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# ADAPTIVE RADIATION AND REGULATORY GENE EVOLUTION IN THE HAWAIIAN SILVERSWORD ALLIANCE (ASTERACEAE)[1]

*Michael D. Purugganan[2] and Robert H. Robichaux[3]*

## ABSTRACT

The Hawaiian silversword alliance is a premier example of adaptive radiation, and serves as a model for exploring the genetic basis of morphological diversification in plants. Molecular analyses at macroevolutionary and microevolutionary scales suggest that rapid and extensive diversification in reproductive and vegetative form in the silversword alliance may be linked to alterations in the tempo and mode of regulatory gene evolution. Unfortunately, the silversword alliance also epitomizes the conservation challenges confronting the biotas of oceanic archipelagos, especially the threats posed by alien species. Taking action now to prevent extinction is key to further exploring adaptive radiation and its link to genome evolution.

*Key words:* adaptive radiation, *APETALA1, APETALA3,* extinction, *GAI,* Hawaiian silversword alliance, regulatory gene evolution.

## ADAPTIVE RADIATION, WILSON'S CONJECTURE, AND THE GENETIC BASIS OF MORPHOLOGICAL DIVERSIFICATION

Adaptive radiation is a key process in the evolution of life's diversity (Carlquist, 1974; Givnish & Sytsma, 1997; Schluter, 2000). Lineages undergoing adaptive radiation are especially prominent in the biotas of oceanic archipelagos and continental rift lakes, where conditions appear to favor unusually high rates of evolutionary innovation. Well-known examples include the Galapagos finches (Lack, 1947; Grant, 1986), Hawaiian honeycreepers (Tarr & Fleischer, 1995), Hawaiian drosophilids (Carson & Kaneshiro, 1976; Kaneshiro et al., 1995), Hawaiian silversword alliance (Carr, 1985; Robichaux et al., 1990), and African rift lake cichlids (Meyer et al., 1990). Such lineages offer a special opportunity for exploring the genetic basis of morphological diversification. The recency and extensiveness of the diversification enhance the prospects for detecting the underlying molecular genetic signatures, thereby allowing for direct comparison of the tempo and mode of morphological and molecular evolution.

Paradoxically, rates of morphological evolution are generally not correlated with rates of molecular evolution (Wilson et al., 1974; King & Wilson, 1975), an early observation reinforced by more recent studies on the evolution of structural genes in lineages exhibiting adaptive radiation. The cytochrome b and mitochondrial control regions of African rift lake cichlids, for example, do not exhibit any significant degree of interspecific molecular divergence despite the spectacular diversity in morphology (and coloration) among the species (Meyer et al., 1990). Such limited differentiation in structural genes is also evident in other lineages exhibiting adaptive radiation (Witter & Carr, 1988; Hodges & Arnold, 1994) and suggests that structural gene evolution may not be the key to rapid and extensive diversification in organismal form.

This apparent paradox led Allan Wilson and others to propose that morphological diversity among species arises primarily from regulatory gene evolution within developmental systems rather than from structural gene evolution (Wilson et al., 1974; King & Wilson, 1975; Wilson, 1976). This conjecture is supported indirectly by comparative developmental work in a number of taxa, which reveals that diversity in form among species arises by transformation of ontogenetic trajectories (Patel, 1994; Carroll, 1995; Warren et al., 1995; Lowe & Wray, 1997). These changes in trajectories result in

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evolutionary innovations in morphology.

Regulatory genes represent a class of loci that control the expression of other genes (Doebley, 1993; Purugganan, 1998). These genes play central roles in the genetic architecture of eukaryotic development, since they guide ontogenetic trajectories and thus specify organismal morphology (Meyerowitz, 1997). Developmental genetic studies in plants have demonstrated that classes of regulatory genes control the differentiation of various reproductive and vegetative structures (Meyerowitz, 1997; McSteen & Hake, 1998). Thus, there has been increasing interest in understanding the evolution of regulatory genes, many of which have been shown to encode sequence-specific, DNA-binding transcriptional activators (Doebley, 1993; Purugganan, 1998). Indeed, if Wilson's conjecture is correct, an assessment of the forces shaping regulatory gene evolution may prove critical to understanding adaptive radiation.

