Accelerated regulatory gene evolution in an adaptive radiation

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The disparity between rates of morphological and molecular evolution remains a key paradox in evolutionary genetics. A proposed resolution to this paradox has been the conjecture that morphological evolution proceeds via diversification in regulatory loci, and that phenotypic evolution may correlate better with regulatory gene divergence. This conjecture can be tested by examining rates of regulatory gene evolution in species that display rapid morphological diversification within adaptive radiations. We have isolated homologues to the Arabidopsis APETALA3 (ASAP3/TM6) and APETALA1 (ASAP1) floral regulatory genes and the CHLOROPHYLL A/B BINDING PROTEIN9 (ASAC9) photosynthetic structural gene from species in the Hawaiian silversword alliance, a premier example of plant adaptive radiation. We have compared rates of regulatory and structural gene evolution in the Hawaiian species to those in related species of North American tarweeds. Molecular evolutionary analyses indicate significant increases in nonsynonymous relative to synonymous nucleotide substitution rates in the ASAP3/TM6 and ASAP1 regulatory genes in the rapidly evolving Hawaiian species. By contrast, no general increase is evident in neutral mutation rates for these loci in the Hawaiian species. An increase in nonsynonymous relative to synonymous nucleotide substitution rate is also evident in the ASCA9 structural gene in the Hawaiian species, but not to the extent displayed in the regulatory loci. The significantly accelerated rates of regulatory gene evolution in the Hawaiian species may reflect the influence of allopolyploidy or of selection and adaptive divergence. The analyses suggest that accelerated rates of regulatory gene evolution may accompany rapid morphological diversification in adaptive radiations.

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ates of morphological evolution are generally not correlated with rates of molecular evolution. This paradoxical observation was highlighted early by Wilson and coworkers (1–3), and subsequent molecular studies in species groups that have undergone recent adaptive radiations, such as African rift lake cichlids (4), columbines (5), and the Hawaiian silversword alliance (6), have documented marked incongruities in rates of morphological and molecular evolution. A proposed resolution to this paradox has been the conjecture that evolutionary changes in regulatory genes, rather than large-scale diversification in structural genes, may be responsible for interspecific variation in organismal morphologies (1–3, 7–9). This conjecture is reinforced by molecular developmental studies that indicate that dramatic shifts in organismal structure may arise from mutations at key regulatory loci (7–9). One test of this conjecture is to examine whether there is a significant acceleration in rates of regulatory gene evolution in species that display rapid morphological diversification within adaptive radiations.

The Hawaiian silversword alliance (Asteraceae: Heliantheae—Madiinae) is a premier example of plant adaptive radiation (6, 10, 11). The silversword alliance comprises 30 species of flowering stalk, the architectural organization of flowering stalks, and the sizes and morphologies of floral organs (11). Even sibling species that appear to have diverged less than 500,000 years ago on the Island of Hawai‘i display significant differences in quantitative inflorescence and floral characters (A. L. Lawton-Rauh, R.H.R., and M.D.P., unpublished observations).

Molecular phylogenetic analyses using chloroplast DNA and rDNA internal transcribed spacer (ITS) sequences (6, 10, 12) have confirmed an early hypothesis that the closest relatives of the Hawaiian silversword alliance can be found among North American tarweeds (Asteraceae: Heliantheae—Madiinae) in the “Madia” lineage (13). Both cytogenetic (14) and allozyme (15) data indicate that the Hawaiian species are tetraploids (n = 13–14), in contrast to the basally diploid condition (n = 6–9) in the most closely related North American species within the “Madia” lineage (13, 16). Results of our recent phylogenetic analyses using two floral homeotic genes have led us to conclude that the Hawaiian silversword alliance descended from an interspecific hybrid between members of the Anisocarpus scabridus and Carlquistia muirii lineages of North American tarweeds (16). Likelihood estimates based on sequence data from the rDNA ITS locus suggest that the most recent common ancestor of the Hawaiian species existed 5.2 ± 0.8 million years ago (mya), contemporaneous with the origin of the Island of Kaua‘i (17). By contrast, the earliest date for the diversification of the North American tarweeds appears to be in the mid-Miocene, 15 mya (17).

