been predominantly driven by in situ speciation rather than repeated colonization from continental sources, making the archipelago an exceptional setting for studying fundamental evolutionary processes including speciation and adaptation. The sequential ages of the islands, which formed by volcanism in a time series [1, 2], coupled with speciation that often parallels island formation, provide a means to estimate DNA substitution rates in endemic Hawaiian lineages. Such estimates of molecular substitution rates are critical to studies of evolution because they provide the timeline from which rates of morphological and behavioral change can be determined. These timescaled data can also be used to investigate the role of life history traits (e.g., metabolism, body size), population dynamics, and cellular activities on evolutionary processes [3].

The Hawaiian honeycreepers (Fringillidae: Drepanidinae) represent one of the most striking adaptive radiations of vertebrates. These colorful songbirds have diversified in bill morphology to an extent that they have not only convergently evolved many of the bill morphologies found in mainland songbirds but in addition evolved several bill forms unknown anywhere else (e.g., akiapolaau, see Figure 1 and Figure 2 in [4]). Unfortunately, without a well-resolved phylogeny, evolutionary insights from the Hawaiian honeycreeper radiation are severely limited. Low levels of sequence divergence among Hawaiian honeycreepers and rapid speciation have thus far prevented adequate resolution of their phylogenetic history (e.g., [5]). These limitations could likely be overcome with a substantially larger data set (in terms of DNA base pairs), as evidenced by an improvement in resolution from early data sets using <1 kb [6, 7] of sequence to more recent data sets of ~2.5 kb [5]. Next-generation sequencing technologies that generate large amounts of sequence data and recent barcoding methods that allow parallel processing of multiple individuals in a single run [8] can efficiently and cost-effectively generate phylogenetic data sets that are orders of magnitude larger than those typically obtained with traditional technology [9].

We sequenced complete mitochondrial genomes (~17 kb) and 13 nuclear loci (8.2 kb) from 19 extant or recently extinct Hawaiian honeycreeper taxa and 28 outgroup taxa (see Table S1 available online) using 454 sequencing technology and sample multiplexing [8, 10] as well as traditional sequencing methods. Further details about DNA sequences are available in the Supplemental Information. In addition to resolving the phylogeny for the 19 extant Hawaiian honeycreepers and related outgroups, we also calibrate evolutionary rates for all regions or genes of the mitochondrial genome and 13 nuclear loci, using Bayesian methods that provide greater accuracy and resolution than in previous work. Two taxon sets were analyzed: the "honeycreeper data set" included all 19 species of recently extant Hawaiian honeycreepers and the common rosefinch as the outgroup, and the "full taxon data set" included all Hawaiian honeycreepers as well as 28 outgroup taxa (with the house sparrow as the outgroup) for a total of 47 taxa (Table S2). These data were intensively analyzed for phylogenetic signal and to compute rates of DNA sequence evolution.

Phylogenetic Analyses

We performed a variety of analyses using data from the two taxon groups described above, and all produced similar topologies, although the best support was recovered with data from whole mitochondrial genomes. As described below, all

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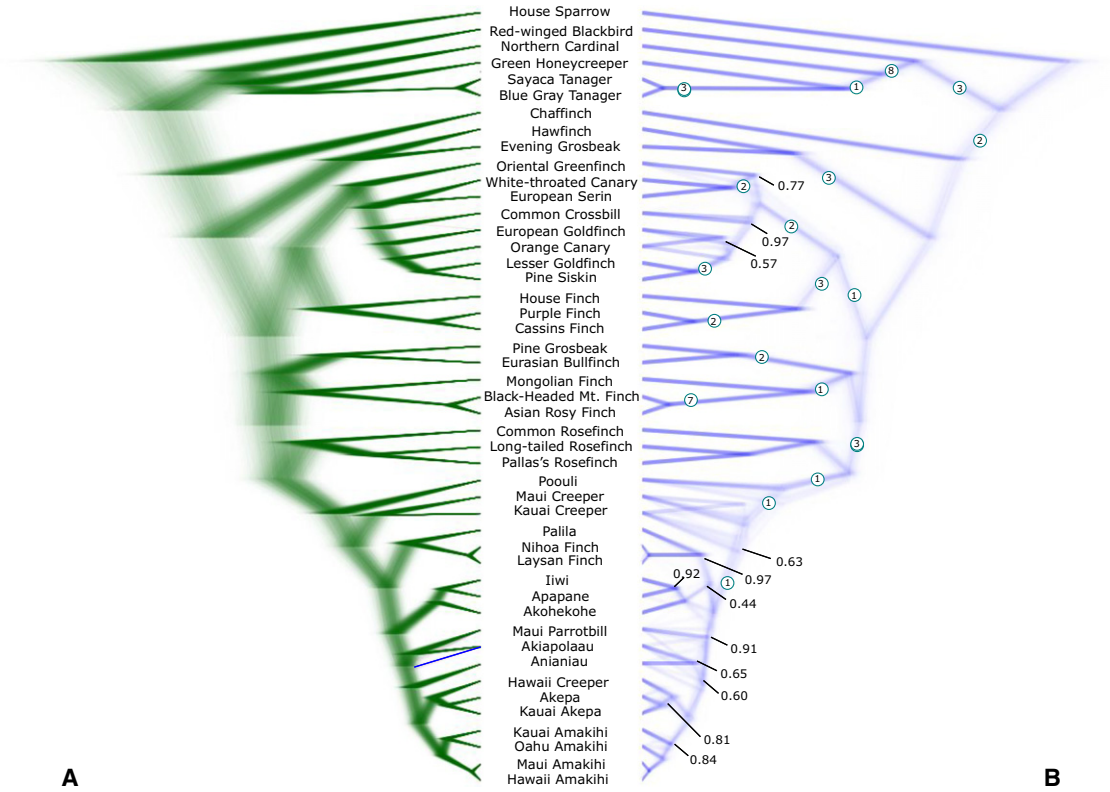

Figure 1. Phylogeny for Hawaiian Honeycreepers and 28 Outgroup Taxa

(A) Cloudogram showing all trees resulting from a Bayesian analysis of whole mitogenomes (19,601 trees; 14,449 bps). Variation in timing of divergences is shown as variation (i.e., fuzziness) along the x axis. Darker branches represent a greater proportion of corresponding trees. All nodes have support values >0.99.

(B) Topogram showing all topologies resulting from Bayesian species tree estimation from 12 nuclear loci and whole mitochondrial genomes (22,389 bps; 1,200 trees). Numbers within circles indicate the number of indels supporting each branch. Darker branches represent a higher number of corresponding topologies. Bayesian posterior probabilities <0.99 are indicated.

analyses conclusively placed the Hawaiian honeycreepers within the cardueline finch clade as sister to Eurasian rosefinches (*Carpodacus*). The branching pattern within the large clade of Hawaiian honeycreepers was fully resolved with mitochondrial data but lacked support from the nuclear intron data at several midlevel nodes corresponding to the placement of a clade of finch-like taxa (palila, Nihoa finch, and Laysan finch) and to the placement of akiapolaau and anianiau (see details below).

Bayesian analyses of the mitochondrial coding sequences alone for all 47 taxa (i.e., the full taxon data set) produced a topology (Figure 1A) that resolved the position of the honeycreepers within the cardueline radiation and all of the internal nodes within the radiation (all Bayesian posterior probabilities [bpp] = 0.99–1.00). In Figure 1A, all post-burn-in trees are shown with their estimated branch-lengths and topologies. The fuzziness of the horizontal plane of the branches reflects the variation in branch lengths among estimated trees. This figure also shows that nearly all post-burn-in trees support the topology shown in green, whereas only a few trees were recovered that support an alternative topology in which the Maui parrotbill and akiapolaau are not sister taxa (shown in blue).

In order to reduce any effects of undetected selection on the phylogenetic reconstruction, additional analyses were conducted on a data set composed of mostly neutral mitochondrial sites (i.e., third codon positions and control region) for the honeycreeper data set. Maximum likelihood and Bayesian analyses of this data set produced topologies identical to that shown in Figure 1A (data not shown). An analysis of only neutral sites was not performed for the larger full taxon data set because there was evidence of substitution saturation for that data set at third codon positions. Those sites were coded as purines or pyrmidines (RY-coded) for analyses of the full taxon data set.

Figure 1B shows a species tree analysis of the combined mitochondrial coding sequences and nuclear loci (22.4 kb of aligned sequence in total) for the full taxon data set. The species tree analysis jointly estimates the underlying phylogeny for each locus and uses this information to estimate the overall phylogeny for the species. Because trees found after burn-in from a Bayesian analysis can vary both in topology and branch lengths, typically, a single representative tree is shown (e.g., the tree with the highest likelihood or consensus tree) and uncertainty is shown as nodal support values indicating the percent of trees in the set that share the node in question. Although this quantitative approach is easily interpretable, it obscures alternative topologies recovered in the analysis. In order to retain information about alternative topologies as well as support, we present all topologies recovered in the post-burn-in analysis for the Bayesian species tree analysis of the mitochondrial coding sequences

and nuclear loci together (Figure 1B, 22.4 kb of aligned sequence in total). For this data set, alternative topologies were not visible when branch length variability was also shown for every tree (see branch length variability on the horizontal plane of Figure 1A), so instead, average branch lengths were calculated and are presented here for each topology (using DensiTree software, [11]). In this analysis, all nodes were resolved with strong support with the exception of a few of the internal nodes within the honeycreeper radiation. The unresolved nodes correspond to the placement of a clade of finch-like taxa (palila, Nihoa finch, and Laysan finch) and the placement of akiapolaau and anianiau. These divergences occur during a particularly rapid time of speciation within the radiation, so it is not surprising that nuclear loci with slower substitution rates and larger population sizes provide less resolution than the mitochondrial data set (see discussion below in Divergence Date Estimation).

Divergence Date Estimation

We assessed the age of the Hawaiian honeycreeper clade and tempo of evolution within the radiation using a Bayesian time-calibrated phylogeny estimated from the whole mitochondrial genomes and using the three island-age calibration points and the rationale from Fleischer et al. [7]. We confirm the assumption that the divergence of the Maui and Kauai creepers reflects the formation of the island of Oahu, because the extinct Oahu and extant Maui creeper are sister taxa based on previous mitochondrial DNA (mtDNA) analyses [12]. Our age estimates (Figure 2) indicate that the ancestral colonists arrived in the Hawaiian Islands sometime between the divergence between Hawaiian honeycreepers and the common rosefinch estimated at 7.2 mya (8.1–6.4 mya 95% highest probability density [HPD]) and the earliest divergence within the Hawaiian honeycreepers at 5.8 mya (6.3–5.2 95% HPD), although these dates might be slightly overestimated because they are generated from a gene tree analysis rather than a species tree analysis [13]. Our calibration also resulted in some novel insights into the pattern and timing of the Hawaiian honeycreeper radiation. In particular, nearly all branches leading to distinctive extant morphological lineages (morphotypes) appear to have diverged between 5.8 and 2.4 mya, a timeframe that overlaps with the formation of the island of Oahu (4.0–3.7 mya) but occurs prior to the formation of the Maui Nui island complex (i.e., Maui, Lanai, Molokai, and Kahoolawe; 2.4–1.9 mya). This suggests that the formation of the new island of Oahu, by providing a second major land area well isolated from Kauai and Niihau, may have enabled a higher rate of adaptation and speciation.

Evolutionary Rate Estimation

Evolutionary rates estimated from three separate mitochondrial and nuclear data sets (see below) in calibrated Bayesian analyses show a broad distribution of locus-specific rates, from 0.00035 substitutions per site per million years (s/s/my; Rag 1) to 0.058 s/s/my (mitochondrial third codon positions).

Evolutionary rates in Figure 3A were estimated in the same analysis from which the Hawaiian honeycreeper divergence dates shown in Figure 2 were estimated, an analysis that partitioned the mitochondrial genome according to functional region (i.e., codon position, noncoding sequence, etc.). To further explore mitochondrial rates of evolution by gene, we conducted an analysis with a separate partition for each of the 13 mitochondrial genes, the three domains of the control region, RNA stems, and RNA loops (Figure 3B). Despite the vastly different number of partitions used in analyses shown in Figure 3A versus Figure 3B, evolutionary rate estimates for partitions shared between these analyses (i.e., control region domain II, RNA stems, and RNA loops) were identical or differed by a value much less than the standard deviation.

Across the complete mtDNA genome, we found an average rate of sequence divergence of 1.8% per million years, similar to estimates from other studies for avian mtDNA (e.g., 1.1%–2.1% for Passeriformes, [14]) and for the *cytochrome-b* gene (2.1%, [15]). Rates of synonymous substitution (i.e., third codon positions) were higher (5.8%, 5.2%–6.3% HPD) than rates of nonsynonymous substitution and closer to rates estimated from within-species comparisons [16]. We found a 14-fold difference between the rates for the RNA stems and domain III of the control region (Figure 3B), with some of the slower rates found for genes that function in oxidative energetics (i.e., the *cytochrome c* complex).

Evolutionary rates for all 13 nuclear loci (Figure 3C) are lower than those of the mitochondrial regions, though the fastest-evolving nuclear locus (*beta-fibrinogen intron* 7, 0.0019 s/s/my) is comparable to the slowest-evolving mitochondrial region (RNA stems, 0.0022 s/s/my). The nuclear introns evolved at an average rate of 0.12% (0.07%–0.20%), somewhat slower than the rate of 0.36% reported by Axelsson et al. [16] from 33 turkey and chicken autosomal introns. We use a younger calibration than the chicken-turkey split used by Axelsson et al. (28 million years) and thus, our data may be more applicable to recent divergences, such as those within songbirds (i.e., Passeriformes, the majority of all extant avian species). The *Rag 1 exon* evolved at a remarkably, though not unexpectedly, slow rate of 0.04% per million years (similar to 0.046%, calculated from [17]).

Discussion

Our results show clearly that next-generation DNA sequence data sets hold tremendous promise for resolving the pattern, process, and timing of island adaptive radiations. The higher rate of substitution and smaller effective population size of mitochondrial compared with nuclear DNA sequences make whole mitochondrial genomes particularly useful for resolving adaptive radiations, at least where mitochondrial introgression is not prevalent [18]. For the Hawaiian honeycreepers, species tree analyses show that the mitochondrial signal agrees with the nuclear signal while providing higher resolution because of the faster average substitution rate compared with nuclear sites. For the mitochondrial genomes, much of the signal derives from the large number of neutral or noncoding sites, which are especially valuable when estimating phylogenies of adaptive radiations because they are more likely to reflect phylogenetic history than is DNA sequence from functional regions that may be confounded by selection. Additionally, variance in mutation rate and branch length variation among gene trees were not pronounced for this group (as shown in Figure 3), although such variance has been shown to be an important potential source of error in estimating phylogenies for recent radiations [19, 20].

Although species tree estimation from the nuclear data alone and the nuclear + mitogenomes produced a well-supported phylogeny, those analyses were unable to resolve all nodes with high support. The mitochondrial sequences, on the other hand, evolved on average ~20 times faster than the nuclear sequences (Figure 3) and produced a fully resolved tree with high support. Including more alleles per taxon might

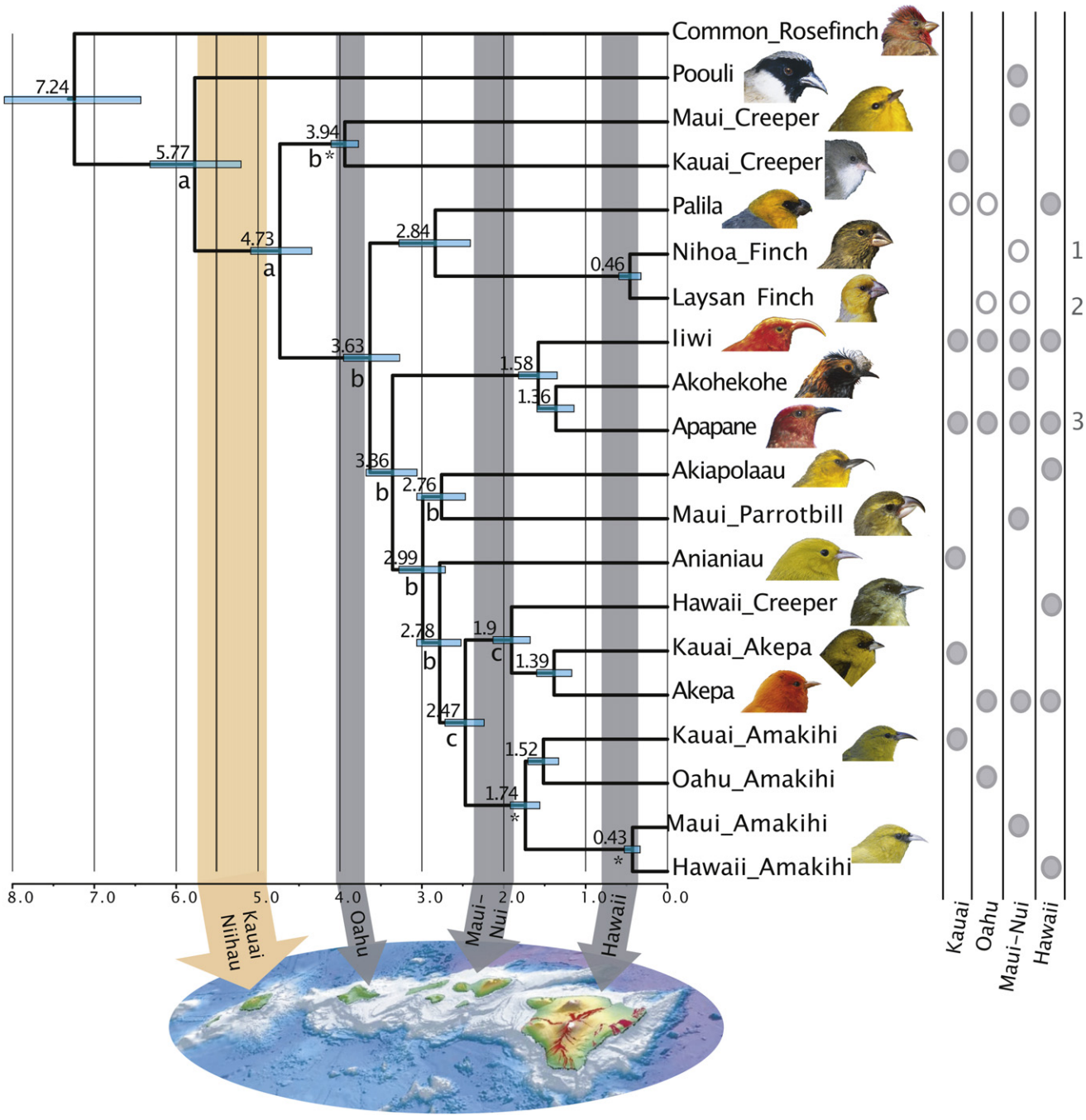

Figure 2. Bayesian Divergence Date Estimates for Hawaiian Honeycreepers from Whole Mitochondrial Genomes Based on Three Island Age Calibration Points [7]

Mean ages are shown above each node, with horizontal bars across nodes representing 95% highest probability density intervals. Shaded vertical bars encompass the estimated subaerial to maximal shield-building dates for the recent Hawaiian Islands [1], where the gray bars indicate island ages used as calibrations, and asterisks (*) identify constrained nodes. Lowercase letters identify divergence of a new morphological lineage before formation of Oahu (a), during or after formation of Oahu (b), or before or during formation of Maui Nui (c). Distributions by island are listed to the right of each taxon where closed circles denote historic and/or extant (and sometimes fossil) distributions, and open circles represent fossil distributions with no known historic or extant populations. (1) The extant population occurs on Nihoa Island, but closely related extinct species mainly differing in size occurred on Kauai, Oahu, and Hawaii Islands. (2) The extant population occurs on Laysan Island, but closely related extinct species mainly differing in sizes occurred on Kauai and Hawaii Islands. (3) A closely related species or subspecies occurred on Laysan Island. Photographs are by Jack Jeffrey.

increase the power for phylogenetic resolution when using the species tree method with nuclear sequences. Although we are confident in the topology presented here, our future research will explore nuclear allelic diversity within Hawaiian honeycreeper taxa in order to more fully utilize modeling of the coalescent process in phylogeny reconstruction.

A broader value of this study is the estimation of rates of sequence evolution across the mitochondrial control region

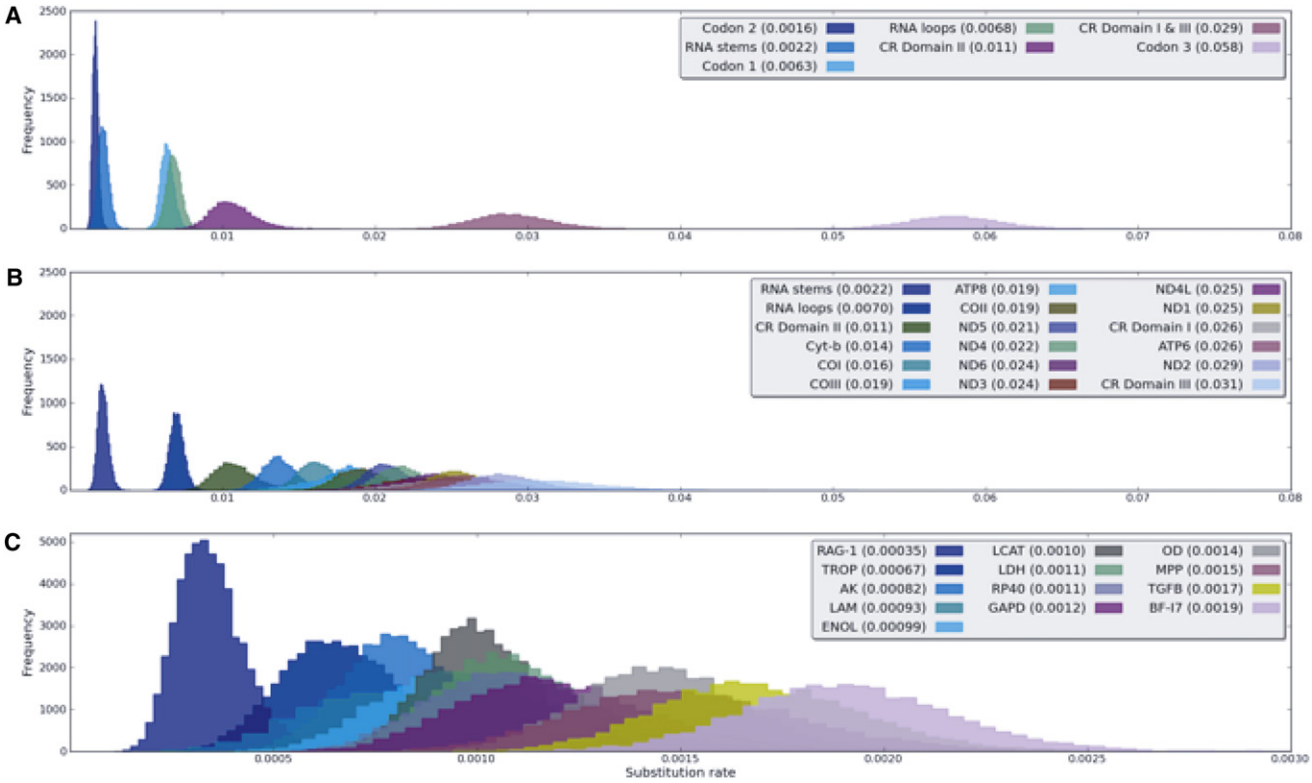

Figure 3. Evolutionary Rates Estimated for Mitochondrial and Nuclear Genes, Introns, and Regions from Three Separate Bayesian Analyses

(A) Seven mitochondrial regions.

(B) Eighteen mitochondrial genes and regions.

(C) Thirteen nuclear loci. Any difference in average substitution rates for regions estimated in multiple analyses (e.g., RNA stems, RNA loops) lies within the standard deviation for the estimates.

and 13 nuclear loci using internal, minimal rate calibrations, rather than external calibrations that may greatly precede the formation of the clade being assessed (Figure S5). In the past, a "2% rule" of avian mitochondrial divergence per million years has often been applied, unfortunately somewhat indiscriminately, in phylogenetics and phylogeography [21]. Our rate estimates will allow researchers to select loci with appropriate rates of evolution for studies of avian phylogeny and population genetics, as well as apply more precise substitution rates specific to the loci under study in order to estimate the time frame for avian evolution. Our rate estimates for several loci and functional regions (e.g., nonsynonymous sites) are largely concordant with other published avian data sets [14–17], suggesting broad applicability of our locus-specific rates for population genetic and phylogenetic studies of avian taxa. For instance, based on our estimates, studies that require high rates of substitution could use ND2 and ATP6 in addition to the more commonly used control region.

Knowledge of the outgroup relationships of an adaptive radiation is essential to interpretations of evolutionary patterns within the radiation; thus, we included a large set of outgroup species in our analysis. This large taxon sampling also allowed us to resolve previously unresolved relationships among outgroups. Most previous studies have identified the cardueline finches (Fringillidae: Carduelinae) as the likely source of the honeycreeper radiation's ancestor (summarized in [22]), but the specific sister group within Carduelinae was unresolved. Previous authors have pointed to crossbills (*Loxia*) and the pine grosbeak (*Pinicola enucleator*) as modern ecological/behavioral analogs for understanding how the continental cardueline ancestor of the Hawaiian honeycreepers may have been able to colonize the remote Hawaiian Islands millions of years ago [23, 24]. In contrast, all of our data sets and methods of analysis (Figure 1; Figure S1) support a sister relationship between Hawaiian honeycreepers and a clade of three rosefinches (Carduelinae: *Carpodacus*), including the common rosefinch.

Common rosefinches share an important life history trait with crossbills and the pine grosbeak: they often move in large mixed-sex groups to new wintering grounds outside their typical range, a behavior termed an "irruption," and they may stay to breed in those new regions [25]. It is possible that colonization by the ancestral species was aided by the arrival of a large mixed-sex flock in the islands, representing a sizable gene pool. Thus, a diverse initial gene pool may have facilitated speciation and the evolution of extreme morphological diversity in the honeycreeper radiation (for another perspective see [4]). Assuming that rosefinches were restricted in distribution to the Old World in the Miocene as they are at present, it is likely that the ancestral stock leading to Hawaiian honeycreepers arrived in Hawaii from the west.