Studies of regulatory gene evolution have focused primarily on macroevolutionary patterns of gene diversification (Purugganan, 1998; Cronk, 2001). Recent phylogenetic analyses of several regulatory gene families reveal that most developmental systems evolve by duplication and divergence of paralogous loci (Sidow, 1992; Atchley et al., 1994; Ruddle et al., 1994; Purugganan et al., 1995; Theissen et al., 1996; Bharathan et al., 1997; Purugganan, 1997). Moreover, regulatory genes appear to evolve faster than typical structural loci (Purugganan & Wessler, 1994; Purugganan, 1998; Ting et al., 1998), and these elevated molecular evolutionary rates may be associated with developmental alterations and morphological diversification. Variation in the expression of regulatory genes has also been observed among divergent lineages. Orthologues of genes that control embryonic development in *Drosophila melanogaster* Meigen, for example, have been examined in a number of insect and crustacean taxa (Patel, 1994; Carroll, 1995; Warren et al., 1995). The studies reveal expression pattern diversity that is correlated with intertaxon variation in developmental patterns and morphological features.

Patterns of regulatory gene evolution evident at the macroevolutionary scale must have their origins in molecular variation that occurs within and among populations. Investigating patterns of variation at the population level, where evolutionary forces play themselves out to determine the fates of alternate alleles (Doebley, 1993; Palopoli & Patel, 1996; Purugganan, 1998, 2000), is thus key to understanding the evolutionary dynamics of these developmental control genes. A molecular population genetic approach provides a framework for assessing how selection and other factors affect variation at regulatory loci, and for delineating mechanisms that lead to evolutionary diversification (Purugganan, 2000).

Recent work in *Arabidopsis thaliana* (L.) Heynh., *Zea mays* L., and *Drosophila melanogaster* suggests that regulatory genes can harbor significant levels of molecular variation (Gibson & Hogness, 1996; Hanson et al., 1996; Richter et al., 1997; Ting et al., 1998; Wang et al., 1999; Olsen et al., 2002), and that selective (Doebley et al., 1997; Purugganan & Suddith, 1998), demographic (Purugganan & Suddith, 1999), and recombinational (Doebley et al., 1997) forces interact to pattern variation at these loci. Analysis of the *Arabidopsis* Heynh. floral developmental pathway reveals contrasting patterns of selection in the coding regions of floral regulatory loci (Olsen et al., 2002). Moreover, there appears to be evidence for selection in the promoter region of at least one gene, the inflorescence architecture gene *TFL1* (Olsen et al., 2002). Analysis of the *tba1* locus in *Zea* L. also highlights the role of selection in shaping promoter variation at a regulatory gene during the evolution of domesticated maize (Wang et al., 1999). In addition, molecular variation at this locus, as well as at *Arabidopsis CAL* (Purugganan & Suddith, 1998) and *Drosophila* Fallén *Scabrous* (Lai et al., 1994), *Delta* (Long et al., 1998), and *Ultrabithorax* (Gibson & Hogness, 1996) loci, is associated with functional variation in regulatory gene activity. These and related studies have begun to shed light on the evolutionary forces that determine the extent and patterning of regulatory gene variation and the phenotypic consequences of diversification at the molecular level.

## MORPHOLOGICAL DIVERSIFICATION AND REGULATORY GENE EVOLUTION IN THE HAWAIIAN SILVERSWORD ALLIANCE

The Hawaiian silversword alliance is a premier example of adaptive radiation (Carr, 1985; Robichaux et al., 1990). The silversword alliance includes 30 species in three endemic genera: *Argyroxiphium* DC., *Dubautia* Gaudich., and *Wilkesia* A. Gray. The species grow in habitats as varied as exposed lava, dry scrub, dry woodland, mesic forest, wet forest, and bog, and range in elevation from less than 100 m to more than 3700 m. In addition to their great ecological diversity, the species exhibit exceptional morphological diversity, extending to most aspects of reproductive and vegetative form.