We have isolated homologues to the Arabidopsis APETALA3 (ASAP3/TM6) and APETALA1 (ASAP1) floral regulatory genes and the CHLOROPHYLL A/B BINDING PROTEIN9 (ASAC9) photosynthetic structural gene from species in the Hawaiian silversword alliance and from related species of North American tarweeds (16). Isolation of the genes from both the rapidly evolving Hawaiian species and their North American relatives provides an opportunity to compare rates of gene evolution between lineages that differ greatly in rates of morphological diversification. Molecular evolutionary analyses indicate significant increases in nonsynonymous relative to syn-
 DXF are copy-specific. Identity of amplified products was confirmed by sequencing.

Fig. 1. (A) Gene maps of ASAP3/TM6 and 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 provide the sizes of the indels. Arrows show positions of PCR primers used to isolate genomic sequences. Circles indicate approximate location of copy-specific RT-PCR primers used in gene expression assays. 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 A. thaliana orthologues. (B) Expression of the ASAP3/TM6 and ASAP1 floral regulatory genes in developing inflorescences of D. arborea. Expression was assayed with gene-specific primers (for the duplicate A and B copies) that amplified 300 nucleotides of cDNA in RT-PCR reactions. Control reactions using cloned A and B gene copies indicate the primers are copy-specific. Identity of amplified products was confirmed by sequencing.

Materials and Methods

Isolation of Genes. The ASAP3/TM6 and ASAP1 regulatory genes were isolated as described (16). The ASCAB9 structural gene was first identified as an expressed sequence tag (EST) in Argro-siphium sandwicense subsp. macropetalum. Ten Hawaiian species were selected to represent the four major lineages in the silversword alliance as previously identified from rDNA ITS trees (13). Six allopolyploidy or from selection and adaptive divergence.

Rate Analyses. Pairwise nonsynonymous (Ka) and synonymous (Ks) nucleotide substitutions in the coding regions of the ASAP3/TM6, ASAP1, and ASCAB9 loci were calculated according to the method of Nei and Gojobori (18), as implemented in MEGA (19). This method gives unbiased estimates when evaluating sequences of low divergences (20). Pairwise Ka/Ks values were calculated for orthologues of ASAP3/TM6, ASAP1, and ASCAB9. The individual pairwise Ka and Ks values reported here can be found in Tables 2–11, which are published as supplemental data on the PNAS web site, www.pnas.org. Significance values were estimated by using 1,000 two-sample bootstrap replicates generated from the joint distribution of Ka/Ks ratios for the Hawaiian and North American species pairs. The Tajima relative rate test was used to examine rate variation between genes in the Hawaiian species and their closest North American relatives, with C. pungens as an outgroup (21). This test has been shown to perform as well as maximum likelihood methods for noncoding region data. Gene phylogenes were reconstructed with PAUP* 4.0b5 (22).
Ancestral nucleotide character states were reconstructed by using BASEML in the PAML program package (23), with the HKY85 nucleotide substitution model (24), and assuming no molecular clock.

**Results and Discussion**

**Regulatory and Structural Genes in the Hawaiian and North American Species.** Homologues to the *Arabidopsis* APETALA3 and APETALA1 floral regulatory genes were isolated from developing flowers of *A. sandwicense* subsp. *macrocephalum*. The two genes are designated as ASAP3/TM6 and ASAP1, respectively (16). The two loci are members of the MADS-box family of plant transcriptional activators, many of which are known to regulate floral and inflorescence development in angiosperms (25). Phylogenetic analysis indicates that the *ASAP3*/TM6 gene is a member of the *AP1* floral homeotic gene group, and appears to be an orthologue of the Lycopersicon TM6 locus (26, 27). The *ASAP1* gene is an orthologue of the *Arabidopsis* AP1 locus. Genetic studies in *Arabidopsis thaliana*, *Antirrhinum majus*, and *Zea mays* indicate that the developmental functions of the floral regulatory genes exhibit broad conservation across the angiosperms (28). *AP1* orthologues control petal and stamen development, and *AP1* orthologues regulate the establishment of floral primordia and control sepal and petal organ identity (28). Molecular genetic studies in *Arabidopsis* further indicate that changes in the activity of *AP1* are correlated with variation in inflorescence branch number and flowering time (29), and QTL mapping studies show that changes in petal and stamen sizes in *Arabidopsis* map to a region that includes *AP3* (30). Because species in the silversword alliance vary in the number of flowers per capitula, flowering time, and the sizes of petals and stamens (11), both ASAP3/TM6 and ASAP1 are candidates for genes that underlie floral and inflorescence morphological and phenological variation among the Hawaiian species.

Gene fragments encompassing exons 1 to 4 of ASAP3/TM6 and exons 3 to 8 of ASAP1 were isolated and sequenced from the Hawaiian and North American species (16). The isolated fragments of the *ASAP3*/TM6 gene contain the coding region for the first 124 amino acids (aa) of the 227-aa encoded protein, including the MADS-box DNA-binding and putative K-box dimerization domains (25). The isolated fragments of the ASAP1 gene contain a region encoding 128 aa of the 242-aa protein, including the K-box and C-terminal domains. The C-terminal domain of MADS-box regulatory proteins is believed to contain the transcriptional activation domain (25). The isolation of different regions of the *ASAP3*/TM6 and ASAP1 genes allows sequence evolution to be examined across different domains of these transcriptional activators.

The *ASAP3*/TM6 and ASAP1 genes are present in duplicate copies (designated as the *A* and *B* copies) in the tetraploid Hawaiian species (16). Duplication of genes may lead to inactivation of one gene copy via pseudogene formation (31). RT-PCR assays demonstrate, however, that the *A* and *B* copies of both genes are transcriptionally active in developing inflorescences of *Dubautia* (see Fig. 1), indicating that pseudogene formation has not generally occurred at these regulatory loci. Only single copies of the genes have been detected in North American species within the “Madi” lineage. Phylogenetic analysis indicates that the *A* and *B* copies of the genes in the Hawaiian species are most closely related to genes in *A. scabridus* and *C. muirii* from North America (16). For the *ASAP3*/TM6 gene, the ranges of sequence divergence are 0.2–1.0% in the Hawaiian species (including both the *A* and *B* copies) and 1.7–5.7% in the North American species. For the *ASAP1* gene, the ranges of sequence divergence are 0.2–1.4% in the Hawaiian species and 2.1–5.2% in the North American species. Plots of estimated vs. observed nucleotide substitutions indicate that neither nonsynonymous nor synonymous sites at these loci are saturated (M.B. and M.D.P., unpublished observations).

The orthologue to the CHLOROPHYLL A/B BINDING PROTEIN9 photosynthetic structural gene was also isolated from *A. sandwicense* subsp. *macrocephalum*, and is designated as ASCAB9. The nuclear *Dubautia* gene encoding a mitochondrial-localized protein ~260 aa in length, which is a portion of the CP26/29 CAB precursor protein in photosystem II (32). A 1.2-kb genomic region encompassing intron 3 to the 3′ untranslated region (UTR) (and including exons 4 to 6) was isolated from ten species in the Hawaiian silversword alliance and six species of North American tarweeds. The Hawaiian species appear to possess three copies of the *ASCAB9* gene. Sequence analyses suggest that intergenic recombination between two of the copies (designated as the *A* and *B* copies) may have given rise to the third copy. All three copies were included in our analyses of rates of sequence evolution. Only one copy of the *ASCAB9* gene has been detected in each of the North American species. For the *ASCAB9* gene, the ranges of sequence divergence are 0.0–0.7% in the Hawaiian species and 0.1–7.4% in the North American species. Because of the small number of nucleotide changes at this locus, especially in the Hawaiian species, the phylogeny of the *ASCAB9* gene is not as highly resolved as the phylogenies of the *ASAP3*/TM6 and ASAP1 genes.

**Accelerated Gene Evolution in the Hawaiian Species.** Molecular evolutionary analyses reveal that the *ASAP3*/TM6 and ASAP1 regulatory genes in species of the Hawaiian silversword alliance are evolving faster than their orthologues in the North American tarweed species. This accelerated evolution is evident when comparing the relative levels of nonsynonymous and synonymous nucleotide substitutions in the coding regions of these loci. Protein-encoding genes evolving at the neutral rate have Ka/Ks ratios equal to 1 (33). Most genes, however, are subject to strong purifying selection, resulting in lower nonsynonymous relative to synonymous substitution rates (i.e., Ka/Ks < 1) (33). The mean Ka/Ks values among orthologues for the *ASAP3*/TM6 and ASAP1 loci in the North American species are 0.12 ± 0.11 (mean ± SD) and 0.29 ± 0.12, respectively (see Fig. 2). These values are comparable to the mean Ka/Ks value of 0.14 observed for several other plant nuclear loci, and 0.11–0.19 observed for MADS-box floral homeotic genes from other species (34).

In contrast to the loci in the North American species, the floral regulatory genes display elevated Ka/Ks ratios in the Hawaiian species. The *ASAP3*/TM6 gene has a mean Ka/Ks value of 0.79 ± 0.52, and the ASAP1 gene has a mean Ka/Ks value of 0.98 ± 0.64 (see Fig. 2). Because there are two copies of each gene in the Hawaiian species, Ka/Ks ratios were calculated separately for orthologues of the *A* and *B* copies (see Tables 2–7); Fig. 2 reports the joint distribution of Ka/Ks ratios for both copies in the Hawaiian species. For each gene, the mean Ka/Ks values for the duplicate copies, when calculated separately, are similar. The increases in Ka/Ks values for the regulatory loci in the Hawaiian species are significantly different from that expected by chance, as assessed by a bootstrap resampling test (*P < 0.001*). Moreover, all but two pairwise comparisons among the genes in the North American species have Ka/Ks < 0.5. By contrast, nearly 30% of the pairwise comparisons among the genes in the Hawaiian species have Ka/Ks > 1. For example, eight of the nine coding region changes between the isolated ASAP1-A gene regions of *Dubautia scabra* and *Dubautia longipedicellata* are nonsynonymous substitutions. High Ka/Ks values have previously been observed for genes under diversifying or directional selection, such as the self-incompatibility, gamete recognition, and MHC loci (35–37).

Unlike the two regulatory genes, the *ASCAB9* structural gene does not exhibit a substantial increase in Ka/Ks ratios in the Hawaiian species.
Hawaiian species. The mean Ka/Ks values for the ASCAB9 gene in the North American and Hawaiian species are 0.14 ± 0.17 and 0.21 ± 0.30, respectively (see Fig. 2 and Tables 8–11). The mean Ka/Ks values do not include pairwise comparisons that have no synonymous substitutions. Reflecting the lower levels of sequence divergence at the ASCAB9 locus, several pairwise comparisons, especially among the Hawaiian species, lack synonymous substitutions, although some have one nonsynonymous substitution.

Accelerated Rate Confined to the Hawaiian Species. Accelerated evolution of the proteins encoded by the floral regulatory genes appears to be confined to the Hawaiian silversword alliance. The number of coding region substitutions was inferred along each branch of the ASAP3/TM6 and ASAP1 gene phylogenies by using maximum likelihood ancestral state reconstructions under the HKY85 model of nucleotide substitutions (see Fig. 3). The total number of nonsynonymous and synonymous substitutions inferred within the gene phylogenies was then partitioned between the Hawaiian and North American species. For both the ASAP3/TM6 and ASAP1 regulatory genes, the number of nonsynonymous substitutions exceeds the number of synonymous substitutions inferred along branches of the gene phylogenies circumscribed by the Hawaiian silversword alliance (see Table 1).

