Interspecific Hybrid Ancestry of a Plant Adaptive Radiation: Allopolyploidy of the Hawaiian Silversword Alliance (Asteraceae) Inferred from Floral Homeotic Gene Duplications

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The polyploid Hawaiian silversword alliance (Asteraceae), a spectacular example of adaptive radiation in plants, was shown previously to have descended from North American tarweeds of the *Madia*/*Raillardiopsis* group, a primarily diploid assemblage. The origin of the polyploid condition in the silversword alliance was not resolved in earlier biosystematic, cytogenetic, and molecular studies, apart from the determination that polyploidy in modern species of *Madia*/*Raillardiopsis* arose independent of that of the Hawaiian group. We determined that two floral homeotic genes, *ASAP3*/*TM6* and *ASAP1*, are found in duplicate copies within members of the Hawaiian silversword alliance and appear to have arisen as a result of interspecific hybridization between two North American tarweed species. Our molecular phylogenetic analyses of the *ASAP3*/*TM6* loci suggest that the interspecific hybridization event in the ancestry of the Hawaiian silversword alliance involved members of lineages that include *Raillardiopsis muirii* (and perhaps *Madia nutans*) and *Raillardiopsis scabrida.* The *ASAP1* analysis also indicates that the two species of *Raillardiopsis* are among the closest North American relatives of the Hawaiian silversword alliance. Previous biosystematic evidence demonstrates the potential for allopolyploid formation between members of the two North American tarweed lineages; a vigorous hybrid between *R. muirii* and *R. scabrida* has been produced that formed viable, mostly tetraporate (diploid) pollen, in keeping with observed meiotic failure. Various genetic consequences of allopolyploidy may help to explain the phenomenal evolutionary diversification of the silversword alliance.

Introduction

Adaptive radiations are among the most spectacular processes in organismal evolution. Radiations are characterized by rapid bursts of evolutionary innovation and are associated with increased speciation rates, elevated levels of morphological diversity, and marked differentiation in ecological characteristics (Givnish 1997). Some of the most dramatic examples of adaptive radiations are found in the Hawaiian archipelago, where isolation, continued island formation, and a high diversity of environmental settings promote species radiations in both plant and animal groups.

The Hawaiian silversword alliance (Asteraceae: Heliantheae–Madiinae) is a premier example of adaptive radiation in plants (Carr 1985; Robichaux et al. 1990; Baldwin and Robichaux 1995; Baldwin 1997). The alliance comprises 30 perennial species in three endemic genera: *Argyroxiphium, Dubautia,* and *Wilkesia* (Carr 1985, 1998*a*, 1998*b*). The species are distributed on six of the eight main islands of the Hawaiian archipelago (Kauai, Oahu, Molokai, Lanai, Maui, and Hawaii), with all but five species being single-island endemics (Carr 1985). Species in the alliance grow in a wide range of habitats, including exposed lava, dry scrub, mesic forests, wet forests, and bogs (Baldwin and Robichaux 1995; Baldwin 1997). The species also display an impressive array of morphological growth forms, including rosette plants, cushion plants, subshrubs, shrubs, trees, and lianas (Baldwin and Robichaux 1995; Baldwin 1997). Furthermore, the silversword alliance is characterized by a high level of chromosomal repatterning, with eight genomic rearrangements distinguished by reciprocal translocations and an aneuploid reduction (Carr and Kyhos 1986).

Understanding the origin of species-rich insular groups is crucial to our attempts to reconstruct evolutionary patterns of ecological and morphological change that characterize adaptive radiations (Givnish 1997). Both cpDNA and rDNA ITS studies have confirmed Carlquist's (1959) hypothesis that the closest relatives of the Hawaiian silversword alliance can be found among the North American tarweeds (Asteraceae: Heliantheae–Madiinae) in the paraphyletic *Madia*/*Raillardiopsis* group (Baldwin et al. 1991; Baldwin 1992, 1996, 1997; Baldwin and Robichaux 1995). A major unanswered question concerns evolution of the polyploid condition found throughout the Hawaiian silversword alliance (Baldwin et al. 1991; Baldwin and Kyhos 1996; Baldwin 1997). Both cytogenetic (Carr and Kyhos 1986; Kyhos, Carr, and Baldwin 1990; Carr, Baldwin, and Kyhos 1996) and allozymic (Witter and Carr 1988) data indicate that the Hawaiian species are tetraploids (n = 13, 14), in contrast to the basally diploid condition (n = 6–9) in each of the most closely related North American lineages of *Madia*/*Raillardiopsis* (Baldwin 1996). Resolution of the origin of polyploidy in the silversword alliance is important for understanding the genomic constitution of the founder species and its descendants, information that could help to identify factors that facilitated this spectacular adaptive radiation.

We report here the isolation of orthologs of two *Arabidopsis* floral homeotic genes, *APETALA1* and

Abbreviations: *AP1, APETALA1*; *AP3, APETALA3*; cpDNA, chloroplast DNA; indels, insertion/deletion mutations; MYA, million years ago; rDNA ITS, nuclear ribosomal DNA internal transcribed spacer.

Key words: MADS-box, inflorescence.

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APETALA3/*TM6*, from members of the Hawaiian silversword alliance and the North American tarweeds. PCR-based sampling indicates that these floral regulatory genes are each present in two copies in the Hawaiian species, but in only one copy in the North American tarweeds examined. Our results suggest that the Hawaiian silversword alliance is an allopolyploid group that descended from a hybrid between members of extant tarweed lineages.

Materials and Methods

Sampling and Nucleic Acid Isolation

Genomic DNA samples were isolated from young leaf tissue collected from 10 species of the Hawaiian silversword alliance and 7 species of North American tarweeds. The Hawaiian species were selected to represent each of the four major lineages in the silversword alliance as previously identified from rDNA ITS trees (Baldwin and Robichaux 1995; Baldwin 1996). Four North American species (*Madia bolanderi*, *Madia nutans*, *Raillardiopsis scabrida*, and *Raillardiopsis muirii*) were chosen to represent each of the four major lineages in the *Madia*/*Raillardiopsis* group as also identified from rDNA ITS trees (Baldwin 1996). Three other North American tarweed species (*Adenothamnus validus*, *Raillardella pringlei*, and *Osmadenia tenella*) are known to fall outside the clade comprising *Madia*/*Raillardiopsis* and the silversword alliance (Baldwin 1996) and were included to serve as the outgroup in the analyses. Genomic DNA was isolated from leaf tissue of *Argyroxiphium sandwicense* subsp. *macrocephalum* using a modified CTAB protocol that reduced the amount of pectin and secondary-product contamination (Friar, Robichaux, and Mount 1996). For all of the North American species and the remaining Hawaiian species, genomic DNA was isolated using the methods of Palmer (1986) or Doyle and Doyle (1987) and further purified on $CsCl_2$ gradients. Total floral RNA was isolated from immature and mature capitula of *A. sandwicense* subsp. *macrocephalum* using a phenol/SDS extraction procedure (Ausubel et al. 1992).