With respect to reproductive traits (Carr, 1985), the species differ greatly in head (or capitulum) and flowering stalk (or capitulescence) architectures. All *Wilkesia* and *Dubautia* species have discoid heads, whereas all *Argyroxiphium* species have radiate heads. Head sizes vary from as few as 2 flowers per head (e.g., in *D. pauciflorula* H. St. John & G. D. Carr) to as many as 650 flowers per head (e.g., in *A. sandwicense* DC. subsp. *macrocephalum* (A. Gray) Meyrat). Flowering stalks vary in shape and number of heads, ranging from as few as 3 heads per flowering stalk (e.g., in *D. ciliolata* (DC.) D. D. Keck subsp. *glutinosa* G. D. Carr) to as many as 600 heads per flowering stalk (e.g., in *A. sandwicense* subsp. *macrocephalum*). The large flowering stalks of *Argyroxiphium* and *Wilkesia* species tend to dominate plants in flower. Floral organs also exhibit qualitative and quantitative differences among the species, including variation in corolla, pappus, and ovary sizes and style morphologies. In addition, the species differ in reproductive mode, with most being polycarpic, but some (e.g., *A. sandwicense* subsp. *macrocephalum* and *W. gymnoxiphium* A. Gray) being predominantly monocarpic.

With respect to vegetative traits (Carr, 1985; Carr et al., 1989; Robichaux et al., 1990), the species exhibit a wide range of growth forms. All *Argyroxiphium* and *Wilkesia* species are rosette shrubs, with the rosettes being sessile in some species (e.g., *A. caliginis* C. N. Forbes) and elevated on woody stems in others (e.g., *W. gymnoxiphium*). Most *Dubautia* species are shrubs, though they range from large, erect forms (e.g., *D. waianapanapaensis* G. D. Carr) to small, spreading forms (e.g., *D. scabra* (DC.) D. D. Keck subsp. *scabra*) and cushion forms (e.g., *D. waialealae* Rock). One *Dubautia* species is a liana (*D. latifolia* (A. Gray) D. D. Keck), and three can grow as small trees (*D. arborea* (A. Gray) D. D. Keck, *D. knudsenii* Hillebr. subsp. *knudsenii*, and *D. reticulata* (Sherff) D. D. Keck). The species also differ greatly in leaf shape, structure, and size. For example, leaves range from as small as 5 mm in length (e.g., in *D. ciliolata* subsp. *ciliolata*) to as large as 500 mm in length (e.g., in *W. gymnoxiphium*).

Molecular phylogenetic analyses provide compelling evidence that the silversword alliance is monophyletic, having descended from species in the "Madia" lineage of North American tarweeds (Baldwin et al., 1991; Baldwin & Robichaux, 1995; Baldwin, 1996, 2003). Cytogenetic data indicate that the Hawaiian species are tetraploids, in contrast to the basally diploid condition in the most closely related species within the "Madia" lineage (Carr & Kyhos, 1981, 1986; Baldwin, 1996; Carr, 2003). Recent analyses of floral homeotic gene duplications further indicate that the Hawaiian species are allotetraploids, deriving from an ancient interspecific hybridization event involving species in the two lineages containing the modern *Anisocarpus scabridus* (Eastw.) B. G. Baldwin and *Carlquistia muirii* (A. Gray) B. G. Baldwin, respectively (Barrier et al., 1999, 2001). Compared to species in the silversword alliance, the North American tarweed species exhibit substantially less morphological and ecological diversity.

The extensive diversification of the silversword alliance appears to have occurred rapidly (Baldwin, 2003). Based on a calibrated rate-constant ITS phylogeny (or "chronogram"), Baldwin and Sanderson (1998) estimated a maximum age for the most recent common ancestor of the silversword alliance of 5.2 ± 0.8 million years, which closely approximates the age of Kauai, the oldest current high island in the archipelago. Using lineage-branching times based on the calibrated ITS phylogeny, Baldwin and Sanderson (1998) further estimated a minimum rate of diversification of 0.56 ± 0.17 species per million years, which is high compared to continental lineages undergoing diversification (Baldwin, 2003).