Neutral Mutation Rates Do Not Display Significant Acceleration in the Hawaiian Species. The large increases in nonsynonymous relative to synonymous nucleotide substitution rates in the ASAP3/TM6 and ASAP1 loci in the Hawaiian species are not correlated with a general acceleration of the neutral mutation rate. Relative rate tests (21) indicate that the regulatory loci do not display a significant increase in nucleotide substitution rates for the largely synonymous third codon positions in the Hawaiian species compared with the North American Anisocarpus and Carlquistia species (Tajima’s test, P = 0.007), with the former having a higher proportion of nonsynonymous substitutions (see Table 1). Unlike the two regulatory genes, however, the ASCAB9 structural gene does not have an excess of nonsynonymous over synonymous substitutions along branches of the gene phylogenies circumscribed by the Hawaiian silversword alliance.

Fig. 2. Distribution of Ka/Ks values for the ASAP3/TM6, ASAP1, and ASCAB9 genes in the North American tarweeds and Hawaiian silversword alliance. Mean values and standard deviations are indicated. Pairwise comparisons that had no synonymous substitutions are not shown in the histograms and were not included in the analyses. Individual pairwise Ka and Ks estimates are shown in Tables 2–11, which are published as supplemental data on the PNAS web site.
The numbers of nonsynonymous and synonymous nucleotide substitutions in the coding regions were inferred along branches of the gene phylogenies using maximum likelihood ancestral state reconstructions (see Fig. 3). Inferred substitutions along the phylogenetic branches at the bases of the A and B copies of the ASAP3/TM6 and ASAP1 loci in the Hawaiian species (denoted in black in Fig. 3) were not included in the analyses, as the substitutions may have occurred in genes of either North American or Hawaiian species. HI, Hawaiian; NA, North American. Significance was calculated by using Fisher's Exact Test (**, P < 0.01; ***, P < 0.001).

**Table 1. Contingency analyses of coding region substitutions in the Hawaiian and North American genes**

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<td>Nonsynonymous</td>
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<td>7 3</td>
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<tr>
<td>Synonymous</td>
<td>15 31</td>
<td>11 64</td>
<td>9 31</td>
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The numbers of nonsynonymous and synonymous nucleotide substitutions in the coding regions were inferred along each branch by maximum likelihood ancestral state reconstructions are given as a ratio (N/S). Levels of bootstrap support are shown next to the nodes. Nodes with less than 70% bootstrap support, and with no coding region nucleotide substitutions inferred along the subtending branch, are collapsed. For the North American species, the generic abbreviations are: A, Anisocarpus; Ad, Adenothamnus; Ca, Carlquistia; Ca, Calycadenia; Ce, Centromadia; D, Deinandra; H, Harmonia; K, Kyhosia; M, Madia; O, Osmadenia; and R, Raillardella. For the Hawaiian species, the generic abbreviations are: A, Argyroxiphium; D, Dubautia; and W, Wilkesia.
the key factor influencing regulatory gene evolution during the adaptive radiation.

Many of the pairwise interspecific Ka/Ks values for the AS4P3/TM6 and AS4P1 loci in the Hawaiian species are greater than 1 (see Fig. 2), which strongly suggests that selection and adaptive divergence may have operated to shape the structure of these transcriptional activators (35–37). The Hawaiian species differ greatly in a suite of reproductive traits, including floral organ size and morphology, inflorescence size, and capitulescence architecture (11). Diversifying or directional selection, acting on variation in these and other reproductive traits, may have led to adaptive divergence of the floral regulatory genes. Further studies may help to clarify whether specific molecular changes at these candidate regulatory genes underlie variation in specific reproductive traits.

In summary, our results suggest that rapid morphological diversification during the adaptive radiation of the Hawaiian silversword alliance has been accompanied by accelerated evolution of genes that regulate developmental processes. Thus, our results may help to resolve a key paradox in evolutionary genetics. Whereas rates of morphological evolution are generally not correlated with rates of structural gene evolution (1–3), they may be correlated with rates of regulatory gene evolution.

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