Age and rate of diversification of the Hawaiian silversword alliance (Compositae)

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ABSTRACT Comparisons between insular and continental radiations have been hindered by a lack of reliable estimates of absolute diversification rates in island lineages. We took advantage of rate-constant rDNA sequence evolution and an "external" calibration using paleoclimatic and fossil data to determine the maximum age and minimum diversification rate of the Hawaiian silversword alliance (Compositae), a textbook example of insular adaptive radiation in plants. Our maximum-age estimate of 5.2 ± 0.8 million years ago for the most recent common ancestor of the silversword alliance is much younger than ages calculated by other means for the Hawaiian drosophilids, lobelioids, and honeycreepers and falls approximately within the history of the modern high islands (≤5.1 ± 0.2 million years ago). By using a statistically efficient estimator that reduces error variance by incorporating clock-based estimates of divergence times, a minimum diversification rate for the silversword alliance was estimated to be 0.56 ± 0.17 species per million years. This exceeds average rates of more ancient continental radiations and is comparable to peak rates in taxa with sufficiently rich fossil records that changes in diversification rate can be reconstructed.

The Hawaiian silversword alliance (*Argyroxiphium*, *Dubautia*, *Wilkesia*; Compositae) has been considered "the best example of adaptive radiation in plants" (1). Life-form diversity among the 28 Hawaiian-endemic species in the group encompasses trees, shrubs, subshrubs, mat-plants, monocarpic and polycarpic rosette plants, cushion plants, and vines that occur across a broad environmental spectrum, from rainforests to desert-like settings (2). Although monophyly of the group is well established (3), absolute ages of the silversword alliance and other Hawaiian lineages have been difficult to estimate. Ages are essential in the reconstruction of the absolute diversification rates—the rate of speciation minus extinction ($S − E$). Availability of such rate estimates would permit comparisons with other insular radiations and with continental radiations, which may exhibit qualitatively or quantitatively different patterns of diversification.

Unfortunately, knowledge of island ages from potassium–argon dating is of minimal value for placing upper limits on ages of lineages in ancient hot-spot archipelagos such as the Hawaiian Islands. The age of terrestrial groups in the Hawaiian archipelago can conceivably date back to the formation of Kure [29 million years ago (Ma)], under the assumption of an unbroken succession of dispersals from older to younger islands since that time (4). An upper age limit of 29 Ma is too high to be of any value for dating island radiations in groups such as Asteraceae—the oldest unequivocal fossil evidence of the family worldwide is more recent, from the mid-Oligocene (5). Moreover, calibration of clock-like divergence of molecular sequences is complicated if individual island ages are used to estimate the age of an entire insular radiation ("internal" calibration). Any error in the placement of one island's age along a branch of the phylogeny will be magnified in age calculations of deeper points in the tree (see ref. 6).

An extraordinary set of conditions allows us to use an "external" calibration to place an evolutionarily meaningful maximum date on the onset of diversification of the silversword alliance. The entire Hawaiian lineage is phylogenetically nested within a western American lineage of herbs in the genera *Madia* and *Raillardiopsis* (7, 8). Conformity of a nuclear rDNA phylogeny of the Hawaiian and western American species with a rate-constant model of molecular evolution and knowledge of the timing of climatic events that gave rise to the continental lineage allow us to place absolute time limits on the diversification of the Hawaiian group. Because branching times can be reconstructed under rate constancy, it is possible to use a statistically efficient estimator of absolute diversification rate, thereby obtaining a much more accurate estimate than would be possible without such information.