## EVOLUTION OF GENES REGULATING REPRODUCTIVE DEVELOPMENT

The molecular genetics of plant reproductive development has been the subject of intense study in recent years (Yanofsky, 1995; Liljegren & Yanofsky, 1996), such that more is likely known about the genetics of floral development than that of any other plant developmental system. Much of this work has been carried out in *Arabidopsis thaliana*, where several genes that regulate floral development have been identified. These regulatory loci have been referred to as floral homeotic genes, with mutational lesions in the genes resulting in the formation of aberrant organ types in flowers. These loci include floral meristem identity genes such as *APETALA1* (*AP1*) (Gustafson-Brown et al., 1994; Mandel et al., 1992; Bowman et al., 1993; Irish, 1998), and floral organ identity genes such as *APETALA3* (*AP3*) (Bowman et al., 1991; Jack et al., 1992; Irish & Yamamoto, 1995).

The *Arabidopsis AP1* gene controls the specification of floral meristem identity as well as sepal and petal organogenesis (Bowman et al., 1993; Irish, 1998). Mutants at this locus display loss of sepals and petals and conversion of floral meristems into partial inflorescence-like shoots (Bowman et al., 1993; Irish, 1998). The *Arabidopsis AP3* gene is a floral organ identity gene that regulates petal and stamen development; mutants at this locus display homeotic transformation of these organs to sepaloid and carpelloid structures (Bowman et al., 1991). Quantitative trait locus (QTL) mapping studies further show that changes in petal and stamen sizes in *Arabidopsis* map to a region that includes *AP3* (Juenger et al., 2000). The function of the related *AP3*-like *Lycopersicon* Miller *TM6* paralogue is unknown, although its expression pattern is consistent with roles in petal and stamen development (Pnueli et al., 1991; Kramer et al., 1998). Orthologues in the Asteraceae to both *AP1* and *AP3* have been isolated in *Gerbera* L., and these loci appear to have functions similar to their *Arabidopsis* counterparts (Yu et al., 1999).

The *AP1* and *AP3* genes are members of the MADS-box regulatory gene family (Riechmann & Meyerowitz, 1997). Members of this gene family are characterized by the presence of a highly conserved 57-amino acid MADS-box, which is also found in human (*SRF*), *Drosophila* (*MEF2C*), and yeast (*MCM1*) transcriptional activators (Purugganan et al., 1995; Riechmann & Meyerowitz, 1997; Alvarez-Buylla et al., 2000). Plant MADS-box genes also contain a moderately conserved 70-amino acid domain called the K-box, which is believed to possess the capacity to form coiled-coil structures. Two other regions (the I- and C-regions) are also found in the encoded proteins, but are poorly conserved in comparisons among different plant MADS-box genes (Purugganan et al., 1995; Riechmann & Meyerowitz, 1997). Functional studies of AP1 and AP3 proteins indicate that plant MADS-box proteins consist of two functional domains: (i) a core domain (MADS-box, I-region, and the 5′ portion of the K-box) involved in DNA-binding and dimerization functions, and (ii) a non-core domain (3′ end of the K-box and the C-region) that may function as the transcriptional activation domain (Riechmann et al., 1996; Krizek & Meyerowitz, 1996; Riechmann & Meyerowitz, 1997).

Recent evidence suggests that rapid morphological diversification in the silversword alliance has been accompanied by an accelerated rate of floral regulatory gene evolution (Barrier et al., 2001). Homologues to *AP1* and *AP3* (designated as *ASAP1* and *ASAP3/TM6*, respectively) have been isolated from developing flowers of *Argyroxiphium sandwicense* subsp. *macrocephalum*. Molecular phylogenetic analyses indicate that the *ASAP1* gene is an orthologue of the *Arabidopsis AP1* locus. The analyses further indicate that the *ASAP3/TM6* gene is a member of the *AP3* floral homeotic gene group, and appears to be an orthologue of the *Lycopersicon TM6* locus (Pnueli et al., 1991; Kramer et al., 1998). The *ASAP1* and *ASAP3/TM6* genes are present in duplicate copies (designated as the *A* and *B* copies, respectively) in the tetraploid Hawaiian species (Barrier et al., 1999), with both copies being transcriptionally active in developing heads (Barrier et al., 2001). By contrast, only single copies of the genes have been detected in North American tarweed species within the "Madia" lineage.

Molecular evolutionary analyses reveal that the *ASAP1* and *ASAP3/TM6* regulatory genes in species of the silversword alliance are evolving faster than their orthologues in the North American tarweed species (Barrier et al., 2001). Ratios of the number of nucleotide substitutions per nonsynonymous site to the number per synonymous site (or Ka/Ks ratios) in the coding regions of the genes are significantly higher among the Hawaiian species than among the North American species. For *ASAP1*, the mean Ka/Ks ratios are 0.98 ± 0.64 and 0.29 ± 0.12 for the Hawaiian and North American species, respectively. For *ASAP3/TM6*, the mean Ka/Ks ratios are 0.79 ± 0.52 and 0.12 ± 0.11 for the Hawaiian and North American species, respectively. (For both loci, the increases in Ka/Ks ratios among the Hawaiian species are significantly different from that expected by chance, as assessed by a bootstrap resampling test [$P < 0.001$].) Many of the pairwise interspecific Ka/Ks values for the *ASAP1* and *ASAP3/TM6* genes in the Hawaiian species are greater than 1, which suggests that positive selection and adaptive divergence may have operated to shape the structure of these transcriptional activators.

Unlike the floral regulatory genes, structural genes in the silversword alliance do not appear to exhibit substantially accelerated rates of evolution. For the *ASCAB9* structural gene, for example, the mean Ka/Ks ratios are 0.21 ± 0.30 and 0.14 ± 0.17 for the Hawaiian and North American species, respectively (Barrier et al., 2001). (The *ASCAB9* gene is an orthologue of the *Arabidopsis CHLOROPHYLL A/B BINDING PROTEIN9* photosynthetic structural gene.) The latter results are in general agreement with previous studies indicating limited genetic differentiation in structural allozyme loci among the Hawaiian species (Witter & Carr, 1988).

Species in the silversword alliance also exhibit variation in the *ASAP1* and *ASAP3/TM6* regulatory genes at the population level (Lawton-Rauh et al., 2003). Molecular population genetic analyses of the genes in *Argyroxiphium sandwicense* subsp. *macrocephalum* and *Dubautia ciliolata* subsp. *glutinosa* reveal significant levels of polymorphism in the introns, especially for the *ASAP1-A*, *ASAP1-B*, and *ASAP3/TM6-A* loci. Within the coding regions of the loci, the type and distribution of polymorphic sites suggest a signature consistent with non-neutral protein evolution. Though polymorphism levels tend to be low in the coding regions, most coding region diversity occurs as nonsynonymous nucleotide substitutions in a majority of the loci. (Across all loci for the two species, there was a total of 14 nonsynonymous and only 3 synonymous substitutions.) The latter trend observed at the microevolutionary scale is consistent with the pattern observed at the macroevolutionary scale, as discussed above (Barrier et al., 2001).

Although the population-level patterning of polymorphic sites in the coding regions of the regulatory genes suggests the action of positive selection, statistical tests for selection cannot reject the possibility that the loci are evolving neutrally (Lawton-Rauh et al., 2003). Given the low number of polymorphic sites in the coding regions, the lack of detectable deviation from neutral-equilibrium expectations may not be surprising. With a low number of polymorphic sites, the commonly used tests for selection tend to have reduced power to reject neutral-equilibrium expectations. Further analyses should help to clarify the role that selective, demographic, and other forces play in shaping variation in the floral regulatory genes at the population level.

### EVOLUTION OF GENES REGULATING VEGETATIVE DEVELOPMENT

A variety of genes affecting shoot growth and form have been identified in *Arabidopsis* and other species (Purugganan et al., 2003). Many of the genes encode transcriptional activators that directly regulate expression of downstream target genes. One group of growth regulatory genes includes *GA INSENSITIVE* (*GAI*) in *Arabidopsis* (Peng et al., 1997) and its homologues in various cereal grasses (Ivandic et al., 1999; