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ECOLOGICAL SPECIATION IN THE EAST MAUI–ENDEMIC *DUBAUTIA* (ASTERACEAE) SPECIES

ELIZABETH A. FRIAR,[1,2] LINDA M. PRINCE,[1] ERIC H. ROALSON,[1,3] MITCHELL E. MCGLAUGHLIN,[1,4] JENNIFER M. CRUSE-SANDERS,[1,5] SARAH J. DE GROOT,[1] AND J. MARK PORTER[1]

[1]*Rancho Santa Ana Botanic Garden, 1500 North College Avenue, Claremont, California 91711*

[2]*E-mail: elizabeth.friar@cgu.edu*

Abstract.—Adaptive diversification can be viewed as a balance between the conservative force of interpopulation gene flow and selection for differential environments. In this paper, we examine ecological, morphological, and genetic differentiation in a small clade consisting of four East Maui–endemic species of *Dubautia*: *D. menziesii*, *D. platyphylla*, *D. reticulata*, and *D. waianapanapaensis*, in the Hawaiian silversword alliance (Asteraceae). The East Maui clade is apparently recently derived (less than 1 million years ago) and is geographically restricted yet displays significant ecological and morphological differences. We used geographic data from historical herbarium specimens, measurements of plant architecture and leaf morphometrics, and measures of genetic differentiation in both microsatellite and nuclear coding loci to examine the correlation of different forms of divergence in this small species flock. We found overlap in large-scale geographic distributions, significant differentiation in most habitat factors, significant microsatellite differentiation, and many shared alleles at nuclear coding loci suggesting on-going lineage sorting. Despite the presence of apparent hybrids in some populations, microsatellite variation is consistent with isolation among species. Using Mantel tests, we compared the direction and extent of diversification among different datasets, to determine whether ecological/morphological divergence was correlated with genetic divergence. Correlations among different datasets showed that habitat was strongly correlated with plant architecture but not leaf morphology. Taken together, these results indicate that ecological and morphological diversification has driven genetic divergence at rapidly evolving microsatellite loci, whereas there is continuing lineage sorting at neutral sites in nuclear coding loci.

Key words.—Ecological speciation, gene flow, Hawaiian silversword alliance, lineage sorting.

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The process of adaptive radiation has been the subject of considerable debate. The ecological theory of adaptive radiation postulates that divergent natural selection drives rapid speciation and phenotypic diversification (Huxley 1942; Mayr 1963; Schluter 1996a). If populations are subjected to different selective regimes as they exploit new environments, they may accumulate phenotypic and genetic differences, leading to speciation. Reduced competition associated with the presence of open resource niches, either from movement into new habitats or the acquisition of a key evolutionary innovation, provides increased opportunities for diversification (Schluter 1996a). Conversely, gene flow among diversifying populations or hybridization between incipient species may retard the process of diversification and homogenize populations (Slatkin 1987; Nosil and Crespi 2004). The process of species diversification in a heterogeneous landscape can thus be viewed as a balance between differential natural selection and gene flow (Endler 1977). Recent work has emphasized that species boundaries can be semipermeable, and, in some cases, low levels of gene flow can increase the rate of speciation in a geographically structured population (Gavrilets et al. 2000; Church and Taylor 2002).

Hawaii is a natural laboratory for the study of adaptive radiation. The archipelago is age structured, with the older current high islands to the northwest, and younger islands to the southeast. This archipelago is also biologically isolated, being located over 3500 km from the nearest continental source for migrants. Restricted opportunities for dispersal to such an isolated archipelago and repeated origins of new islands have apparently led to ample open niches for exploitation and species divergence. Additionally, the landscape is varied, with steep ecological gradients in a number of factors, including elevation, rainfall, and solar radiation. The result has been a number of large-scale adaptive radiations in both plants and animals. In their survey of Hawaiian biogeography, Funk and Wagner (1995) observed several common trends in Hawaiian adaptive radiations. One pattern is the presence of multiple nested within-island species radiations. They observed that, although interisland dispersal events were not typically associated with habitat shifts, within-island species radiations were associated with habitat diversification for many groups of species.

The Hawaiian silversword alliance (Asteraceae) is a premier example of adaptive radiation in plants. The 30 species in three genera (*Argyroxiphium* DC., *Dubautia* Gaudich., and *Wilkesia* A. Gray) inhabit localities that vary from 75 to 3750 m in elevation and from 400 mm to over 12,000 mm in annual rainfall (Carr 1985; Robichaux et al. 1990). Phylogenetic analysis of the alliance has suggested an older-to-younger island direction of dispersal (Baldwin and Robichaux 1995; Baldwin 1997; Baldwin and Sanderson 1998). There are also a number of monophyletic within-island species radiations, each of which exhibits a shift between wet and dry habitats, demonstrating a repeating phylogenetic pattern (Baldwin and Robichaux 1995).

One recently evolved example of a monophyletic single-island species radiation into a small species flock is the group

[3] Present address: School of Biological Sciences and Center for Integrated Biotechnology, Washington State University, Pullman, Washington 99164.

[4] Present address: Department of Biology, University of South Dakota, 414 East Clark Street, Vermillion, South Dakota 57069.

[5] Present address: Department of Biology, Salem College, P.O. Box 10548, Winston-Salem, North Carolina 27108.

TABLE 1. Morphological variation in East Maui–endemic species of *Dubautia* (Carr 1985).

Species	Plant height (m)	No. of capitulae	Florets per capitulum	Leaf nerves	Phyllotaxy	Leaf length (cm)	Leaf width
D. menziesii	2.5	5–35	12–25	3–5	whorled, opposite, or alternate	2–5	4–17 mm
D. platyphylla	3.5	9–90	12–30	7–11	opposite or whorled	4–9	1–3.8 cm
D. reticulata	8	5–100	4–12	5–7	opposite	3–7	4–13 mm
D. waianapanapaensis	1–4	5–30	7–30	5–9	opposite	4–12	5–22 mm

of East Maui–endemic species of *Dubautia*: *D. menziesii* (A. Gray) Keck, *D. platyphylla* (A. Gray) Keck, *D. reticulata* (Sherff) Keck, and *D. waianapanapaensis* G. Carr (formerly treated as *D. dolosa* [Degener and Sherff] G. Carr). These four species in *Dubautia* section *Railliardia* (Gaudich.) G. Carr form a monophyletic group in nuclear internal transcribed spacer (nrITS) and chloroplast phylogenies (Baldwin et al. 1990; Baldwin and Robichaux 1995; E. A. Friar, unpubl. data). Although the monophyly of these species is moderately well supported in the combined nrITS-chloroplast phylogeny (bootstrap = 89%), there is very little divergence among them at allozyme or DNA sequence level markers (Witter and Carr 1988; Baldwin and Robichaux 1995). The low level of sequence variation is not entirely unexpected due to the recent divergence of the species (Baldwin and Robichaux 1995). If the basal node for this group of species does not predate their habitat, the maximal date of divergence is the age of Haleakala volcano, or about 0.75 million years (Carson and Clague 1995).

Despite low genetic variation, there is considerable morphological and ecological variation in this group of taxa (Carr 1985). *Dubautia menziesii* grows as a large, densely branched shrub in high elevation dry scrubland between 1800 and 3075 m in elevation and flowers between July and December. *Dubautia platyphylla* typically grows as a large, loosely branched shrub on slopes of ravines between 1725 and 2750 m in elevation and flowers between mid-June and August and is currently known from only two localities. *Dubautia reticulata* typically grows as a single-trunked small to medium-sized tree in mesic to wet forest habitats between 1575 and 2300 m elevation, flowers between September and January, and has a very restricted distribution with only two to three known populations with more than 10 individuals (Carr 1985). *Dubautia waianapanapaensis* occurs as a large shrub in wet forest habitats between 1525 and 2275 m in elevation, flowers between August and December, and is only known from one locality. These four species also differ in the number of florets per capitulum, the number of capitulae per compound inflorescence, and leaf length (Carr 1985; Robichaux et al. 1990). Many of these traits are overlapping in their distribution, although the species are generally considered distinctive in overall presentation (Table 1; Carr 1985; Robichaux et al. 1990; Kirchoff et al. 2004).

These four species typify the pattern within the silversword alliance of a small, single-island monophyletic lineage that exhibits evidence of at least one ecological shift between wet and dry habitats (Baldwin and Robichaux 1995). Despite the close geographic proximity of populations of different species, they have been described as having different habitat preferences. *Dubautia menziesii* occurs in subalpine to alpine dry shrublands, which are characterized by low precipitation falling predominantly between November and March, and low vegetative cover on gravel to cinder substrates (Gagne and Cuddihy 1990). Subalpine to alpine dry shrubland habitats also experience frequent frosts. *Dubautia platyphylla* grows in more sheltered situations (e.g., gulches) in subalpine dry to mesic shrubland habitats that have higher annual average rainfall. The soil in this habitat tends to be thin with many rock outcroppings (Gagne and Cuddihy 1990). *Dubautia reticulata* grows in montane mesic to wet forests characterized by year-round rainfall, an uneven tree canopy, and an understory of native ferns (Gagne and Cuddihy 1990). *Dubautia waianapanapaensis* occurs in montane wet forest habitats with high year-round rainfall and fog drip moisture; substantial tree overstory; and an understory of shade-tolerant shrubs, ferns, and mosses (Gagne and Cuddihy 1990). In their analysis of ecological diversification of the silversword alliance, Baldwin and Robichaux (1995) classified *D. menziesii* and *D. platyphylla* as ''dry'' habitat species and *D. reticulata* as a ''wet'' habitat species (*D. waianapanapaensis* was not available in that study, but it would be classed as a wet habitat species as well; R. Robichaux, pers. comm.). These designations were based in part on ecophysiological measurements of bulk tissue elastic modulus, that is, the change in turgor for a given change in intracellular water, or, roughly, the ability of a plant to withstand increased water stress without wilting. Bulk tissue elastic modulus is significantly different between dry- and wet-habitat species, but not within each pair of ecologically similar species (Robichaux 1985; Robichaux and Canfield 1985; R. Robichaux, pers. comm.).

Like many of the species in the Hawaiian silversword alliance, the East Maui–endemic species of *Dubautia* are interfertile (Carr 1985). Hybridization between *D. reticulata* and *D. waianapanapaensis* in contact zones creates a continuous grade of morphological variation and eliminates discontinuities between taxa (Carr 1985). *Dubautia reticulata* also hybridizes with *D. menziesii* and *D. platyphylla*, as well as the more distantly related *D. scabra* and *D. plantaginea* (Carr 1985). *Dubautia menziesii* and *D. platyphylla* also hybridize in contact zones, where morphological differences between these two species are also largely obscured. Despite the possibility of introgression, however, individuals with obviously intermediate morphology are only found in contact zones between populations. Therefore, it is currently unclear whether these taxa represent (1) morphological and ecological variants from the same basic gene pool (i.e., products of phenotypic plasticity), (2) separate species with ongoing large-scale gene flow that fails to obscure morphological species boundaries, possibly via selection (i.e., Petit et al. 2004), or (3) separate species with only minimal gene flow at population boundaries. In this paper, we examine the ecological, morphological, and genetic variation within one population

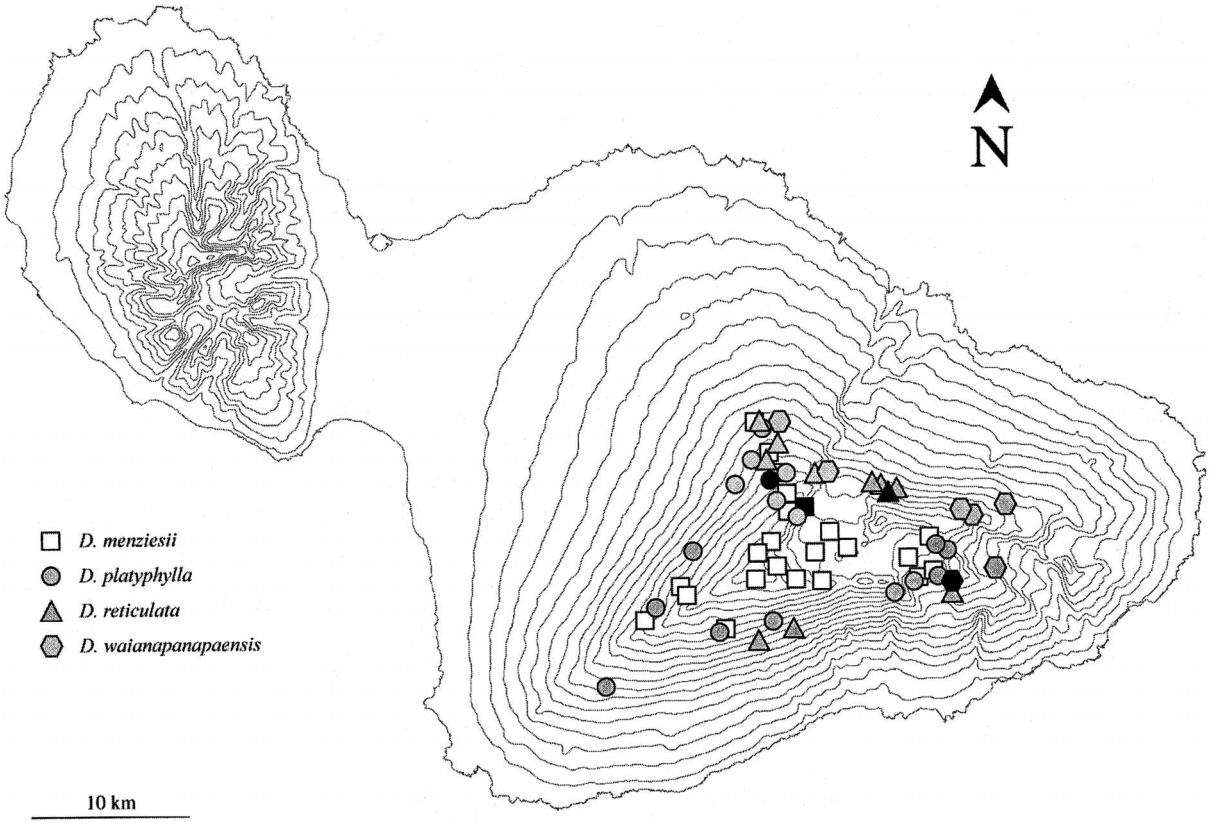

FIG. 1. Map of the island of Maui showing historical collection localities for *Dubautia menziesii* (squares), *D. platyphylla* (circles), *D. reticulata* (triangles), and *D. waianapanapaensis* (hexagons). Localities collected for this study are indicated by filled symbols.

of each species and among the sampled populations. Using these data, we examine the correlation in species divergence in these parameters and use our results to infer the impact of gene flow and natural selection on species boundaries.

MATERIALS AND METHODS

Due to the rarity of these species and the intensity of data collection, we were only able to sample one population per species. For instance, *D. waianapanapaensis* is only known from one locality, and the sampled population of *D. reticulata* is the only remaining population of this decimated species not known to be highly introgressed. Because of the potential for hybridization obscuring species differences, each sampled population was chosen to minimize the impact of recent hybridization on our dataset. This sampling design allows us to examine species differentiation and large-scale introgression and draw inferences about the relative roles of hybridization, lineage sorting, and ecological differentiation in the history of these species.

Geographic and Ecological Differentiation

Historical localities identified as belonging to one of the four East Maui species were identified using herbarium label information, as presented in Carr (1985). These localities were placed on a Hawaii base map in ArcView GIS (ver. 3.3; ESRI Inc. Redlands, CA). Apparent recollections from the same locality were combined into a single locality, and information from the most recent collection date was used to place the locality onto the base map, using GPS points or detailed geographical descriptions. Localities are shown in Fig. 1.

Using layers developed by the State of Hawaii Office of Planning (http://www.hawaii.gov/dbedt/gis/index.html), each historical locality was scored for elevation, rainfall, solar radiation, environment, and canopy type. Rainfall was recorded as median annual rainfall in millimeters. Solar radiation was recorded as estimated solar calories per square centimeter per day.

On the vegetation map layers, environments were coded as species association types, as given by the GIS layers, and included: ''no vegetation,'' ''dry,'' ''mesic,'' or ''wet.'' Likewise, canopy types were coded as the percent tree canopy crown cover, and included: none (0% tree crown canopy cover), scattered (5%–25% cover), or closed or open forest (25%–60% cover). For these analyses, codings were given nominal scores for comparison. Environments for each locality were coded as no vegetation = 1, dry = 2, mesic = 3, and wet = 4. Likewise, canopy types were coded as none = 1, scattered = 2, and closed or open forest = 3. Comparisons among species were performed using nonparametric Mann-Whitney *U*-tests. These data were also analyzed with principal components analysis (PCA) in StatView (ver. 5.0.1 1998, SAS Institute, Inc., Cary, NC). Each variable was compared among species using ANOVA as implemented in StatView. In addition, each PCA axis was also compared

using ANOVA to assess species differentiation. To test pairwise species differences among all pairs of taxa, mean centroid values for each axis were compared using unpaired *t*-tests, and variances in each axis were compared using *F*-tests. Bonferroni corrections were used to correct for multiple comparisons (Rice 1989).

For all other analyses, one population of each species, as identified above, was used. These localities are identified in Fig. 1 as closed symbols and described below.

Dubautia menziesii.—This species was collected from near the Kalahaku Overlook (KH), on the upper west slope of Haleakala Crater on Maui. These plants occur in alpine scrub habitat and grow as small to large shrubs. This population is extensive, and numbers in the thousands. It does not come into contact with populations of other *Dubautia* species at this location.

Dubautia platyphylla.—This species was collected from Upper Waikamoi Gulch (WK). These plants occur as large, sprawling shrubs, growing along the steep edges of a ravine. This population numbers approximately 30–50 individuals. It comes into contact with some individuals of *D. menziesii* on the upper slopes of Waikamoi Gulch. Several apparent hybrids were seen between *D. menziesii* and *D. platyphylla* in this population, but were not sampled.

Dubautia reticulata.—The Koolau Gap (KG) locality of *D. reticulata* was chosen for sampling because it is the largest known population. These plants occur as small to large trees in mesic to wet forest habitat. This population occurs across two connected drainages and numbers in the hundreds of individuals.

Dubautia waianapanapaensis.—This taxon was collected from near Lake Waianapanapa (LW). The plants grow as large shrubs in wet forest and shrubland habitat. This population numbers in the hundreds, though the individuals are scattered throughout wet forest.

For population genetic analysis, we collected leaf material from 30 haphazardly selected individuals of each population for *D. menziesii*, *D. platyphylla*, and *D. reticulata*. For *D. waianapanapaensis*, only 24 individuals were sampled, which represented all known plants at the sample locality. Approximately 1 g of leaf material was collected from each individual, kept cold, and shipped to Rancho Santa Ana Botanic Garden (RSABG) on ice, to be stored at −80C until DNA extraction. DNA was extracted from leaves using the methods of Friar et al. (2000) or the nucleon phytopure protocol described in Friar (2005).

Plant Architecture

A number of measures of plant architecture were taken in the field for 15 individuals each of *D. menziesii*, *D. platyphylla*, and *D. reticulata*. *Dubautia waianapanapaensis* could not be sampled for this analysis because of the remote locality. Plant height was measured as the vertical distance from the base of the plant to the highest point. Plant length was measured as the distance from the base of the plant to the top of the longest branch on the plant. These two measurements are identical for a plant growing vertically; however, plants growing at a significant angle may be very different in height and length. Plant height at first branch was measured from the plant base to the bottom side of the first branching point. Plants that branched at or below the soil level were recorded as having a height at first branch of zero. Stem circumference and stem diameter were recorded at the base of the first branch. For plants that branched at or below the soil level, this was measured at the soil line. Both circumference and diameter were recorded as the stems at this point were often highly irregular. Stem diameter was also measured at 1/2 and 1/4 total plant height, on the largest stem at that height. Stem circumference was not measured at these points, because the stems were mostly round. Whole-plant diameter was measured as the longest measures covered by plant material at two orthogonal directions. Analyses were performed with both measures included, or with them multiplied to generate plant area. *Dubautia* plant canopy density was estimated as the amount of whole plant area encompassed by leaf surface area.

As above, these data were analyzed using ANOVA of each trait separately and with PCA as implemented in StatView. Each PCA axis was analyzed with ANOVA and pairwise species comparisons for each axis were compared using unpaired t-tests, and variances in each axis were compared using F-tests. Bonferroni corrections were used to correct for multiple comparisons (Rice 1989).

Leaf Morphometric Measurements

Leaf morphometeric measurements were made on the same individuals from which whole-plant measurements were collected. One branch was selected per individual for 15 individuals and placed into coolers and/or refrigerators until analysis. For each stem, node 0 was defined as the node with the youngest fully expanded leaf, or the lowermost leaf of the cluster of leaves at the shoot apex at the distal position of the branch. Positive nodes were defined as those older nodes down the stem. Negative nodes were defined as those toward the tip of the branch with still developing leaves. For these analyses, only mature leaves at positive nodes were used.

Shortly after collection, a single fresh leaf from each positive node was placed adaxial side down on a Hewlett-Packard (HP; Palo Alto, CA) ScanJet ADF flatbed scanner, scanned at 300 dpi in grayscale with the HP Precision Scan Pro software version 1.4.2, and images saved as jpeg files. All leaves were scanned within 24 h of their collection in the field, and within 1 h of removal from the stem. Individual images of leaves at nodes 1, 6, 10, and 15 were created and rotated to be oriented as close as possible to vertical in Adobe Photoshop version 5.5 (Adobe Systems Inc., San Jose, CA).

Landmarks were placed on individual leaf images using the program TPSDIG (ver. 1.31; Rohlf 2001) at the base, apex, 1/8, 1/4, midpoint, 3/4, 7/8, and 15/16 of the total leaf length. TPS files were created for the program to open all leaf images from an individual at the same time. At each increment, three landmarks were placed: one on each margin and one on the midvein. Datapoints were saved as separate TPS files for each leaf.

Datapoints were then converted from TPS format to CS format by the program CoordGen6 (of the IMP package; Sheets 2002), to enable use of the program tmorphogen6 (Strauss and Bookstein 1982). tmorphogen6 was used to mea-

sure 62 interlandmark distances per leaf to generate a truss (Strauss and Bookstein 1982). Distances were imported into StatView or SPSS version 11.0.2 (SPSS, Inc., Chicago, IL) for PCA. Independent analyses were performed for leaves at each node, as well as for all data combined. Differences in PCA axes were assessed as above using ANOVA and multiple *t*-tests for species pairwise comparisons. Bonferroni corrections were used to correct for multiple comparisons (Rice 1989).

DNA Loci

A total of seven microsatellite loci were scored for each population. Microsatellite locus development was described in Friar et al. (2000). Three loci, AP3MS1, AP3MS2, and AP3MS3 were developed from DNA sequence data from the *ASAPETALA3* (*ASAP3*, *APETALA3* homologue) gene, and are located within 1 kb of one another (Barrier et al. 2000). MKMS2, MKMS3, and MKMS4 were developed de novo from genomic DNA from species of *Argyroxiphium*, and are not assumed to be linked. Locus 42-2 was adapted from *Helianthus* as described in Friar et al. (2000).

Nuclear gene-specific primers for DNA sequence analysis used in this study were developed specifically for *Dubautia* species and their close relatives. Because all *Dubautia* species are allotetraploid (Barrier et al. 1999), copy-specific primers were developed to amplify only one of the two homeologues of the *ASAP3* gene, designated the *ASAP3*-A or *ASAP3*-B locus.

DNA Amplification

Microsatellite loci were amplified in 15 μl reactions containing 3 μl genomic DNA (at 5 ng/μl), 0.6 μl each primer (at 10 pm), 0.9 μl dNTPs (at 2.5 mM), 0.5 U Taq (Promega), and 1.5 μl 10× amplification buffer (0.1M Tris-HCl, 0.5M KCl, 5% v/v glycerine, pH 8.3). Annealing temperatures and $MgCl_2$ concentrations were independently optimized for each population for each primer. Primers were as in Friar et al. (2000). Forty cycles of amplification were carried out in an MJ Research Inc. (Hercules, CA). PTC-100 thermalcycler. Amplification products were examined on 1.5% agarose gels to verify fragment size. Once amplification conditions were optimized, loci were amplified using fluorescently labeled primers (FAM or HEX) for analysis on an ABI3100 Genetic Analyzer platform (Applied Biosystems, Inc., Foster City, CA). Amplification products were coloaded with ROX-350 size standard according to manufacturer's specifications. Electrophoretic results were analyzed with GeneScan software (ver. 3.7.1, Applied Biosystems).

For sequence analysis of the *ASAP3* loci, samples were amplified with common forward and A- or B-copy-specific reverse primers (*ASAP3*-3F: TACAAACAGGCAGGTGAC ATACTC; AP3A-1067R: TTGCCTGCAAACCCATTCAR GTT; AP3B–1284R: GCACACCCATTCAAATTAAGTTT ATCA) and an annealing temperature of 56–64° in 10 μL reactions. *Pfu* polymerase (Stratagene) was used to minimize polymerase error with an ammonium sulfate polymerase chain reaction (PCR) buffer. Some reactions also included up to 5% dimethysulfoxide, which improved product yield and specificity. PCR products were cleaned using an abbreviated version of the polyethylene glycol precipitation protocol (Johnson and Soltis 1995) and cloned using the Zero-Blunt Topo TA Kit (Invitrogen Life Technologies, Carlsbad, CA). Minipreps of liquid cultures were cleaned using Eppendorf Plasmid MiniPrep Kits, quantified using a Biospec-1601 spectrophotometer (Shimadzu Biotech, Columbia, MD), and cycle sequenced using 1/8 concentration ABI BigDye III (Applied Biosystems) on PTC-100 thermal cyclers (MJ Research). A large number of internal primers were used in addition to M13F and M13R primers for sequencing, as previously published (Barrier et al. 2001; Lawton-Rauh et al. 2003). Samples were visualized on an ABI 3100 platform (Applied Biosystems) at RSABG.

Microsatellite Scoring and Analysis

Each population was characterized by the observed number of alleles (A_O), the effective number of alleles (A_E), observed heterozygosity (H_O) and expected heterozygosity under Hardy-Weinberg expectations (gene diversity; H_E). One-tailed *t*-tests to determine significance in differences of these estimates and *F*-tests to determine significance of differences in variances among them were performed using StatView version 5.0.1. Bonferroni corrections were used to correct for multiple comparisons (Rice 1989). Tests for fit to Hardy-Weinberg expectations and pairwise linkage disequilibrium were performed using Arlequin version 2.000 (Schneider et al. 2000).

Hickory version 1.0 (Holsinger 1999; Holsinger and Wallace 2004) was used to provide Bayesian estimates of *f*, an analog of F_{IS}, a measure of inbreeding within a population. The default Monte Carlo Markov chain (MCMC) parameters were used for all analyses. Each run was performed three times to verify convergence of estimates. For single-population estimates of *f*, the $\theta^B = 0$ model was used.

To test the hypothesis that the recent origin of this species flock and/or ongoing large-scale introgression has led to a lack of genetic differentiation, F_{ST} and R_{ST} pairwise genetic distances among species were also calculated using Arlequin. AMOVA was used to assess the significance of differentiation among populations using both F_{ST} and R_{ST} distances (Arlequin). Genetic differentiation among taxa was tested using BayesAss version 1.2 (Wilson and Rannala 2003), which uses an MCMC method to estimate recent gene flow among populations, using three million iterations, a sampling frequency of 2000, and discarding the first 500 trees as burn-in. Changing the maximum percentage of migrants up to 50% per population did not alter the results.

DNA Sequence Alignment and Analysis

Vector fragments were trimmed and sequences were edited in Sequencher version 4.2.1 (Gene Codes Corp., Ann Arbor, MI). Edited sequences were aligned manually using Se-Al version 2.0a11 (Rambaut 1996) and exported into a large, concatenated (interleaved) NEXUS file for analysis in PAUP* 4.0 (Swofford 2002). Ambiguously aligned regions (including microsatellites) were excluded from all analyses. In *ASAP3*-A, a single parsimony-informative gap of 49 base-pairs was coded separately as a multistate character at the end of the concatenated matrix. *ASAP3*-B had no gaps in the

TABLE 2. Ecological data for historical occurrences of East Maui–endemic species of *Dubautia*. Historical populations were placed onto base maps with locality information taken from herbarium labels. Data were generated from GIS overlays for elevation, rainfall, solar radiation, environment (scored as no vegetation = 1, dry = 2, mesic = 3, wet = 4), and canopy type (scored as none = 1, scattered = 2, closed or open forest = 3). Species means that share a letter superscript are not significantly different.

Species	*N* (no. of populations scored)	Elevation (m ± SE)	Rainfall (mm/year ± SE)	Solar radiation (calories/cm²/day ± SE)	Environment (mean score)	Canopy type (mean score)
D. menziesii	23	2219 ± 76.5 m[a]	1870 ± 197[a]	407 ± 7.6[a]	1.5[a]	1.5[a]
D. platyphylla	16	1972 ± 53.0 m[b]	2380 ± 314[a]	394 ± 8.3[a]	2.1[a,b]	2.1[a,b]
D. reticulata	12	1664 ± 63.4 m[c]	2060 ± 239[a]	362 ± 9.0[b]	2.6[b,c]	2.5[b]
D. waianapanapaensis	6	1753 ± 94.5 m[c]	4000 ± 447[b]	325 ± 11.2[c]	3.4[c]	3.0[b]

aligned data matrix once microsatellite regions were removed. The aligned data matrix is available from TreeBase (accession no. SN2872). GenBank accession numbers for *ASAP3*-A sequences are DQ010853–DQ010880; for *ASAP3*-B they are DQ010881–DQ010907.

Estimates of DNA sequence diversity were calculated using Arlequin (ver. 2.000). Haplotype networks among alleles were calculated using TCS (Clement et al. 2000) using a 95% cutoff criterion, except where noted. Haplotype networks have been used primarily in within-species analyses, but the close genetic relationships and large number of shared haplotypes make this a reasonable technique for these species. Phylogenetic relationships among haplotypes were estimated using maximum likelihood (ML) and maximum parsimony (MP) criteria, as well as Bayesian estimation. Samples of *Argyroxiphium sandwicense* subspecies *macrocephalum* and *Dubautia laxa* subspecies *hirsuta* were used as outgroups. Modeltest (Posada and Crandall 1998) was used to determine the best model for ML (GTR + γ). Maximum parsimony and maximum likelihood analyses used ACCTRAN character state optimization, tree bisection-reconnection branch swapping, and employed 10 random-addition replicates, holding 100 trees per step. Statistical support for nodes was estimated using 1000 bootstrap pseudoreplicates with 10 random-addition replicates each for MP and ML. Maximum likelihood and MP trees were constructed using PAUP* 4.0 (Swofford 2002). In addition, relationships among haplotypes were evaluated using Bayesian posterior probabilities generated by one million generations and a sampling frequency of 1000, also using a GTR + γ model of evolution (MrBayes ver. 3.1.2; Ronquist and Huelsenbeck 2005). Burn-in times were specific to each dataset and were determined using the variance of splits feature. The first 500–1000 estimates were discarded using a criterion of stability below a variance of splits of 0.01.

Dataset Comparisons

To compare divergence among taxa, mean pairwise distances between taxa were calculated for each dataset. For those datasets in which PCA analysis was performed (leaf morphology, plant architecture, habitat), mean pairwise distances between taxa were calculated as the two- or three-dimensional vector distance between PCA centroids. For microsatellite data, both F_{ST} and R_{ST} genetic distances were calculated. For DNA sequence data, mean population genetic distances were calculated using Kimura two-parameter distances as implemented in Arlequin. For comparisons with ecological inferences, mean geographic distances among species were calculated as the mean geographic distance among historically reported localities, shown in Fig. 1 (see above for placement of localities). For other comparisons, actual geographic distances between the sampled populations were used.

Comparisons among pairwise distance matrices were performed using Mantel tests as implemented in IBD on the Web (http://www.bio.sdsu.edu/pub/andy/IBD.html; Jensen et al. 2005). Significance of association was determined using 1000 bootstrap pseudoreplicates. For each pairwise comparison, four analyses were performed, employing direct comparisons or log-transforming one or both variables.

RESULTS

Geographic and Ecological Differentiation among Species

Mean pairwise geographic distances between populations of different species were not significantly higher between taxa compared to distances between populations of the same species (data not shown). Several aspects of plant habitat differed significantly among the East Maui taxa, based on reports from historical localities. Species means for each variable are presented in Table 2. These four species show significant differences for most of the measured variables. ANOVAs showed significant differences among species for all five of the variables ($P < 0.0001$ to $P = 0.0067$).

Two significant factors describing ecological variation among historical localities of these species were extracted using PCA. These axes described 74.4% and 12.7% of the variance in habitat scores, respectively. Habitat factor 1 had high positive factor loadings for increased rainfall (0.704), wet habitat designation (0.937), and increased canopy cover (0.928) and high negative factor loadings for elevation (−0.816) and solar radiation (−0.899). Habitat factor 2 also had high factor loadings for rainfall ($r = 0.689$).

ANOVAs showed significant differentiation in habitat factor 1 ($P < 0.0001$), but not habitat factor 2 ($P = 0.324$). Most pairwise species comparisons showed significant differences for centroids for habitat factor 1, except for the comparisons between *D. platyphylla* and *D. menziesii* ($P = 0.059$) and *D. platyphylla* and *D. reticulata* ($P = 0.136$). Comparisons of centroids for habitat factor 2 showed only two significant differences: between *D. menziesii* and *D. reticulata* ($P = 0.0001$) and between *D. reticulata* and *D. waianapanapaensis* ($P = 0.0045$). Variances in these measures were not significantly different among taxa. A PCA graph showing

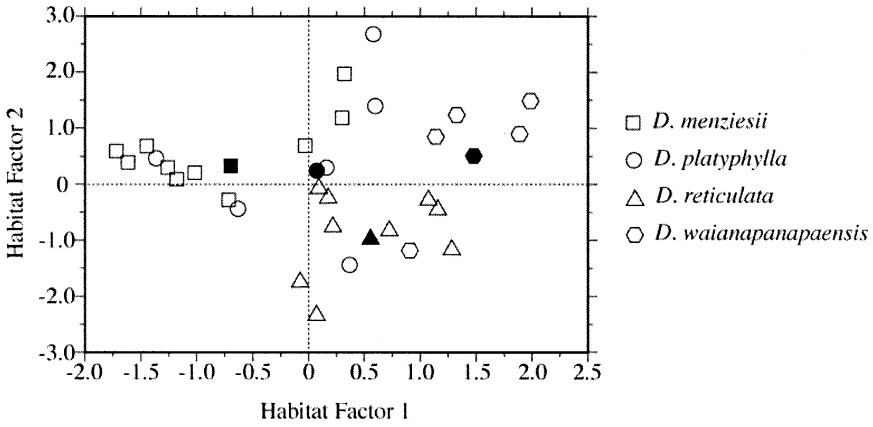

FIG. 2. Principal components analysis of habitat variables for the East Maui species of *Dubautia*: *D. menziesii*, *D. platyphylla*, *D. reticulata*, and *D. waianapanapaensis*. Species centroids are indicated by black symbols.

ecological differentiation among the species for habitat factors 1 and 2 is shown in Figure 2.

Plant Architecture

The three species sampled showed significant differences in most variables of plant architecture as measured in the field. ANOVA showed significant differentiation in all measures except stem circumference at first branch ($P = 0.094$). Pairwise species comparisons indicate that *D. reticulata* is larger in overall plant size and more arborescent than the other two species. Species means for the measured plant architecture variables are presented in Table 3.

Two factors describing differences in plant architecture among *D. menziesii*, *D. platyphylla*, and *D. reticulata* were extracted using PCA. These factors described 65.4% and 18.7% of the variation in plant architecture, respectively. Architecture factor 1 had high factor loadings for all measures of plant size (0.802–0.948). Architecture factor 2 had high factor loadings for stem circumference and diameter at first branch (0.874 and 0.769, respectively) and percent canopy density (0.543).

ANOVA showed significant differentiation in architecture factor 1 among species ($P < 0.0001$), but not architecture factor 2 ($P = 0.195$). Centroids for the three measured taxa were significantly different from one another for architecture factor 1 ($P < 0.0001$ for all comparisons). *Dubautia platyphylla* was significantly different from *D. reticulata* for architecture factor 2 ($P = 0.008$). Variances in the axes differed among taxa, as well. For architecture factor 1, *D. menziesii* had significantly higher variance than either other species (*D. platyphylla*, $P = 0.002$; *D. reticulata*, $P = 0.008$). For architecture factor 2, *D. reticulata* had significantly higher variance than *D. platyphylla* ($P = 0.007$). The PCA for architecture factors 1 and 2 is shown in Figure 3.

Leaf Morphometric Measurements

A total of three significant factors was extracted from the leaf morphometric measurements, representing 80.7%, 9.3% and 5.6% of the variation, respectively. Approximately the same factors were found for each individual leaf position and for all leaves combined. Leaf factor 1 had high factor loadings for all measures of leaf size (0.891–0.994). Leaf factor 2 had high factor loadings for several measures of leaf width below the midpoint (0.533–0.802). Leaf factor 3 had high positive factor loadings for several measures of leaf width above the midpoint (0.477–0.721), and high negative factor loadings for measures of leaf width at 1/8 leaf length (−0.401–0.415). These patterns were consistent for leaves at all nodes, so only the results from all leaves combined are presented below.

ANOVA showed significant differentiation in all leaf factors ($P < 0.001$ for all). All pairwise comparisons of PCA centroids between species were significant for all leaf factors

TABLE 3. Diversity in the East Maui–endemic species of *Dubautia* in measurements on plant architecture. *Dubautia waianapanapaensis* could not be sampled for these measurements because of restrictions on its remote locality. Species means that share a letter superscript are not significantly different.

Species	Height (cm ± SE)	Length (cm ± SE)	Height at first branch (cm ± SE)	Stem circumference at first branch (mm ± SE)	Stem diameter at first branch (mm ± SE)
D. menziesii	91.60 ± 4.916[a]	96.13 ± 5.033[a]	0.09 ± 0.093[a]	86.87 ± 16.608[a]	25.00 ± 4.817[a]
D. platyphylla	196.93 ± 6.774[b]	232.07 ± 6.208[b]	1.13 ± 0.675[a]	61.93 ± 7.256[b]	20.77 ± 2.177[b]
D. reticulata	478.67 ± 24.835[c]	532.80 ± 19.819[c]	72.53 ± 12.583[b]	95.81 ± 6.417[a]	33.53 ± 2.257[a]

Species	Stem diameter at 1/4 height (cm ± SE)	Stem diameter at 1/2 height (cm ± SE)	Plant area (m^2 ± SE)	Canopy density
D. menziesii	1.85 ± 0.121[a]	1.48 ± 0.156[a]	1.76 ± 0.266[a]	21.67 ± 4.752[a]
D. platyphylla	1.71 ± 0.118[a]	1.26 ± 0.065[a]	4.43 ± 0.532[b]	7.47 ± 1.359[b]
D. reticulata	17.11 ± 2.009[b]	8.42 ± 0.701[b]	27.92 ± 2.682[c]	29.67 ± 2.865[a]

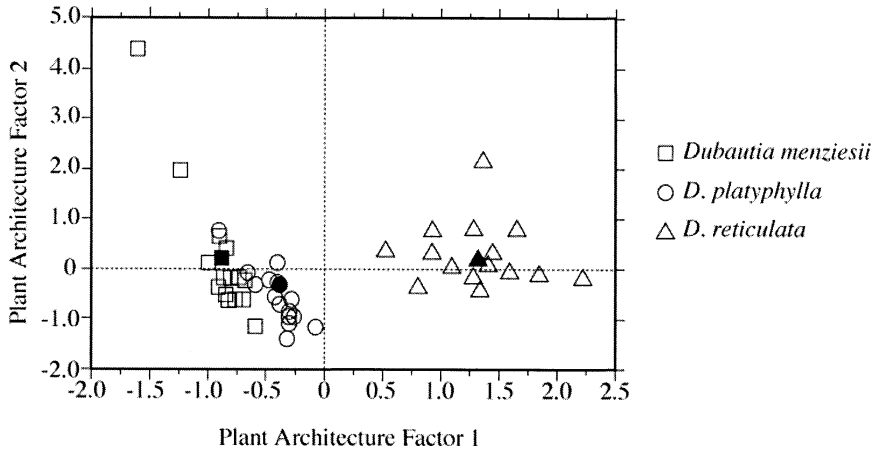

FIG. 3. Principal components analysis of plant architecture for *Dubautia menziesii*, *D. platyphylla*, and *D. reticulata*. Species centroids are indicated by black symbols.

except for the comparison between *D. platyphylla* and *D. reticulata* for leaf factor 1 ($P = 0.642$), and the comparison between *D. menziesii* and *D. waianapanapaensis* for leaf factor 3 ($P = 0.717$). Variances among species were largely not significantly different for any of the leaf factors. The PCA graph for leaf factors 1 and 2 is shown in Figure 4.

Microsatellite Diversity

Based on our population sampling, the most variable microsatellite locus in *D. menziesii*, *D. reticulata*, and *D. waianapanapaensis* was AP3MS1, with 11, 10, and 4 alleles, respectively. The most variable locus in *D. platyphylla* was MKMS2 with four alleles, though AP3MS1 was the second most variable locus with three alleles. The remaining loci were far less diverse with one to three alleles each. Allele frequencies were largely skewed, with one or two alleles at high frequency, and the rest being rare (data not shown). Two loci (AP3MS1 and MKMS2) had noncontinuous allele size distributions, with alleles of intermediate sizes not represented for some of the species (data not shown).

Average observed heterozygosity (H_O), expected heterozygosity (H_E), observed number of alleles (A_O), effective number of alleles (A_E), and f are presented in Table 4. The species do not differ significantly in any measures of genetic diversity within populations, once Bonferroni corrections for multiple comparisons are applied ($\alpha = 0.008$). However, there is a trend for *D. platyphylla* to be less variable than the rest of the species, and for *D. reticulata* to be slightly more variable.

Variances in population diversity estimators among loci differed among populations. *Dubautia platyphylla* showed significantly less variance in A_E compared to all other populations (*D. menziesii*, $P < 0.0001$; *D. reticulata*, $P = 0.001$; *D. waianapanapaensis*, $P = 0.005$), and in H_O compared to all other populations (*D. menziesii*, $P = 0.006$; *D. reticulata*, $P = 0.002$). *Dubautia waianapanapaensis* showed significantly lower variance in A_E and A_O compared to *D. menziesii* ($P = 0.023$ and $P = 0.011$, respectively).

One locus, AP3MS1, showed significant deviation from Hardy-Weinberg expected frequencies in three of the species: *D. menziesii*, *D. platyphylla*, and *D. reticulata*. MKMS2 showed significant deviations from Hardy-Weinberg frequencies in two populations: *D. platyphylla* and *D. reticulata*. AP3MS3 showed significant deviations from Hardy-Wein-

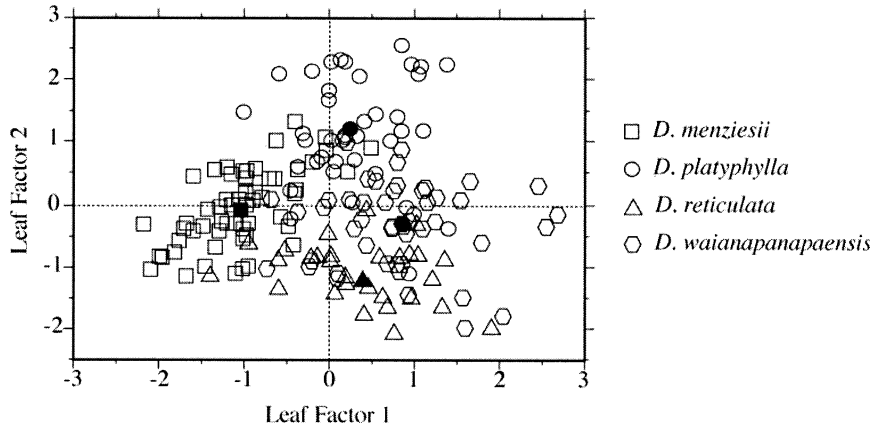

FIG. 4. Principal components analysis of leaf morphometric data for the East Maui species of *Dubautia*: *D. menziesii*, *D. platyphylla*, *D. reticulata*, and *D. waianapanapaensis*. Species centroids are indicated by black symbols.

TABLE 4. Diversity estimates at microsatellite loci for each population of East Maui–endemic species of *Dubautia*.

Species	H_O (SD)	H_E (SD)	A_O (SD)	A_E (SD)	f (SD)
D. menziesii	0.161 (0.208)	0.218 (0.298)	2.62 (3.420)	1.73 (1.487)	0.308 (0.0776)
D. platyphylla	0.047 (0.057)	0.081 (0.108)	1.88 (1.126)	1.10 (0.143)	0.481 (0.1258)
D. reticulata	0.235 (0.250)	0.290 (0.298)	2.62 (3.021)	1.85 (1.363)	0.229 (0.0683)
D. waianapanapaensis	0.154 (0.173)	0.201 (0.235)	2.12 (1.126)	1.39 (0.540)	0.270 (0.1017)

berg frequencies in *D. waianapanapaensis*. In all cases, there was a significant reduction in the frequency of heterozygotes. Bayesian estimates of *f*, an estimator of population inbreeding, were significantly greater than zero for all species, indicating significant inbreeding and showed significant differences among species (Table 4). Based on the criteria of Holsinger and Wallace (2004), *D. platyphylla* had significantly higher inbreeding than any of the other species. None of the other pairwise comparisons showed significant differences. This estimate of inbreeding was very similar to traditional estimates of F_{IS}, though *f* for all species was slightly larger than F_{IS} (data not shown).

Tests for pairwise gametic phase disequilibrium among microsatellite loci showed no significant disequilibrium among loci for most populations. Locus 42–2 showed significant linkage with Ap3MS3 in the *D. menziesii* population and with Ap3MS2 in the *D. reticulata* population. It is possible that 42–2 is physically linked to the *ASAP3* gene in these taxa; however, it seems likely that this result is due to deviations from Hardy-Weinberg equilibrium at these loci (Schneider et al. 2000). The lack of significant linkage disequilibrium among the three microsatellite loci derived from the *ASAP3* gene is more puzzling. The most likely explanation is that the scored microsatellite loci come from different homeologues of the *ASAP3* locus. Because primers were designed to amplify only a single locus but were not identified as to homeologue, all of these microsatellite loci may not be physically linked, despite being derived from a single gene sequence.

Pairwise F_{ST} and R_{ST} genetic distance estimates between all pairs of taxa are significantly different from zero (Table 5). The two estimators are considerably different in their estimates of genetic distance and are not significantly correlated in a Mantel test ($r = 0.31$, $P = 0.34$). AMOVA using both F_{ST} and R_{ST} distances also showed significant differentiation among species, although the inferred distribution of variances into among-population and within-population components differed. Using F_{ST} distances, 46.98% of the variation was attributed to among-population variation and 53.02% to within-population variation. Using R_{ST} distances, 70.27% of the variation was among populations and 29.73% within populations. The difference between distance estimators is likely due to their differing in assumptions on microsatellite evolution. F_{ST} assumes an infinite allele model, in which mutational events between any pair of alleles are equally likely. R_{ST} assumes a stepwise mutational model, in which only mutational changes of one length unit are allowed. There has been considerable debate regarding the most appropriate model for microsatellite data. In this case, both models suggest significant differentiation between all sampled populations. Likewise, the Bayesian θ^B estimate for the group was 0.163 (SD = 0.210) and significantly greater than zero. A Bayesian assignment method detected no significant recent gene flow among the four sampled populations (Table 6).

DNA Sequence Diversity within and among Species

The A and B homeologues of the *ASAP3* locus can be distinguished by several large insertion/deletion events in introns 2 and 3. Aligned sequence length among all individuals was 1022 nucleotides for the *ASAP3*-A locus and 1230 nucleotides for the *ASAP3*-B locus.

Despite our attempts to not sample hybrid individuals, several *ASAP3*-A sequences were found to be more closely related to sequences of a more distantly related species, *D. plantaginea*, which has been sampled in other ongoing studies of the Hawaiian silversword alliance. This species occurs near *D. reticulata* and *D. waianapanapaensis*. These sequences may have resulted from hybridization or possibly PCR contamination and were removed from all subsequent analyses.

Measures of within-population sequence variation and the *ASAP3*-A and *ASAP3*-B loci are presented in Table 7. All measures of genetic diversity within species at these loci are low, as is consistent with other studies in this group (Lawton-Rauh et al. 2003; A. Lawton-Rauh, R. Robichaux, and M. Purugganan, unpubl. ms.). Diversity at the *ASAP3*-B locus is lower than that at the *ASAP3*-A locus, as measured by the number of segregating sites and the number of haplotypes, again consistent with previous findings (Lawton-Rauh et al. 2003; unpubl. ms.).

As was found with the microsatellite data, *D. platyphylla* shows lower levels of within-population diversity at *ASAP3*-B than the other East Maui taxa, in terms of nucleotide diversity (π), number of haplotypes, number of segregating sites, and haplotype diversity, though this pattern does not hold for *ASAP3*-A (Table 7). At the *ASAP3*-A locus, *D. waian-*

TABLE 5. F_{ST} (above diagonal) and R_{ST} (below diagonal) distances between pairs of East Maui–endemic species of *Dubautia*.

	D. menziesii	*D. platyphylla*	*D. reticulata*	*D. waianapanapaensis*
D. menziesii	—	0.684	0.394	0.270
D. platyphylla	0.850	—	0.355	0.638
D. reticulata	0.649	0.105	—	0.330
D. waianapanapaensis	0.852	0.546	0.310	—

TABLE 6. Recent gene flow between East Maui–endemic species of *Dubautia* based on Bayesian assignment methods (Wilson and Rannala 2003).

To:	From:			
	D. menziesii	*D. platyphylla*	*D. reticulata*	*D. waianapanapaensis*
D. menziesii	0.989 ± 0.0111	0.003 ± 0.0051	0.004 ± 0.0057	0.004 ± 0.0064
D. platyphylla	0.004 ± 0.0059	0.988 ± 0.0110	0.004 ± 0.0056	0.004 ± 0.0058
D. reticulata	0.004 ± 0.0064	0.007 ± 0.0110	0.984 ± 0.0162	0.005 ± 0.0072
D. waianapanapaensis	0.006 ± 0.0094	0.005 ± 0.0075	0.005 ± 0.0070	0.984 ± 0.0151

apanapaensis is considerably more diverse in all measures than the rest of the taxa. At the *ASAP3*-B locus, the most diverse taxon is *D. reticulata*. For both loci, species show a subset of the overall diversity in the group (Table 7).

Using Kimura two-parameter genetic distances, most pairwise comparisons among taxa show significant genetic differentiation at the *ASAP3*-A locus except for *D. menziesii* versus *D. platyphylla* ($P = 0.99$). By contrast, at the *ASAP3*-B locus, only the comparisons of *D. platyphylla* versus *D. reticulata* ($P = 0.003$) and *D. reticulata* versus *D. waianapanapaensis* ($P = 0.001$) are significantly different.

Estimates of Tajima's *D* for all species at both loci are also presented in Table 7. None of these estimates is significantly different from zero, indicating that the distribution of polymorphisms within species is largely consistent with neutral-equilibrium model expectations (Tajima 1989).

Haplotype networks for the *ASAP3*-A and *ASAP3*-B loci are shown in Figure 5. In neither network do all of the alleles from a given species form a monophyletic clade. *ASAP3*-A shows considerably more evolutionary distance among haplotypes, particularly in *D. reticulata* and *D. waianapanapaensis*, with three haplotypes from those taxa showing considerable divergence from the others. All *ASAP3*-A haplotypes from *D. menziesii* and *D. platyphylla* (haplotypes 1–5) are connected by one to two mutational events, while the haplotypes from *D. reticulata* and *D. waianapanapaensis* (haplotypes 1, 6–8) show greater mutational differences among haplotypes. In contrast, the haplotype network for *ASAP3*-B shows more shared haplotypes among taxa.

Maximum likelihood phylogenies of *ASAP3*-A and *ASAP3*-B haplotypes are shown in Figure 6, along with estimates of MP bootstrap support shown over the relevant nodes (estimates of ML bootstrap support are virtually identical; data not shown). For *ASAP3*-A, parsimony analysis found six most parsimonious trees of length 22. For *ASAP3*-B, one most parsimonious tree was found of length 16. As in the haplotype networks, in neither phylogeny do all haplotypes from a given species form a monophyletic lineage. Bayesian analysis recovered the same tree as MP and ML analyses. Posterior probabilities for each node are also shown in Figure 6.

In the *ASAP3*-A phylogeny, the base of the tree has no statistical support; however, two smaller clades are well supported. All of the haplotypes that occur in the two dry habitat species, *D. menziesii* and *D. platyphylla*, form a well-supported clade that includes haplotype 1, which is found in all species except *D. platyphylla*. The haplotypes found in the wet habitat species (*D. reticulata* and *D. waianapanapaensis*), on the other hand, show greater phylogenetic diversity, being present in both well-supported clades, suggesting that these species may be the oldest species in the lineage. The *ASAP3*-B haplotype phylogeny is less resolved, with only the clade of haplotypes from the East Maui species flock receiving strong statistical support.

Dataset Comparisons

Inspection of the pattern of significant pairwise differences for the various measures of divergence among these species shows a complex distribution of differentiation. In a comparison of the nine datasets (habitat factor 1, habitat factor 2, architecture factor 1, architecture factor 2, leaf factor 1, leaf factor 2, microsatellite genetic distance [F_{ST}], *ASAP*3-A [Kimura two-parameter distances], and *ASAP3*-B [Kimura two-parameter distances]), no pair of taxa differed significantly in all datasets, and no pair of taxa showed no significant differences in any dataset. *Dubautia reticulata* and *D. waianapanapaensis* showed the most significant differences in all of the datasets, lacking significant differentiation only in the *ASAP3*-A dataset. The comparison of *D. menziesii* and *D. platyphylla* had the least number of significant differences (five of 10).

TABLE 7. DNA sequence diversity at the AP3A and AP3B loci in the East Maui–endemic species of *Dubautia*, including haplotypic diversity (*Hd*) and nucleotide diversity (π).

Species	Segregating sites	Haplotypes	*Hd* (mean ± SD)	π (mean ± SE)	Tajima's *D*
Overall AP3A	12	8	0.806 ± 0.051	0.00454 ± 0.00070	1.10
D. menziesii	2	3	0.667 ± 0.160	0.00103 ± 0.00025	1.17
D. platyphylla	2	3	0.667 ± 0.160	0.00084 ± 0.00025	0.21
D. reticulata	9	3	0.700 ± 0.218	0.00412 ± 0.00168	−0.20
D. waianapanapaensis	10	3	0.833 ± 0.222	0.00491 ± 0.00231	−0.83
Overall AP3B	6	6	0.775 ± 0.055	0.00120 ± 0.00016	−0.11
D. menziesii	2	3	0.667 ± 0.160	0.00085 ± 0.00021	1.17
D. platyphylla	2	2	0.286 ± 0.196	0.00046 ± 0.00032	−1.24
D. reticulata	5	3	0.733 ± 0.155	0.00212 ± 0.00042	1.05
D. waianapanapaensis	1	2	0.571 ± 0.119	0.00046 ± 0.00010	1.34