MATERIALS AND METHODS

Sample data [except for *Madia madioides* (Nutt.) E. Greene, *Baldwin 488* (DAV), Napa County, CA] and methods for DNA extraction, PCR, and sequencing of the internal transcribed spacer region (ITS) of nuclear rDNA were reported by Baldwin and Robichaux (9). ITS sequences from 11 Hawaiian populations, including all representatives of five species (*Argyroxiphium grayanum*, *A. kauense*, *Dubautia linearis*, *D. platyphylla*, and *Wilkesia hobdyi*), studied by Baldwin and Robichaux (9) were excluded from the phylogenetic analyses because of complete identity with a sequence included in our study. Only three of the 28 species of the silversword alliance [*A. virescens* (presumed extinct), *D. dolosa,* and *D. waialealae*] recognized by Carr (2) were unavailable and not sampled. ITS sequences (ranging from 638 to 643 bp) were analyzed from 29 in-group samples of 25 Hawaiian populations (representing 24 taxa) of *Argyroxiphium*, *Dubautia*, and *Wilkesia* and the four perennial North American species of *Madia* and *Raillardiopsis*. Annual species in the *Madia/Raillardiopsis* group were excluded because of results from a battery of rate-constancy tests (refs. 10 and 11; detailed below) on minimum-length parsimony trees of annuals alone, perennials alone, and combinations of annuals and perennials. The results confirmed expectations (see ref. 12) of generally higher and more heteroge-

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neous rates of ITS evolution in the annuals than in the perennials.

Phylogenetic trees were estimated by using Fitch parsimony analysis and "branch-and-bound" search algorithms in β test versions of PAUP* 4.0 (versions d56–d59) provided by D. L. Swofford (Smithsonian Institution). Reliability of clades was assessed by bootstrapping (100 replicates with a heuristic search strategy and 20 random taxon-addition sequences each). The HKY85 (with Γ-distributed rate variation) model of sequence evolution (13) was chosen after performing a series of likelihood ratio (LR) tests on progressively more complex models. Variation in evolutionary rates across sites (14) was examined in an LR test that compared a Γ-distributed model in which the shape parameter was estimated, to one in which the shape parameter was set to infinity (equivalent to constant rates across sites, yielding a test with one degree of freedom). Variation in rates across lineages was examined by using a tree-wide LR test (10, 11) to compare rate-constant and rate-variable models in each of the four minimum-length ITS trees of the silversword alliance and their closest perennial relatives. Standard formulas for determining degrees of freedom for this test (10) assume a fully resolved tree and must be corrected for any polytomies. Degrees of freedom for the test of rate constancy across lineages are equal to the difference between the parameters in the rate-constant model (18 internal node ages plus one rate parameter) and the rate-variable model (one parameter for each branch length on the unrooted tree = 46 parameters), which equals 27. All likelihoods were determined with PAUP* 4.0.

Maximum likelihood estimates of divergence times were obtained after discovery of lineage rate constancy. Standard errors of the divergence times were estimated by a three-step nonparametric bootstrap procedure (15) as follows: (*i*) 100 resampled data matrices were generated by using the SEQBOOT program in PHYLIP 3.57 (16), (*ii*) the matrices were imported into PAUP* 4.0 and divergence times were obtained on the tree of Fig. 1 for each data matrix, by using the model of sequence evolution described above. Resulting trees with node times were exported to a tree file. (*iii*) The tree file was processed by the program R8S (available from M. J. Sanderson by anonymous ftp to loco.ucdavis.edu), which summarizes the bootstrap distribution of divergence times for each node. The error estimates (Fig. 2) reflect the stochastic error caused by having sampled only a finite number of characters.

Error in divergence time estimates attributable to mistaken tree topology was estimated from trees generated by parsimony analysis of 25 bootstrap replicates of the ITS data. The trees obtained from the bootstrap replicates represent a broad sample of the topologies that are not in strong conflict with the ITS data set. Divergence times were calculated from each of the trees under a rate-constant model of sequence evolution across lineages. The mean and standard deviation of the resultant divergence time estimates, in comparison with estimates from the most parsimonious trees, provide an indication of the error from inaccuracy of tree reconstruction.

We also evaluated the possibility of error in the tree topologies (and branch lengths) stemming from recombination between divergent ITS sequences, under the scenario of an allopolyploid (hybrid) origin of the silversword alliance from the primarily diploid lineages of *Madia/Raillardiopsis* (see ref. 17). No evidence of highly divergent intra-genomic ITS repeats was found in the silversword alliance (9), but the possibility of homogenization of different parental ITS copies via concerted evolution (see ref. 18) before diversification of the group cannot be dismissed. None of the ITS changes that map to the branch uniting members of the silversword alliance occurs in parallel within *Madia/Raillardiopsis*, based on ACCTRAN reconstructions of character evolution on the most parsimonious ITS trees using PAUP*. The absence of any characters exhibiting this pattern suggests that ITS sequences in the silversword alliance are not recombinant between divergent ITS sequences from different extant lineages in *Madia/Raillardiopsis*. This result is consistent with an origin of the tetraploid condition by wide allopolyploidy followed by unidirectional concerted evolution (19) or by autopolyploidy.

A conservative approach to calibrating the rate-constant ITS trees was undertaken to obtain a maximum age estimate for the most recent common ancestor of the silversword alliance. Fossil evidence (20–22) and corroborative paleoclimatic data (23) demonstrate that the shift from a summer-wet to summer-dry climate began abruptly in western North America at 15 Ma and continued through the late Miocene. All but one of the 86 species in the continental tarweed group (including *Madia/Raillardiopsis*) that gave rise to the silversword alliance occur in the summer-dry California Floristic Province, with the vast majority of species restricted to the region (24, 25). Nesting of the silversword alliance lineage well within the California tarweed phylogeny (25) dictates that the most recent common ancestor of the silversword alliance could not have arisen before the diversification of tarweeds in western North America, an event that must postdate the onset of mid-Miocene summer drying. We therefore used 15 Ma as our calibration date—a conservative maximum age—for the most recent common ancestor of the clade comprising silversword alliance and its Californian ITS sister group.

The rate of species diversification in the silversword alliance was obtained by using maximum likelihood estimators and a plot of number of lineages through time, derived from Fig. 1 (Fig. 3). Knowledge of branching times, provided by the clock-like divergence of ITS, affords two important statistical advantages in the estimation of diversification rates. (*i*) Average error and bias are reduced compared with estimators that rely only on the age of the clade and the number of extant taxa (26). (*ii*) Given a large enough sample size, these inferred times contain information that allows separate inferences of speciation and extinction rates, S and E (27, 28).

For purposes of comparison to other taxa and model testing, we examined both a Yule, or pure birth, model of diversification (speciation but no extinction) and a birth–death (BD; speciation and extinction) model (29). Although an LR test suggested a slightly better fit of the BD model to the data ($-2 \ln LR = 4.5$; $P < 0.05$), computer simulation showed that the variance and bias of the estimators of S and E were about four times higher than for the Yule model (as expected; see refs. 26 and 30), rendering them nearly useless. LR tests of models with and without extinction [using equation 21 in Nee *et al.* (28) with and without $\mu = 0$] were also highly sensitive to taxon sampling, suggesting that the assumption of asymptotic behavior of the test (convergence to χ^2) was not met with this small number of taxa. For estimation purposes, we therefore assumed the simpler Yule process and used the Kendall/Moran estimator (31, 32), $\hat{S} = (N - 2)/B$, where B is the summed durations of all branches descended from the most recent common ancestor of all species in the clade and N is the number of extant lineages. Two estimators of the variance of $\hat{S}$ make different assumptions about the population of stochastic processes under study. Kendall's estimator, $\mathrm{var}(\hat{S}) = S^2/2(e^{ST} - 1)$, assumes a population of stochastic processes all persisting a fixed amount of time, T. Moran's estimator, $\mathrm{var}(\hat{S}) = S^2/(N - 2)$, which is 50% lower for our data, assumes a population of processes that all terminate with the same number of taxa, N. We used the larger more-conservative value. There is an additional source of error, of course, caused by the uncertainty in the divergence time estimates themselves. The Kendall/Moran estimator assumes the divergence times are known without error. We accounted for this error by recalculating the diversification rate estimates over all the bootstrapped character data sets that were used to generate Fig. 2.
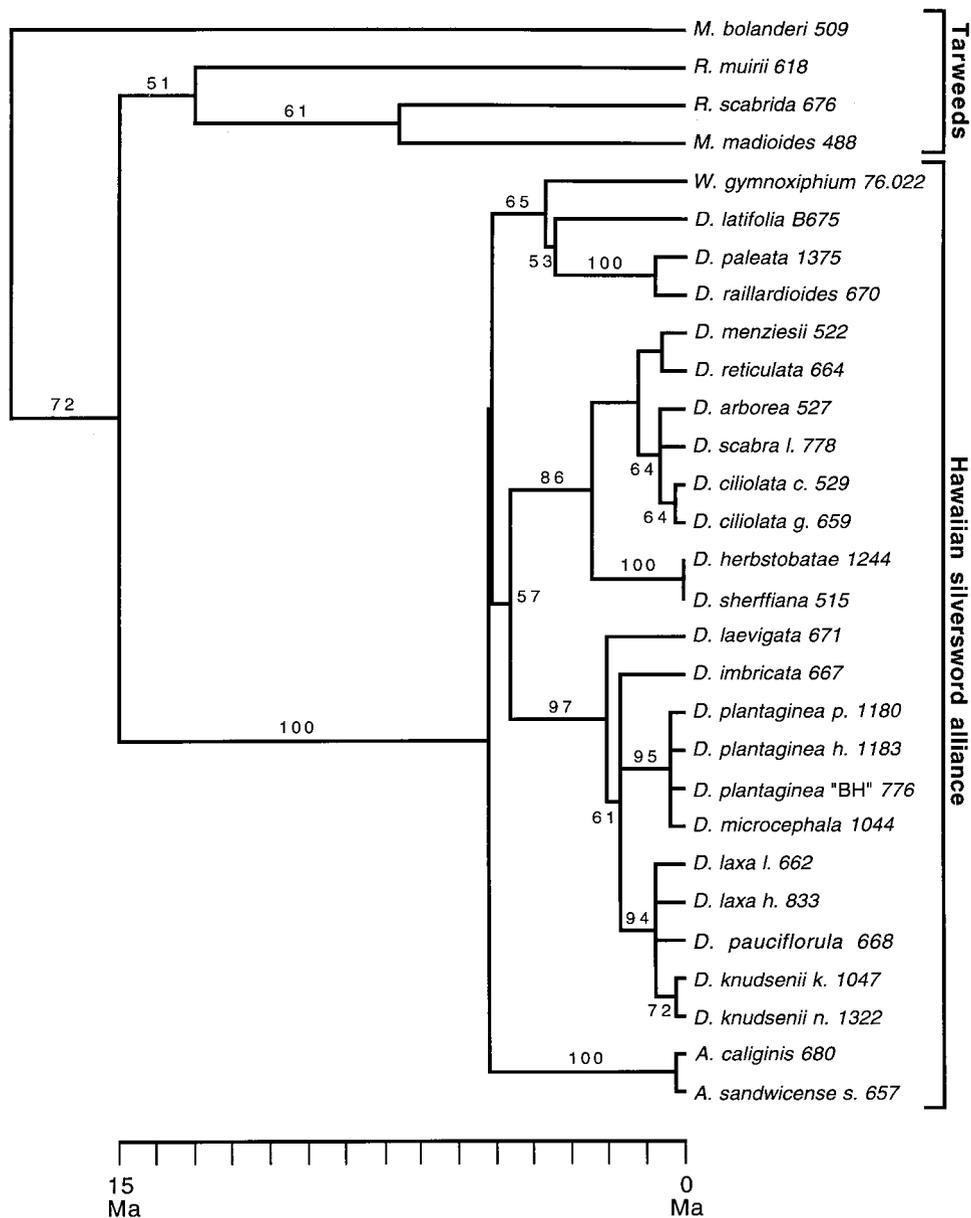
FIG. 1.   Time-calibrated phylogeny of one of four most-parsimonious rDNA ITS trees of the Hawaiian silversword alliance (*Argyroxiphium*, *Dubautia*, and *Wilkesia*) and closest continental perennial relatives in *Madia* and *Raillardiopsis* (8, 9). Outgroup tarweed taxa, *Adenothamnus validus* and *Raillardella pringlei*, are not shown. Bootstrap values are shown along branches. Consistency index = 0.81. Retention index = 0.85. The tree shown is a clade that nests within the much larger tarweed radiation (ca. 114 species in 17 genera) (25).

RESULTS AND DISCUSSION

A sequence evolution model with constant rates across sites is strongly rejected in favor of one with variable rates ($-2 \ln LR = 47.12$ or 47.74, depending on whether lineage rate constancy is assumed or not, respectively: $P \ll 0.001$). Estimated transition–transversion ratios are consistently about 1.81 and shape parameters of the Γ distribution are about 0.40.

On the other hand, the hypothesis of equivalent rates of ITS evolution across lineages throughout the minimum-length trees cannot be rejected at the conventional $\alpha = 0.05$ level (e.g., for the tree in Fig. 1, $-2 \ln LR = 36.30$, 27 df, $P < 0.10$; for all four trees, $-2 \ln LR$ ranged from 36.2 to 38.6, all under the 40.11 test value for $\alpha = 0.05$). This result permitted estimation of internal node times via maximum likelihood. Calibration of the most recent common ancestor of the silversword alliance and the ITS sister lineage of California tarweeds (*Madia madioides*, *Raillardiopsis muirii*, *Raillardiopsis scabrida*)

at the upper limits of conceivable age for the Californian group, 15 Ma, yields an age of 5.2 ± 0.8 Ma for the most recent common ancestor of the Hawaiian group, i.e., at about the time of origin of Kaua'i (5.1 ± 0.2 Ma, ref. 33)—the oldest high Hawaiian island (Fig. 1). Placement of younger, perhaps more realistic, dates at the same calibration point leads, of course, to estimates of younger age for the silversword alliance ancestor. Our age estimate is robust to phylogenetic uncertainty associated with the ITS data set; the mean age estimate from 25 bootstrap trees for which we repeated the age-estimation procedure outlined above is 5.5 ± 0.3 Ma.

Our results demonstrate that the exceptional diversification of the silversword alliance has occurred within the time span encompassed by the modern high islands of the Hawaiian archipelago. Other comparable adaptive radiations in the Hawaiian Islands have been estimated by other methods to be of much greater antiquity: Hawaiian *Drosophila* (>10 Ma; ref. 34), Hawaiian lobelioids (15 Ma; ref. 35), and honeycreepers
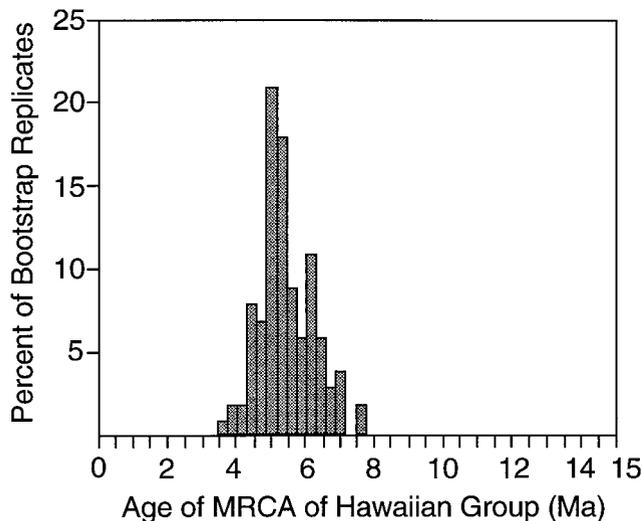
FIG. 2. Bootstrap (15) distribution of age estimates for the silver-sword alliance using a 15 Ma calibration, accounting for the stochastic sampling error in the sequence data used to infer ages. MRCA, most recent common ancestor.