Eurasian rosefinches and the other close outgroups share the typical finch-like bill shapes of continental cardueline finches, confirming that the Hawaiian honeycreepers evolved their astonishing diversity of bill forms from an ancestor with a finch-like bill and emphasizing the importance of conditions

on the Hawaiian Islands in stimulating such a diverse radiation. However, although all Eurasian rosefinch species resemble one another in phenotype, particularly in bill morphology [26], the oldest divergences in the honeycreeper radiation lead to species that differ from rosefinches and other continental cardueline finches in bill morphology and feeding niche. The recently extinct poouli had a superficially finch-like bill but with a modified hard palate; it fed not on seeds but on snails and other invertebrates [27]. The Kauai and Maui creepers have straight thin bills and feed on arthropods; the Maui creeper in particular is very warbler-like [24, 28]. The three species of honeycreepers in our analysis that closely resemble continental cardueline finches in their conical bills, cranial osteology, and feeding habits (the palila, Nihoa finch, and Laysan finch) are unexpectedly more recently diverged. That the earliest diverging Hawaiian honeycreeper lineages lead to taxa that differ in morphology and niche from continental cardueline finches suggests that directional selection early in the radiation favored adaptation to an invertebrate diet, an evolutionary pattern also observed in the Galapagos finches [24, 29]. Our phylogenetic results are consistent either with a single evolutionary loss and subsequent gain of the finch-like morphology and feeding niche or with the persistence of a finch-like lineage with at least two gains of more thin-billed and warbler-like morphologies. In either case, the resolved molecular phylogeny reveals a more complex pattern of morphological evolution than would be expected based on classic papers about the radiation, which proposed phylogenetic patterns that minimized the morphological distance between related taxa [23, 30].

The timing of the earliest divergence within the honeycreepers corresponds closely to the emergence from the sea of the islands of Niihau (5.7–5.3 mya) and Kauai (5.4–4.9 mya; Figure 2). In this early time period before the formation of Oahu, two divergences occurred, each separating a distinct morphological lineage from the rest of the radiation (first the poouli and second the Oahu and Maui creeper and relatives). Biogeographically, the placement of poouli (a Maui taxon) as an early divergence in Figure 2 suggests that the poouli or its relatives formerly occurred on older islands. In the rich fossil record of Hawaiian honeycreepers, the poouli occurs only on Maui; however, it may be that extinct taxa, such as Xestospiza, that occur on older islands are relatives of the poouli [24, 31]. DNA sequences from extinct subfossil honeycreepers might resolve this issue.

Within the honeycreeper radiation, a burst of cladogenesis accompanied by morphological diversification occurred between 5.8 and 2.4 million years ago, a time period that encompasses the formation of Oahu, yet precedes the formation of Maui Nui. During this time frame, six of ten major extant morphological lineages evolved (nodes labeled "b" in Figure 2). That only two morphological lineages evolved after this time frame emphasizes the importance of the formation of Oahu, more so than Maui Nui, to the present-day morphological diversity of Hawaiian honeycreepers. This result is surprising, given the larger size and greater ecological complexity of Maui Nui as well as other work describing the importance of Maui Nui for diversification in other Hawaiian radiations [32]. For groups with more limited dispersal than the Hawaiian honeycreepers, ecological conditions on Maui Nui, and particularly the breakup of the Maui Nui complex of volcanoes into several islands, probably played a greater role in speciation. In contrast, repeated colonization and isolation between Kauai and Oahu appears to be pivotal in spurring cladogenesis of the Hawaiian honeycreepers. Oahu, as a newly formed island initially without avian residents, likely provided a blank slate allowing ecological and morphological differentiation [33].

Supplemental Information

Supplemental Information includes two figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.cub.2011.09.039.

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