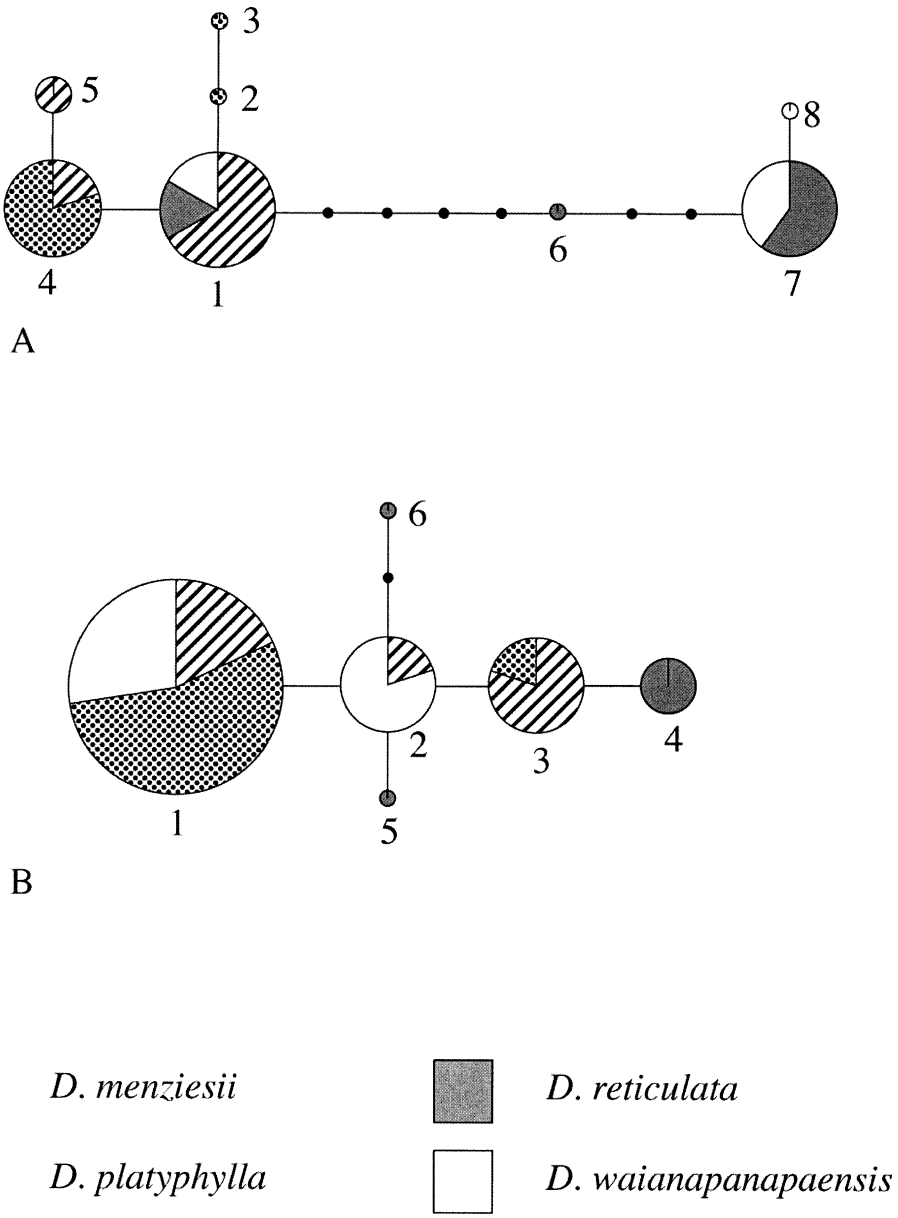

FIG. 5. Haplotype networks for *ASAP3*-A (A) and *ASAP3*-B (B) loci in the East Maui–endemic species of *Dubautia*. Relative circle sizes indicate haplotype frequency, and the colored slices represent the proportion of the haplotype in each species. The number of inferred steps between haplotypes is indicated by small filled circles. Each haplotype is indicated by number.

Several comparisons among datasets showed significant correlations. As might be expected, variation in whole plant morphology and habitat were tightly correlated ($r = 0.8912$, $P < 0.001$). Genetic distances calculated from microsatellite variation were not significantly correlated with any other measures. Genetic distances from DNA sequence data were correlated with several measures of plant morphology. Distances from the *ASAP3*-A dataset were significantly correlated with leaf morphometric measures ($r = 0.6363$, $P < 0.001$).

DISCUSSION

In this study, we found significant ecological, morphological, and microsatellite differentiation among the closely related East Maui–endemic species of *Dubautia*. These species differences have apparently been maintained despite geographic proximity between populations of different species. We detected no recent gene flow among the sampled populations. This indicates that widespread introgression or landscape level gene flow is not occurring, because all of the sampled populations are geographically closer to populations of other taxa than to other populations of the same species. Ongoing hybridization among these species on the boundaries of populations, as indicated by previous reports of morphological intermediates (Carr 1985), is apparently insufficient to lead to a lack of genetic differentiation, as shown by the significant microsatellite differentiation. However, haplotypes of two nuclear loci exhibited significant allele

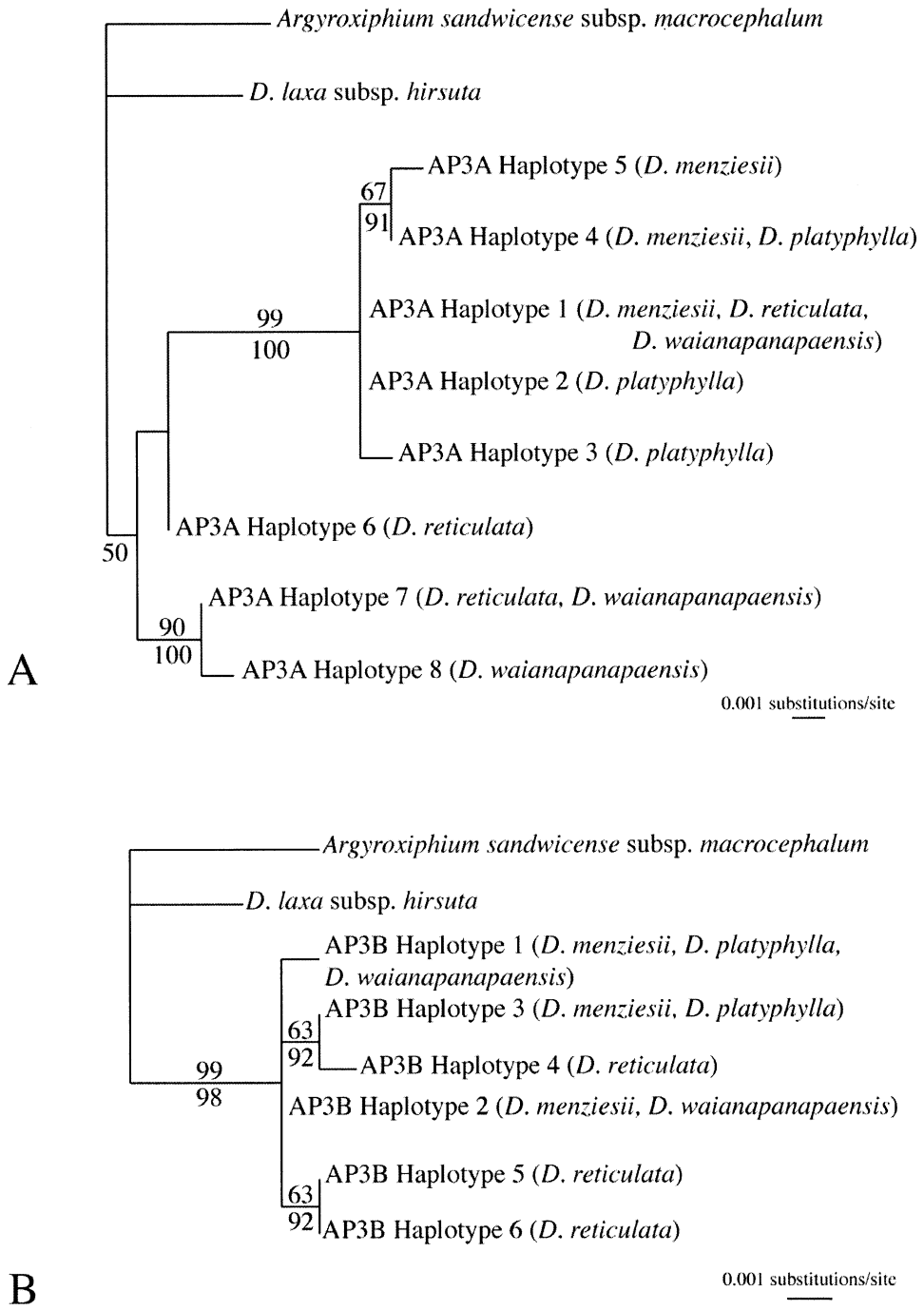

FIG. 6. Maximum-likelihood phylogeny for *ASAP3*-A (A) and *ASAP3*-B (B) loci for the East Maui–endemic species of *Dubautia*. Numbers over the nodes represent parsimony bootstrap support. Numbers below the nodes represent Bayesian posterior probabilities. Species containing each haplotype are listed after the haplotype number.

sharing across species, indicating the presence of incomplete lineage sorting. There was a complex pattern of correlations among species divergence in different datasets, suggesting that a complex history of natural selection on ecological adaptation and lineage sorting has maintained species differences despite the potential for ongoing gene flow. Our sample sites were chosen to minimize the impact of current or historical hybridization, so these results may underestimate the role of introgression or hybridization in the evolutionary history of these species. Many plant species have been shown to be de facto reproductively isolated, despite the lack of intrinsic barriers to hybridization (Grant 1981; Church and Taylor 2002; Rieseberg et al. 2003; Tovar-Sanchez and Oyama 2004). In these cases, it is very likely that reinforcing selection for ecological specialization, either directly on the plants, through impacts on pollinators, or selection against

intermediates (Campbell et al. 1997; Wolf et al. 2001; Wu and Campbell 2005) is responsible for the development and maintenance of species barriers.

Morphological and Ecological Differentiation

Our data show significant morphological and ecological differentiation among these four closely related species in the East Maui *Dubautia* species flock. Ecological and morphological divergence in this species flock seems to follow the steep moisture clines in the East Maui landscape, as indicated by significant differences in moisture habitat among historical species localities (Fig. 2, Table 2). These moisture clines, at least in part, follow the altitudinal gradient surrounding the Haleakala volcano. Habitats with very different moisture regimes may be in physical proximity, potentially allowing gene flow among diverging populations. These taxa have thus maintained their differing ecological and morphological adaptations despite geographic proximity.

Measurements of tissue elastic modulus in these species showed that the dry habitat *D. platyphylla* and *D. menziesii* species pair do not differ significantly in this important measure of tissue water relations, but both taxa differ significantly from the wet habitat *D. reticulata* and *D. waianapanapaensis* species pair, which, in turn, do not differ significantly from one another (Robichaux 1985; Robichaux and Canfield 1985; R.H. Robichaux, pers. comm.). The dry habitat species show measures of tissue elastic modulus similar to other taxa in the Hawaiian silversword alliance that are endemic to dry habitats, whereas the wet habitat species show intermediate tissue elastic modulus. These data were used to suggest that the dry habitat species pair was evolutionarily basal, and that the wet species pair was derived and recently invaded the wetter habitats (Robichaux 1985; Robichaux and Canfield 1985; R. H. Robichaux, pers. comm.).

It has been suggested that the degree and speed of evolutionary divergence is determined by genetic constraints, and that evolutionary divergence will tend to follow the lines of least genetic resistance (Schluter 1996b; Stebbins 1974). It has also been suggested that the vector of greatest phenotypic variance is a reasonable substitute for the vector of greatest genetic variance, although whether this assumption holds true within as well as among species is still debatable (Cheverud 1988; Willis et al. 1991; Schluter 1996b). In this case, we see an example of a repeated trend of diversification into wet and dry habitats, suggesting that the silversword alliance has a particularly large genetic variance in these traits, allowing for rapid differentiation along moisture habitat gradients (Carr 1985; Baldwin and Robichaux 1995).

Genetic Divergence

Genetic divergence among these species was measured in two ways: microsatellite and DNA sequence divergence. Microsatellites have a very high mutational rate, up to 10^{-4} events/locus/year, compared to 10^{-9} substitutions/site/year for DNA sequences, and a shallow coalescence time, and thus their diversity reflects recent evolutionary events (Balloux et al. 2000). Nucleotide substitutions have a much lower rate, and thus can reflect deeper evolutionary divergences, though the rate of divergence can be locus specific. In this study, the microsatellite and DNA sequence data provided different views of the evolutionary history of the East Maui *Dubautia* species flock, perhaps as a result of sampling different evolutionary time depths. The microsatellite data show complete population divergence, with no gene flow among the populations, while both sequence datasets show significant amounts of haplotype sharing. These results may be biased by the deliberate avoidance of hybrids in our sampling design. However, this sampling scheme allows us to address more historical aspects of species divergence in these taxa. Current hybridization could confound these more historical aspects, if obvious hybrids had been sampled. The fact that the microsatellite data show no evidence for ongoing gene flow among populations suggests that the DNA sequence data is reflecting incomplete lineage sorting in these very recently diversifying taxa, rather than ongoing hybridization.

These four species do not differ significantly in amounts of genetic diversity within each population, either from one another or from other species in the silversword alliance (Friar et al. 2000, 2001). Species in the silversword alliance in general tend to have lower genetic diversity than continental species, probably because of recent divergence events (Friar et al. 2000, 2001).

Genetic diversity within populations does not correlate with current population size, or apparent historical population distribution, based on herbarium collections (Carr 1985). However, the sampled populations do differ significantly in estimates of inbreeding within populations. Most species in the Hawaiian silversword alliance apparently are pollinated primarily by generalist solitary bees and are self-incompatible, and thus require a pollinator visit in order to set seeds (Carr et al. 1986; Forsyth 2003). Seeds are likely to have been bird dispersed, particularly between islands within the Hawaiian archipelago, given their apparent adaptations to animal dispersal. However, wind or water dispersal may play a role in within-island seed movement (Baldwin and Robichaux 1995). Several self-incompatible species of the silversword alliance have been studied in depth, and estimates of inbreeding have typically been very low for those taxa (Friar et al. 2000, 2001). However, at least one species of *Dubautia*, *D. scabra*, has reverted to self-compatibility and has very low within-population genetic diversity and high estimated inbreeding compared with other silversword alliance species (E. A. Friar, M. McGlaughlin, and J. Cruse-Sanders, unpubl. ms.). *Dubautia platyphylla* has a significantly higher inbreeding estimate within the sampled population than the other species sampled here or other silversword taxa (Friar et al. 2001). The significant inbreeding estimate in *D. platyphylla* may reflect the loss of self-incompatibility in this species, leading to an increase in both uniparental and biparental inbreeding, as has been seen in other species in the Hawaiian silversword alliance (E. A. Friar et al., unpubl. ms.). Its breeding system is currently being examined in more detail in greenhouse-grown populations (E. A. Friar, M. McGlaughlin and J. Cruse-Sanders, unpubl. data).

Unlike the microsatellite data, the DNA sequence data at both the *ASAP3*-A and *ASAP3*-B loci do not show significant differentiation among the East Maui–endemic species of *Dubautia*. The degree of haplotype sharing differs between

ASAP3-A and *ASAP3*-B, with *ASAP3*-B being the more conserved locus, with more shared haplotypes, fewer mutations, and shorter phylogenetic branches.

The haplotype networks show considerably more diversity at *ASAP3*-A than at *ASAP3*-B. In particular, *D. reticulata* and *D. waianapanapaensis* have greater sequence diversity than *D. menziesii* and *D. platyphylla* at *ASAP3*-A. This pattern is less clear in the *ASAP3*-B haplotype network, perhaps because of the overall lower sequence diversity. This pattern of haplotype sharing is very similar to that found previously using *ASAP3*-A and *ASAP3*-B in other members of the Hawaiian silversword alliance by A. Lawton-Rauh et al. (unpubl. ms.). They found significant allele sharing at *ASAP3*-A between *D. arborea* and *D. ciliolata*, two species endemic to the Mauna Kea volcano on the Island of Hawaii.

There is also the suggestion in the *ASAP3*-A phylogeny that the haplotypes present in the dry habitat taxa are derived relative to those present in wet habitat taxa. The pattern at *ASAP3*-B is less clear, perhaps because of the overall lower diversity at that locus, but there is also the tendency for the alleles from *D. menziesii* and *D. platyphylla* to be derived, possibly suggesting that the common ancestor of the East Maui *Dubautia* species flock was a wet habitat species. This finding conflicts with the inferences based upon ecophysiological measurements, in which the wet habitat species were posited to be evolutionarily derived (Robichaux 1985; Robichaux and Canfield 1985; R. H. Robichaux, pers. comm.). Given the lack of well-supported phylogenetic resolution, inferences on the basal ecological setting of this species are still inconclusive.

Comparison of Ecological, Morphological, and Genetic Divergence

In these data, we show that species of the East Maui *Dubautia* group are significantly diverged in ecological habitat and some aspects of morphology. Comparisons among datasets have shown significant correlation between ecological habitat and plant architecture, but not leaf morphometric measurements. There was a significant association between leaf morphology and divergence at the *ASAP3*-A locus.

Because these four species probably share the same generalist pollinators (Forsyth 2003), the significant ecological, morphological, and microsatellite divergence implies that divergent selection along physical environment gradients has driven genetic divergence. This pattern is similar to those found in other members of the silversword alliance with more in-depth population sampling, where gene flow across a landscape is more closely correlated with ecological differences than with geographic distance (i.e., landscape model of gene flow sensu Sork et al. 1999; E. A. Friar et al., unpubl. ms.).

Several different mechanisms for the process of ecological speciation associated with adaptive radiation have been proposed (Schluter 2001). Populations in allopatry or near allopatry may accumulate significant differences and diverge as a by-product of differential adaptation (Mayr 1942; Dobzhansky 1951; Turelli et al. 2001; Via 2001). Sympatric populations may be able to speciate due to selection against intermediate phenotypes (Wilson and Turelli 1986; Dieckmann and Doebeli 1999; Hatfield and Schluter 1999). Ecological speciation would suggest that divergence in ecological traits would drive reproductive isolation (Schluter 1996a; Lu and Bernatchez 1999).

These proposed mechanisms differ in their predictions of the relative roles of natural selection and cessation of gene flow in promoting reproductive isolation in diverging species, which has been the subject of an ongoing debate (Crespi and Sandoval 2000; Hendry et al. 2001, 2002; Schluter 2001; Lenormand 2002; Saint-Laurent et al. 2003; Hendry and Taylor 2004; Nosil and Crespi 2004). Particularly in animals, an inverse relationship has been shown between gene flow and morphological divergence in many systems (Hendry and Taylor 2004; Hendry et al. 2002; Lu and Bernatchez 1999; Riechert et al. 2001). However, in plants, there have been numerous examples of a lack of connection between divergence at genetic markers and divergence in ecological, morphological, and/or geographic traits (Lerceteau et al. 1997; Persson and Gustavsson 2001). Brunell and Whitkus (1997) showed that random amplified polymorphic DNA diversity in *Eriastrum densifolium* showed continuous variation, and was not correlated with morphology or elevation, but rather with geographic distance. Likewise, Heywood and Levin (1984) found that allozyme diversity correlated with geographical distance rather than morphology. These authors interpreted their results to suggest that gene flow among populations was homogenizing their genomes, whereas divergent selection was maintaining different morphological patterns. Saint-Laurent et al. (2003) found that sympatric species of fish had significant haplotype sharing despite morphological and ecological divergence.

The complex patterns of correlation among ecology, morphology, and two different genetic datasets for the East Maui *Dubautia* group points to natural selection maintaining the species boundaries in spite of the potential for ongoing gene flow. In particular, the fact that plant architecture and ecological habitat were correlated, and yet neither was correlated with significant differentiation in leaf morphology or genetic divergence, suggests that correlating ecological and morphological divergence is important for understanding the traits upon which natural selection is acting.

Conclusions

We found significant and consistent morphological and ecological differentiation and genetic differentiation at microsatellite loci in this small group of four closely related species. We can use these data to reject the hypothesis of ongoing large-scale gene flow, which is not consistent with the level of differentiation observed. Instead, these results support previous delimitations of species and indicate that large-scale introgression among these morphologically defined species may be minimal. Thus, the presence of apparent hybrids in several of the populations may not indicate that these species are effectively interbreeding (notwithstanding evidence for local introgression, which may be quite extensive in *D. reticulata*), and that they may be de facto reproductively isolated based on their ecological adaptations rather than actual lack of interfertility.

Thus, our data support ecological speciation among these four closely related species of *Dubautia*, given significant

ecological, morphological, and recent genetic differentiation, although there is still continuing lineage sorting. We found differentiation among historical populations in ecological parameters, which were correlated with morphological differences. Genetic differentiation appears to be complete at the more rapidly evolving microsatellite loci, while lineage sorting is still ongoing at the deeper coalescence times as shown at nuclear coding loci. This evidence together suggests that a common ancestor gave rise to a series of populations differing in their ability to exploit a resource, in this case probably rainfall, which then differentiated morphologically and genetically, with the neutral genetic differentiation still ongoing. This study is unusual in showing ecological speciation in the absence of geographic differentiation.

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