[15–20 Ma (36); but younger age estimates (7–8 Ma, ref. 37; 3.5 Ma, ref. 38) have been calculated from other molecular data]. Our results do not preclude the possibility that the original Hawaiian founder from western North America may have arrived long before the origin of Kaua'i, but our data do show that if such ancient arrival did occur, only one surviving lineage no older than Kaua'i gave rise to the extraordinary diversity of morphological and ecological forms in existence today.

This information on timing of the radiation sets the stage for analysis of absolute diversification rates. By using the Kendall/Moran estimator and an age of origin of the Hawaiian clade at

5.2 Ma, we estimate the diversification rate to be $\hat{S}= 0.56 \pm 0.17$ species per million years. The standard error is that because of the finite number of "observations" (the divergence times) on the stochastic branching process. An additional component of error is caused by uncertainty in the divergence time estimates themselves and was estimated (via boot-strapping) to be ± 0.07 species per million years. Note also that these diversification rates are minimum estimates because we used a maximum date for the tree calibration.

Most estimates of diversification rates in other groups also assume a Yule process but use the simpler estimator $[\ln (N) - \ln(N_0)]/T$, based on initial diversity, N_0 (usually $N_0 = 1$), standing diversity, N, and inferred clade age, T (39–42). This estimator is not as accurate as the Kendall/Moran estimator (31, 32), because it is based on only two items of data, the numbers N_0 and T, rather than up to $N_0 - 2$ items—the divergence time estimates. Although the calculated diversification rate for the silversword alliance is a minimum value, it exceeds rate estimates (derived from standing diversities) for radiations of putatively tachytelic continental groups, such as angiosperm families [median of 0.12 species per million years and a maximum of 0.39 (39)], rodent families [mean of 0.22 and maximum of 0.35 (40)], and recent African large-mammal genera [range, 0.0–0.39 (41, 42)].

However, these are all average rates that may not reflect early periods of rapid radiation in the history of the clades. Without a detailed fossil record or reliable clock-based divergence time estimates, detection of episodes of unusually high or low diversification is difficult. The case of Neogene horses is instructive in this regard because a sufficiently rich fossil record exists to permit estimates of speciation and extinction rates over time (43). Neogene horses underwent an early period of extremely rapid radiation, with $\hat{S}= 0.5 - 1.4$ (43), which is comparable to the rate inferred in Hawaiian silverswords, especially given the likelihood that our estimate is low. Thus, the tempo of the silversword alliance radiation over the last 5 million years does not just exceed average rates for continental radiations, it may well be comparable to peak rates during these radiations. Additional quantitative estimates of diversification rates in both island and continental radiations are needed to test this hypothesis further.

FIG. 3. Natural logarithm (ln) of species diversity through time based on the tree in Fig. 1, with scoring of additional species that are identical in ITS sequence with species included in the tree [At all ITS sites, A. grayanum (BGB 661) and A. kauense (BGB 773) are identical to Argyroxiphium sandwicense ssp. sandwicense (BGB 657); D. linearis (BGB 516) is identical to Dubautia arborea (BGB 527); D. platyphylla (BGB 524) is identical to Dubautia menziesii (BGB 522); W. hobdyi (GDC 1150) is identical to Wilkesia gymnoxiphium (Char 76.022) (see ref. 9)]. Ages are based on a 15-Ma calibration point for the most recent common ancestor of the Hawaiian silversword alliance and its Californian ITS sister group.

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