Isolation of the *Argyroxiphium sandwicense AP3*/*TM6* and *AP1* Genes

MADS-box loci can be readily identified using sets of degenerate PCR primers that specifically amplify MADS-box sequences (Rounsley, Ditta, and Yanofsky 1995). The isolation of MADS-box sequences from *A. sandwicense* subsp. *macrocephalum* by this method allowed us to obtain sequence information that permitted the design of 5′ nonoverlapping, nested PCR primers specific to the *ASAP3*/*TM6* and *ASAP1* genes. These gene-specific primers were used to isolate cDNAs utilizing the PCR-based rapid amplification of cDNA ends (RACE) protocol (Frohman, Dush, and Martin 1988). First-strand cDNA was synthesized from *A. sandwicense* subsp. *macrocephalum* floral RNA with AMV reverse transcriptase following standard protocols (Boehringer Mannheim), and amplified cDNA was cloned using the TA procedure (In Vitrogen) and sequenced.

PCR primers to amplify *ASAP3*/*TM6* and *ASAP1* genomic regions were designed based on the cDNA sequences. The primers ASAP3-2 and ASAP3-3R allowed PCR amplification of a region spanning exons 1–4 of the *ASAP3*/*TM6* gene. Primers were designed to isolate both size-differentiated copies of this gene (1.1 kb vs. 1.4 kb) in the Hawaiian silversword alliance species. For *ASAP1*, primers ASAP1/3X and ASAP1/8XR were designed to isolate both *A* and *B* copies of this gene; the length difference between the duplicate copies was too small, however, for reliable size differentiation. To isolate the two copies of *ASAP1*, gene-specific primer pairs (ASAP1-F1A/ASAP1-RB and ASAP1-AF/ASAP1-R) were constructed to amplify sequences from exons 3–8 of different duplicate copies of the gene within the Hawaiian species. The primers were used in PCR amplifications using the error-correcting rTth polymerase formulation (Perkin-Elmer) in standard buffer with 40 cycles of 95°C for 1 min, 55°C for 1 min, and 72°C for 4 min. The nucleotide error rate for this formulation is less than 1 bp in 7 kb of sequence (unpublished observations). In experiments designed to determine whether other copies of these loci are present, an annealing temperature 5–10°C below primer melting temperature was used. PCR-amplified DNA was cloned using the TA cloning kit (In Vitrogen) and sequenced using automated sequencers (Iowa State University Sequencing Facility, NCSU DNA Sequencing Facility). Sequencing was done with nested primers, with multiple sequencing reactions conducted for divergent sequences. All sequence changes were rechecked visually against sequencing chromatograms and are deposited in GenBank (accession numbers AF147210–AF147258).

Phylogenetic Analyses

Nucleotide sequences were aligned visually. Phylogenetic analyses were conducted using maximum-parsimony techniques implemented in PAUP*4.0d54 (Swofford 1998). Both substitution and insertion/deletion (indel) differences were used and weighted equally in the analyses, with the indels separately coded (as additional characters) to reflect nonindependence of continuous gaps. Parsimony analyses were conducted using the heuristic search procedure, with random taxon addition (10 replicates), tree bisection-reconnection branch swapping, and MULPARS in effect. Clade support was estimated by parsimony analysis of 500 bootstrap replicates of the data set using the search procedures outlined above.

Interspecific Hybridizations

In addition to a hybrid combination previously reported between *R. muirii* and *R. scabrida* (Baldwin 1989; Kyhos, Carr, and Baldwin 1990), hybrids were produced in the combinations *Madia madioides* × *R. scabrida* and *Dubautia knudsenii* × (*M. madioides* × *R. scabrida*) (unpublished data). Cross-pollinations were performed by rubbing the stylar surfaces of pollen-shedding capitula together. Full cypselae from crosses were hydrated overnight on wet filter paper, and F_1 embryos were surgically excised and germinated on wet filter pa-

per. The F_1 plants were grown to flowering maturity under cool, low-humidity greenhouse conditions at the University of California–Berkeley. Pollen fertility and pollen morphology of hybrid plants were examined by staining with cotton blue in lactophenol. Chromosomal association at meiotic metaphase I was examined, using phase microscopy, in squashed microsporocytes stained with acetocarmine mixed with Hoyer's solution.

Results and Discussion

Floral Homeotic Genes as Sources of Characters for Plant Phylogeny Reconstruction

In recent years, the molecular genetics of floral and inflorescence development have been the subject of intense study (Riechmann and Meyerowitz 1997). Much of this work has been carried out on *Arabidopsis thaliana* (Brassicaceae), in which several genes that control flower development have been identified (Riechmann and Meyerowitz 1997). The loci are referred to as floral homeotic genes, with mutational lesions in the genes resulting in the formation of aberrant organ types in flowers.

Molecular studies have revealed that most of the floral homeotic genes isolated to date belong to the MADS-box regulatory gene family of transcriptional activators (Ma, Yanofsky, and Meyerowitz 1991; Purugganan et al. 1995; Riechmann and Meyerowitz 1997). In *A. thaliana,* at least 25 members of this gene family have been isolated, and most of these appear to regulate differing aspects of flower development (Purugganan et al. 1995; Riechnmann and Meyerowitz 1997). Many of the duplications that resulted in the growth of this gene family appear to have occurred fairly early in the evolutionary history of the vascular plants (Purugganan 1997), and the different paralogous genes are readily distinguishable. Some members of the gene family, however, appear to have arisen from more recent duplications, possibly as a result of polyploidization events that occurred during evolution (Mena et al. 1996; Lowman and Purugganan 1999). The MADS-box floral homeotic genes are among the fastest-evolving plant nuclear loci observed thus far, with variable regions that are evolving at 3.7×10^{-9} nonsynonymous substitutions per site per year (Purugganan et al. 1995). This rate is comparable to the mean rate for synonymous substitutions for other plant nuclear genes (4.7×10^{-9} substitutions per site per year) (Purugganan et al. 1995). The rapid evolution of the MADS-box floral homeotic genes, including their introns, makes them attractive as potential sources of new molecular characters for plant phylogenetic investigations at lower taxonomic levels.

AP3/*TM6* and *AP1* Orthologs in the Hawaiian Silversword Alliance

Homologs of the *Arabidopsis* floral homeotic genes *AP3* and *AP1* were successfully isolated from developing flowers of *A. sandwicense* subsp. *macrocephalum* utilizing RT-PCR techniques. The isolated *AP3* homolog cDNA is 837 bp long and contains a long open reading frame encoding a putative protein of 226 amino acids (aa) that encodes a MADS domain (see fig. 1). The protein shows strong similarity to the *Arabidopsis AP3* and *Antirrhinum DEFICIENS* proteins, with overall similarities of 84% and 88%, respectively, at the peptide level. Phylogenetic analysis indicates that the isolated gene is a member of the *AP3* group of genes (Purugganan et al. 1995; Purugganan 1997) and appears to be an ortholog of the tomato *TM6* locus (Kramer, Dorit, and Irish 1998). We refer to the gene as *ASAP3*/*TM6.*

A

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                1         10         20         30
ASAP3/TM6-A     MGRGR VETRK IENNT NRQVT YSKRR NGIFK
AP3             .A..K IQIKR ...Q. ..... ..... ..L..
DEF             .A..K IQIKR ...Q. ..... ..... ..L..
TM6             ???.K I.IK. ...S. ..... ..... .....

                31        40         50        57
ASAP3/TM6-A     KAHEL TVLCD AKVPL IMFSN TGKFH EY
AP3             ..... ..... .R.SI ....S SN.L. ..
DEF             ..... S.... ...SI ..I.S .Q.L. ..
TM6             .RK.. ..... ..IS. ..L.S .R.Y. ..
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B

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               1         10         20         30
ASAP1-A        MGRGK VQLRR IENKI NRQVT FSKRR GGLLK
AP1            ....R ...K. ..... ..... ..... A....
SQUA           ..... ...K. ..... ..... ..... .....

               31        40         50        57
ASAP1-A        KAHEI SVLCD AEVAL IVSSS KGKLF EF
AP1            ..... ..... ..... V.F.H ..... .Y
SQUA           ....L ..... ..... ..F.N ..... .Y
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FIG. 1.—MADS-box sequences of isolated floral homeotic gene orthologs. The amino acid sequences inferred from the *Argyroxiphium sandwicense* subsp. *macrocephalum* (*A*) *ASAP3*/*TM6-A* and (*B*) *ASAP1-A* cDNA sequences are shown and compared with homologs in *Arabidopsis thaliana* (*AP3, AP1*), *Antirrhinum majus* (*DEF, SQUA*), and *Lycopersicon esculentum* (*TM6*).

The isolated *A. sandwicense* subsp. *macrocephalum AP1* (or *ASAP1*) cDNA is 839 bp long, with an open reading frame encoding a putative protein of 231 aa and possessing a 57-aa MADS domain (see fig. 1). The encoded protein shows strong similarity (80%–85%) to the *Arabidopsis AP1* and *Antirrhinum SQUAMOSA* proteins, and phylogenetic analysis confirms that *ASAP1* is the ortholog of the *Arabidopsis AP1* locus (results not shown).

Duplicate Copies of the Floral Homeotic Genes in the Hawaiian Silversword Alliance

Using primers designed from cDNA sequences, *ASAP3*/*TM6* genomic sequences spanning exons 1–4 were amplified by PCR. Two bands of 1.1 and 1.4 kb, were obtained from species in the Hawaiian silversword alliance, suggesting that *ASAP3*/*TM6* is present in two distinct copies in each species. In contrast, only one PCR band was amplified from each North American tarweed species examined. The 1.1-kb fragment from the Hawaiian species is designated *ASAP3*/*TM6-A,* and the larger 1.4-kb product is *ASAP3*/*TM6-B.* We successfully isolated both gene copies of *ASAP3*/*TM6* from nine Hawaiian silversword alliance species, but only *ASAP3*/*TM6-A* from *Dubautia plantaginea.* The two gene copies from *A. sandwicense* subsp. *macrocephalum* are minimally divergent from one another (93% similarity). The introns of *ASAP3*/*TM6-A* and *ASAP3*/*TM6-B* have

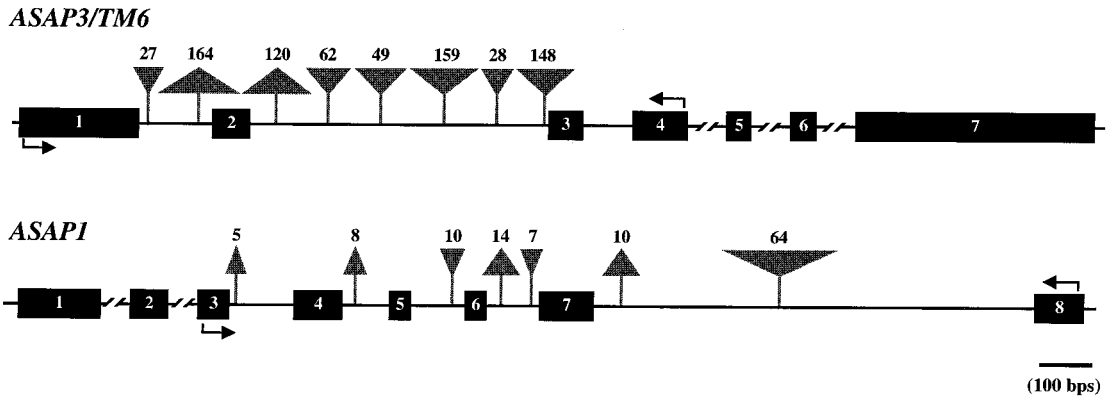

FIG. 2.—Gene maps of (*A*) *ASAP3*/*TM6* and (*B*) *ASAP1* loci. Exons are shown as numbered boxes. The gene maps depicted are for the *A* copies of the loci. Upright and inverted triangles represent major deletions and insertions, respectively, that characterize the *B* duplicate copies. Numbers above the triangles indicate the sizes of the indels. Arrows show positions of PCR primers used to isolate genomic sequences. A 100-bp scale bar is provided. Relative sizes of exons and introns in the amplified regions were derived from comparison of genomic and cDNA sequences. Exon sizes outside the amplified regions are estimates based on comparison with data from *Arabidopsis thaliana* orthologs.

diverged by 20 indels ranging in size from 1 to 369 bp (see fig. 2). In contrast, orthologous sequences within the *ASAP3*/*TM6-A* and *ASAP3*/*TM6-B* gene groups in the Hawaiian taxa are 99% and 97%–98% similar, respectively.

The *ASAP1* gene was also amplified using primers designed from cDNA sequences and (like *ASAP3*/*TM6*) was present in duplicate copies in the genomes of the Hawaiian species. PCR amplification of exons 3–8 of *ASAP1* yielded two copies that were both approximately 1.8 kb in length. In contrast, PCR amplification produced only one gene copy in each of the North American species examined. The duplicate copies of the gene in the Hawaiian species, which differ only slightly in size, are designated *ASAP1-A* and *ASAP1-B.* We successfully isolated both copies from nearly all Hawaiian silversword alliance species, with the exception of the *B* copy from *Dubautia laevigata* and *Dubautia latifolia.* We also isolated the *ASAP1* loci from a representative of each of the four major lineages within the North American *Madia*/*Raillardiopsis* group, as well as from *O. tenella*. As with *ASAP3*/*TM6-A* and *ASAP3*/*TM6-B*, the two gene copies of *ASAP1* from *A. sandwicense* subsp. *macrocephalum* show 93% similarity. The two gene copies are differentiated by 30 indels that range in size from 1 to 61 bp (see fig. 2). Orthologous sequences within the *ASAP1-A* and *ASAP1-B* gene groups in the Hawaiian taxa were 98%–99% and 95%–96% similar, respectively.

The presence of two distinct *ASAP3*/*TM6* and *ASAP1* genes in the Hawaiian species is not unexpected—as mentioned above, both cytogenetic (Carr and Kyhos 1986; Kyhos, Carr, and Baldwin 1990; Carr, Baldwin, and Kyhos 1996) and allozyme (Witter and Carr 1988) studies indicate that the Hawaiian species are tetraploids ($n = 13, 14$), in contrast to the basally diploid condition of each of the most closely related North American tarweed lineages ($n = 6–9$) (Baldwin 1996). We utilized a permissive PCR-based approach to examine whether the members of the North American *Madia*/*Raillardiopsis* group possessed only a single gene copy of these two floral homeotic loci. A PCR-based approach was necessitated by the difficulties in conducting DNA blot analyses with these species, which appear to have both large genomes and high methylation levels (unpublished data).

For permissive PCR, primers were designed based on the conserved regions of both *A* and *B* copies of each gene and used to amplify sequences in the *Madia*/*Raillardiopsis* species using permissive annealing temperatures (below primer melting temperature [5–10°C]). The design of these primers around conserved regions ensures the amplification of both gene copies if they are present in the North American taxa. This permissive PCR approach resulted in the detection of nine new sequences of >500 bp. Analyses of these novel amplicons against GenBank identified two of these sequences: (1) a *Tyl*/*copia*-like retrotransposon and (2) a plant extensin-like gene. The other seven sequences could not be identified from database searches. No duplicates of the *ASAP1* or *ASAP3*/*TM6* genes were detected in the *Madia* and *Raillardiopsis* genomes using this permissive PCR approach. Together with the previous cytogenetic and allozyme studies, these results suggest that these floral homeotic loci are present in only single copies in the immediate North American relatives of the Hawaiian silversword alliance.

ASAP3/*TM6* Gene Tree

Figure 3*A* depicts a gene phylogeny for the *ASAP3*/*TM6* loci obtained from maximum-parsimony analysis. The tree demonstrates that the *ASAP3*/*TM6-A* and *ASAP3*/*TM6-B* genes from the Hawaiian silversword alliance each form a monophyletic group. The *ASAP3*/*TM6-A* sequences group together with 83% bootstrap support, and the *ASAP3*/*TM6-B* copies constitute a monophyletic group with 100% support. The phylogeny of the loci also suggests that the presence of the *A* and *B* copies in the Hawaiian species is the result of evolutionary reticulation, rather than duplication following divergence from the North American tarweeds; the *A* and *B* copies are not resolved as sister to one another,

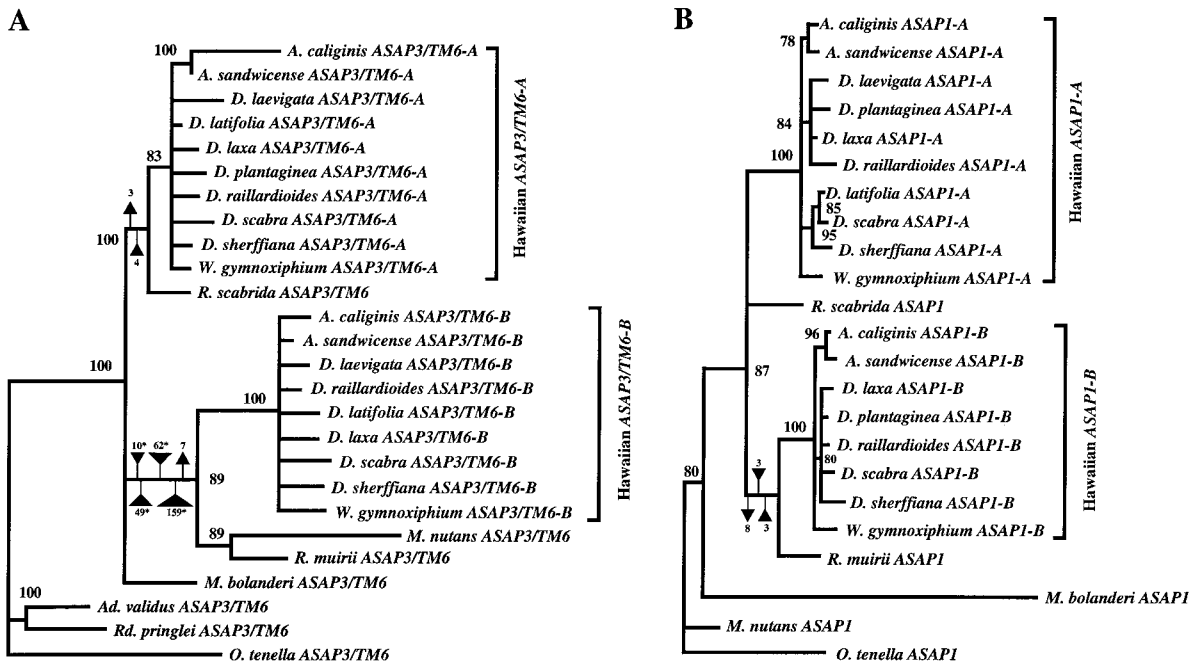

FIG. 3.—Phylogenetic reconstruction of (*A*) *ASAP3/TM6* (tree length = 343 steps, consistency index [CI] = 0.860) and (*B*) *ASAP1* loci (tree length = 211 steps, CI = 0.863) in the Hawaiian silversword alliance and North American tarweeds using maximum-parsimony analyses. Numbers next to the nodes give bootstrap support for branches. Indels (>1 bp) that support the grouping of *Raillardiopsis scabrida* and *Raillardiopsis muirii* with *A* and *B* gene copies are mapped onto the phylogeny. The starred indels in the *ASAP3/TM6* tree are those length variants found in both *R. muirii* and *ASAP3/TM6-B*, but not *Madia nutans*. Abbreviations: *A* = *Argyroxiphium*, *D* = *Dubautia*, *W* = *Wilkesia*, *R* = *Raillardiopsis*, *M* = *Madia*, *Ad* = *Adenothamnus*, *Rd* = *Raillardella*, and *O* = *Osmadenia*.

but rather as sister to genes from different species or species groups of North American tarweeds. The *ASAP3/TM6-A* genes appear to be closely related to the gene in *R. scabrida*, with the association showing 100% bootstrap support. In contrast, the *ASAP3/TM6-B* sequences appear most closely related to the sequences of *R. muirii* and *M. nutans*, with 89% bootstrap support. In the *ASAP3/TM6* phylogeny, the *R. muirii* and *M. nutans* genes also group together with 89% bootstrap support.

A large number of indels differentiate the *ASAP3/TM6* loci in the different species examined. A minimum of 176 indel events in the *ASAP3/TM6* introns can account for the length differences observed and scored for analysis. Many of the indels are found in only one species, but several appear to be shared by multiple taxa; e.g., the indels diagnosing the *ASAP3/TM6-A* and *ASAP3/TM6-B* gene lineages in the silversword alliance. The indels range in size from 1 bp to a large deletion of 1.2 kb restricted to *M. nutans*. Five microsatellite loci also contribute to some of the length variation.

Several indels provide support for distinct North American tarweed ancestries of the *ASAP3/TM6-A* and *ASAP3/TM6-B* genes (see fig. 3*A*). Two indels, a 4-bp insertion and a 3-bp deletion in intron sequences, unambiguously group the *R. scabrida* locus with the Hawaiian *ASAP3/TM6-A* sequences. Moreover, at least four indels, ranging in size from 10 to 159 bp, are shared between the *R. muirii* gene and the Hawaiian *ASAP3/TM6-B* sequences.

Length variation in *ASAP3/TM6* exhibits homoplasy that partly complicates identification of the North American tarweed gene lineages most closely related to those of the Hawaiian silversword alliance. None of the four length variants shared between the Hawaiian *ASAP3/TM6-B* sequences and the *R. muirii* gene is found in the *M. nutans* sequence, although a separate 7-bp deletion is shared by all three lineages. Three indels can also be found that are shared by the *ASAP3/TM6-B* genes and the sequences from *R. muirii* and *M. bolanderi*.

ASAP1 Gene Tree

The *ASAP1* gene provides an additional set of nuclear sequences for analysis of relationships between the Hawaiian silversword alliance and the North American tarweeds. Like the *ASAP3/TM6* gene, *ASAP1* was found in duplicate copies in the Hawaiian species, yet appears to be present in only one copy in the genomes of North American members of the *Madia/Raillardiopsis* group. Each of the copies found in the Hawaiian species, *ASAP1-A* and *ASAP1-B*, forms a monophyletic group with 100% bootstrap support (see fig. 3*B*). Moreover, a larger clade containing the genes of *R. scabrida* and *R. muirii* with both Hawaiian *ASAP1-A* and *ASAP1-B* loci is supported by bootstrap analysis at the 87% level. Thus, the *ASAP1* tree corroborates the finding from the *ASAP3/TM6* phylogeny that the genes of *R. scabrida* and *R. muirii* are more closely related to those of the

Hawaiian species than are the genes of other sampled species of the *Madia*/*Raillardiopsis* group (see fig. 3*B*).

Unlike the *ASAP3*/*TM6* phylogeny, however, the *ASAP1* phylogeny does not provide strong resolution of the relationships among the Hawaiian *ASAP1-A* and *ASAP1-B* genes and the *ASAP1* genes of *R. muirii* and *R. scabrida.* Several indels provide evidence for a lineage comprising the *R. muirii* gene and *ASAP1-B* sequences; three indels ranging in size from 3 to 8 bp are shared by only the *R. muirii ASAP1* gene and the *ASAP1-B* sequences. However, a 9-bp insertion is shared by the *R. muirii* gene and both Hawaiian gene lineages, and two small deletions place both the *R. muirii* and *R. scabrida ASAP1* sequences closer to the *ASAP1-A* lineage than to the *ASAP1-B* lineage. The incomplete resolution within the lineage of Hawaiian and *Raillardiopsis* genes may be due to the effects of homoplasy accompanying the high rate of molecular evolution of the *ASAP1* gene, which appears to evolve faster than the *ASAP3*/*TM6* locus (unpublished data).

A Hybrid (Allopolyploid) Ancestry of the Hawaiian Silversword Alliance

The origin of the Hawaiian silversword alliance, considered ''the best example of adaptive radiation in plants'' (Raven, Evert, and Eichorn 1992), was ill-defined until Carlquist (1959) suggested that the group was related to North American tarweeds. Molecular systematic investigations using both cpDNA restriction site data (Baldwin et al. 1991) and rDNA ITS sequences (Baldwin and Robichaux 1995; Baldwin 1997) have confirmed and extended Carlquist's (1959) hypothesis by showing that the Hawaiian species are phylogenetically nested within the paraphyletic *Madia*/*Raillardiopsis* group and originated after considerable diversification of the North American tarweeds (Baldwin 1996; Baldwin and Sanderson 1998). Previous investigations have not, however, determined whether the species of the silversword alliance are auto- or allopolyploids (see Baldwin 1997).

Our molecular phylogenetic analyses using two nuclear-encoded floral homeotic genes provide evidence that species in the Hawaiian silversword alliance are allopolyploids that descended from a hybrid between species of two extant lineages in the *Madia*/*Raillardiopsis* group. Based on the *ASAP3*/*TM6* analysis, the interspecific hybridization event in the ancestry of the Hawaiian silversword alliance involved members of lineages that include *R. muirii* (and perhaps *M. nutans*) and *R. scabrida.* The *ASAP1* analysis also suggests that the two species of *Raillardiopsis* are among the closest North American relatives of the silversword alliance. Moreover, the earlier finding that *R. muirii* cpDNA is more closely related to the Hawaiian cpDNAs than is *R. scabrida* cpDNA (Baldwin et al. 1991) suggests that a member of the lineage represented by *R. muirii* was the maternal parent of the hybrid ancestor of the silversword alliance (see fig. 4). The phylogenetic position of the lineage represented by *M. nutans* remains uncertain because of apparent topological conflict between the *ASAP3*/*TM6* and *ASAP1* trees. The *ASAP3*/*TM6* tree

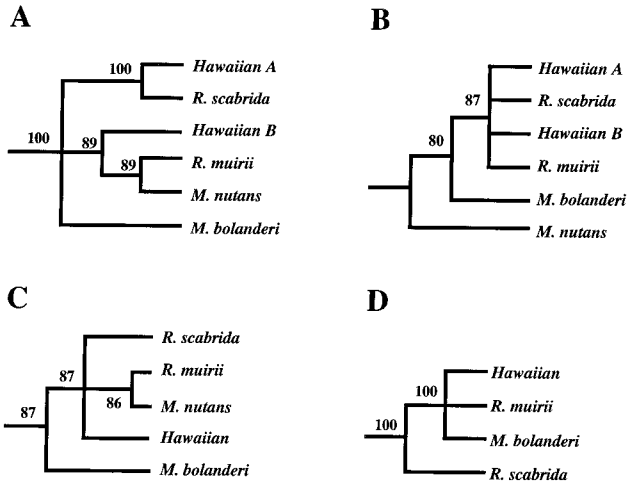

FIG. 4.—Comparison of nuclear and cpDNA trees of the Hawaiian silversword alliance and North American tarweeds. The trees summarize relationships resolved from analyses of (*A*) *ASAP3*/*TM6,* (*B*) *ASAP1,* (*C*) rDNA ITS, and (*D*) cpDNA restriction site data. Tree *C* is the result of a reanalysis of the rDNA ITS data (Baldwin 1992), with sampling restricted to the same set of ingroup taxa examined in the present study (unpublished data). Tree *C* is topologically congruent with published rDNA ITS trees of similar sampling (Baldwin 1992), except that *Madia stebbinsii* was included in the earlier study instead of the closely related *Madia nutans.* Tree D was published previously (Baldwin et al. 1991). Numbers next to the nodes give bootstrap support levels. All nodes with less than 50% support are shown collapsed.

placement of *M. nutans* and *R. muirii* as sister taxa receives support, however, from the rDNA ITS phylogeny, which is topologically congruent with the *ASAP3*/*TM6* results (fig. 4).

Phylogenetic analyses of nuclear genes that have not undergone interlocus concerted evolution have proven crucial to our identification of a hybrid ancestry of the silversword alliance. Both cpDNA and rDNA ITS analyses, which have provided the most widely used molecular data for investigations of plant phylogeny, can sometimes prove insufficient for identifying reticulate evolution (see fig. 4). Uniparental cpDNA trees are useful for aiding resolution of hybridization events only in comparison with phylogenetic data from the nuclear genome (e.g., Baldwin 1997). Analyses of rDNA ITS sequences in the Hawaiian silversword alliance did not reveal evidence of divergent or recombinant ITS copies within species (Baldwin and Robichaux 1995; Baldwin 1997; Baldwin and Sanderson 1998), a result that may be explained by rapid, unidirectional concerted evolution following the ancient hybridization event resolved here (Hillis et al. 1991; Baldwin and Sanderson 1998). Strongly conflicting signal between cpDNA and rDNA ITS data, another possible indication of hybrid ancestry, was not found from comparisons of the two character sets using the partition homogeneity test (Farris et al. 1995), as implemented in PAUP* (Baldwin 1992; unpublished data).

Plausibility of the Inferred Evolutionary Reticulation Based on Evidence from Artificial Hybrids

Although up to 5–6 Myr may have elapsed since the onset of diversification of the silversword alliance

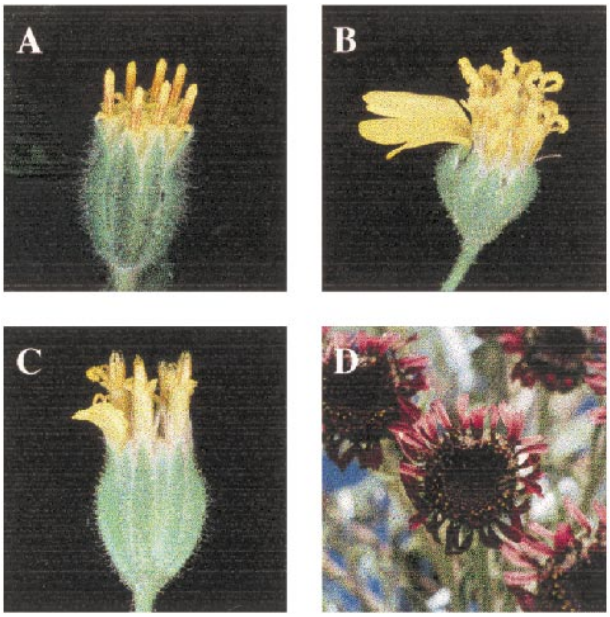

FIG. 5.—Flowering capitula of (*A*) *Raillardiopsis muirii*, (*B*) *Raillardiopsis scabrida*, and (*C*) their interspecific hybrid (*R. muirii* × *R. scabrida*). Note the presence of the ray flower with expanded limb in *R. scabrida*, the absence of ray flowers in *R. muirii*, and the presence of one ray flower with a short limb in the hybrid. The capitulum of the Haleakala silversword, *Argyroxiphium sandwicense* subsp. *macrocephalum* (*D*), is included for comparison.

(Baldwin and Sanderson 1998), enough genetic similarity remains between *R. muirii* and *R. scabrida*, representatives of the two apparent ancestral lineages of the Hawaiian group, to permit formation of hybrids (see fig. 5). A cross between *R. muirii* and *R. scabrida* yielded a vigorous hybrid individual of low fertility (13.5% pollen stainability) (Baldwin 1989). The high proportion of unstained, inviable pollen probably arose from the extensive meiotic failure observed at the chromosomal level (Baldwin 1989; Kyhos, Carr, and Baldwin 1990). One third of the stainable, presumably viable pollen grains in this hybrid were unusually large and tetraporate (rather than triporate), a condition correlated with unreduced ploidy in Asteraceae (Baldwin 1989; Kyhos, Carr, and Baldwin 1990). Although production of synthetic allopolyploids was not attempted, the *R. muirii* × *R. scabrida* individual (see fig. 5) displayed sufficient vigor and apparent diploid-pollen fertility to have promising prospects for generating an allopolyploid lineage. The hypothesis of an allopolyploid origin of the silversword alliance based on hybridization between lineages including *R. muirii* and *R. scabrida* thus appears to be biologically plausible.

Hybrids between members of the silversword alliance and each of four perennial species of the *Madia*/*Raillardiopsis* group have also been produced (Baldwin 1989; Kyhos, Carr, and Baldwin 1990; Baldwin et al. 1991; Carr, Baldwin, and Kyhos 1996). The intergeneric hybrid progeny between *R. muirii* and *D. laevigata* were vigorous with high pollen fertility (pollen stainability 49%), although most of the stainable grains were large, tetraporate, and presumably unreduced (Carr, Baldwin, and Kyhos 1996). A cross between *R. scabrida* and a *D. knudsenii* × *Dubautia laxa* hybrid produced a single plant, which died before flowering (Carr, Baldwin, and Kyhos 1996). Hybrids have also been produced between *D. knudsenii* and a *M. madioides* × *R. scabrida* hybrid. Based on results of rDNA ITS analysis (Baldwin 1996), *M. madioides* and *R. scabrida* are sister species belonging to the same lineage in the *Madia*/*Raillardiopsis* group. Hybrids between *D. knudsenii* and the *M. madioides* × *R. scabrida* hybrid were vigorous, with low pollen fertility (pollen stainability ca. 1%) and mostly univalents at meiotic metaphase I. Despite low fertility of the plants, the ability to produce these vigorous intergeneric hybrids indicates that the Hawaiian species and their closest relatives among North American tarweeds continue to retain considerable genetic similarity after millions of years of evolutionary divergence.

Genomic Constitution of the Hawaiian Founder and Extinction Implications

Our evidence for allopolyploidy of the silversword alliance leads us to conclude that the tarweed founder, not just the most recent common ancestor, of the Hawaiian group was an allopolyploid. Otherwise, we must hypothesize (1) ancient long-distance dispersal of two (diploid) North American tarweed species to the same location in the Hawaiian Islands, (2) hybridization and formation of an allopolyploid lineage in situ, and (3) subsequent extinction of the two diploid species in Hawaii (Baldwin 1997). The alternative scenario of dispersal of an allopolyploid species from western North America is simpler, requiring only one dispersal to the Hawaiian Islands and extinction of one lineage—the North American allopolyploid group (Baldwin 1997). Although multiple polyploid lineages are known within the $n = 8$ *Madia*/*Raillardiopsis* lineage, strongly-supported rDNA ITS relationships show that the extant North American polyploids most closely related to the Hawaiian species originated well after divergence of the silversword alliance from the North American tarweeds (Baldwin 1996; unpublished data). We conclude that despite the phenomenal evolutionary success of the silversword alliance ancestor in the Hawaiian setting, allopolyploids of the same genomic constitution in North America proved to be an evolutionary dead end. A similar allopolyploid extinction in western North America following successful long-distance dispersal (to South America) appears to have occurred in the Asteraceae genus *Blennosperma* (Ornduff 1961).

Genetic Consequences of Allopolyploidy in the Hawaiian Silversword Alliance

The inferred hybrid constitution of the Hawaiian founder may have promoted adaptive radiation of the silversword alliance. The presence of two divergent genomes in the colonizing ancestor of the Hawaiian group may have endowed the ancestor with more genetic variation and a greater ability to respond to selection (Jiang et al. 1998) than would be found in a diploid progenitor. Any intergenomic (allosyndetic) chromosome pairing that may have occurred in the early Hawaiian allopoly-

ploids would have allowed for wide genetic segregation and a broad array of progeny phenotypes (see Grant 1975), thereby conceivably enhancing the prospects for success in diverse island habitats. The impact of such extensive recombination on diversification of the silversword alliance may have been great, particularly in light of the ecological and morphological diversity of the North American lineages implicated in the formation of the hybrid ancestor of the Hawaiian group.

In general, the increased number of different loci correlated with allopolyploid formation provides novel avenues for molecular evolutionary and phenotypic divergence (Soltis and Soltis 1995). Allopolyploidy may impart upon plants a greater degree of genetic variation (Levin 1983; Soltis and Soltis 1995), increased redundancy of developmental pathways (Gibson and Spring 1998), and potential for structural and functional divergence of duplicated loci (Ohno 1970). We conclude that a variety of genetic factors associated with allopolyploidy may have been critically important in the spectacular adaptive radiation of the Hawaiian silversword alliance.

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LITERATURE CITED

AUSUBEL, F. M., R. BRENT, R. E. KINGSTON, D. D. MOORE, J. G. SEIDMAN, J. A. SMITH, and K. STRUHL. 1992. Short protocols in molecular biology. John Wiley, New York.

BALDWIN, B. G. 1989. Chloroplast DNA phylogenetics and biosystematic studies in Madiinae (Asteraceae). Ph.D. dissertation, University of California, Davis.

———. 1992. Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: an example from the Compositae. Mol. Phylogenet. Evol. **1**:3–16.

———. 1996. Phylogenetics of the California tarweeds and the Hawaiian silversword alliance (Madiinae: Heliantheae *sensu lato*). Pp. 377–391 *in* D. J. N. HIND and H. J. BEENTJE, eds. Compositae: systematics. Royal Botanic Gardens, Kew, England.

———. 1997. Adaptive radiation of the Hawaiian silversword alliance: congruence and conflict of phylogenetic evidence from molecular and non-molecular investigations. Pp. 103–128 *in* T. J. GIVNISH and K. J. SYTSMA, eds. Molecular evolution and adaptive radiation. Cambridge University Press, Cambridge, England.

BALDWIN, B. G., D. W. KYHOS, J. DVORAK, and G. D. CARR. 1991. Chloroplast DNA evidence for a North American origin of the Hawaiian silversword alliance (Asteraceae). Proc. Natl. Acad. Sci. USA **88**:1840–1843.

BALDWIN, B. G., and R. H. ROBICHAUX. 1995. Historical biogeography and ecology of the Hawaiian silversword alliance: new molecular phylogenetic perspectives. Pp. 259–287 *in* W. L. WAGNER and V. A. FUNK, eds. Hawaiian biogeography: evolution on a hot spot archipelago. Smithsonian Institution Press, Washington, D.C.

BALDWIN, B. G., and M. J. SANDERSON. 1998. Age and rate of diversification of the Hawaiian silversword alliance (Compositae). Proc. Natl. Acad. Sci. USA **95**:9402–9406.

CARLQUIST, S. 1959. Studies on Madiinae: anatomy, cytology and evolutionary relationships. Aliso **4**:171–236.

CARR, G. D. 1985. Monograph of the Hawaiian Madiinae (Asteraceae): *Argyroxiphium, Dubautia* and *Wilkesia*. Allertonia **4**:1–123.

———. 1998*a*. A new species of *Dubautia* (Asteracea-Madiinae) from Kaua'i, Hawaiian Islands. Novon **8**:4–7.

———. 1998*b*. Another new species of *Dubautia* (Asteraceae-Madiinae) from Kaua'i, Hawaiian Islands. Novon **8**:8–11.

CARR, G. D., B. G. BALDWIN, and D. W. KYHOS. 1996. Cytogenetic implications of artificial hybrids between the Hawaiian silversword alliance and North American tarweeds (Asteraceae, Heliantheae-Madiinae). Am. J. Bot. **83**:653–660.

CARR, G. D., and D. W. KYHOS. 1986. Adaptive radiation in the Hawaiian silversword alliance: II. Cytogenetics of artificial and natural hybrids. Evolution **40**:969–976.

DOYLE, J. J., and J. L. DOYLE. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem. Bull. **19**:11–15.

FARRIS, J. S., M. KALLERSJO, A. G. KLUGE, and C. BULT. 1995. Constructing a significance test for incongruence. Syst. Biol. **44**:570–572.

FRIAR, E. A., R. H. ROBICHAUX, and D. W. MOUNT. 1996. Molecular genetic variation following a population crash in the endangered Mauna Kea silversword, *Argyroxiphium sandwicense* ssp. *sandwicense* (Asteraceae). Mol. Ecol. **5**:687–691.

FROHMAN, M. A., M. DUSH, and G. R. MARTIN. 1988. Rapid production of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. Proc. Natl. Acad. Sci. USA **85**:8998–9002.

GIBSON, T. J., and J. SPRING. 1998. Genetic redundancy in vertebrates: polyploidy and persistence of genes encoding multidomain proteins. Trends Genet. **14**:46–49.

GIVNISH, T. J. 1997. Adaptive radiation and molecular systematics: issues and approaches. Pp. 1–54 *in* T. J. GIVNISH and K. J. SYTSMA, eds. Molecular evolution and adaptive radiation. Cambridge University Press, Cambridge, England.

GRANT, V. 1975. Genetics of flowering plants. Columbia University Press, New York.

HILLIS, D. M., C. MORITZ, C. A. PORTER, and R. J. BAKER. 1991. Evidence for biased gene conversion in concerted evolution of ribosomal DNA. Science **251**:308–310.

JIANG, C. X., R. J. WRIGHT, K. M. ELZIK, and A. H. PATTERSON. 1998. Polyploid formation created unique avenues for response to selection in *Gossypium* (cotton). Proc. Natl. Acad. Sci. USA **95**:4419–4424.

KRAMER, E. M., R. L. DORIT, and V. F. IRISH. 1998. Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. Genetics **149**:765–783.

KYHOS, D. W., G. D. CARR, and B. G. BALDWIN. 1990. Biodiversity and cytogenetics of the tarweeds (Asteraceae). Ann. Mo. Bot. Gard. **77**:84–95.

LEVIN, D. A. 1983. Polyploidy and novelty in flowering plants. Am. Nat. **122**:1–25.

LOWMAN, A. C., and M. D. PURUGGANAN. 1999. Duplication of the *Brassica oleracea APETALA1* gene and the evolution of domesticated cauliflower. J. Hered. (in press).

MA, H., M. F. YANOFSKY, and E. M. MEYEROWITZ. 1991. *AGL1-AGL6,* an *Arabidopsis* gene family with similarity to floral homeotic and transcription factor genes. Genes Dev. **5**:484–495.

MENA, M., B. A. AMBROSE, R. B. MEELEY, S. P. BRIGGS, M. F. YANOFSKY, and R. J. SCHMIDT. 1996. Diversification of the C-function activity in maize flower development. Science **274**:1537–1540.

OHNO, S. 1970. Evolution by gene duplication. Springer-Verlag, New York.

ORNDUFF, R. 1961. Experimental studies in two genera of Helenieae (Compositae): *Blennosperma* and *Lasthenia.* Q. Rev. Biol. **38**:141–150.

PALMER, J. D. 1986. Isolation and structural analysis of chloroplast DNA. Methods Enzymol. **118**:167–186.

PURUGGANAN, M. D. 1997. The MADS-box floral homeotic gene lineages predate the origin of seed plants: phylogenetic and molecular clock estimates. J. Mol. Evol. **45**:392–396.

PURUGGANAN, M. D., S. D. ROUNSLEY, R. J. SCHMIDT, and M. F. YANOFSKY. 1995. Molecular evolution of flower development—diversification of the plant MADS-box regulatory gene family. Genetics **140**:354–356.

RAVEN, P. H., R. F. EVERT, and S. E. EICHORN. 1992. Biology of plants. 5th ed. Worth, New York.

RIECHMANN, J. L., and E. M. MEYEROWITZ. 1997. MADS domain proteins in plant development. Biol. Chem. **378**:1079–1101.

ROBICHAUX, R. H., G. D. CARR, M. LIEBMAN, and R. W. PEARCY. 1990. Adaptive radiation of the Hawaiian silversword alliance (Compositae-Madiinae)—ecological, morphological and physiological diversity. Ann. Mo. Bot. Gard. **77**: 64–72.

ROUNSLEY, S. D., G. S. DITTA, and M. F. YANOFSKY. 1995. Diverse roles for MADS-box genes in *Arabidopsis* development. Plant Cell **7**:1259–1269.

SOLTIS, D. E., and P. S. SOLTIS. 1995. The dynamic nature of polyploid genomes. Proc. Natl. Acad. Sci. USA **92**:8089–8091.

SWOFFORD, D. L. 1998. PAUP* 4.0 beta. Sinauer, Sunderland, Mass.

WITTER, M. S., and G. D. CARR. 1988. Adaptive radiation and genetic differentiation in the Hawaiian silversword alliance (Compositae: Madiinae). Evolution **42**:1278–1287.

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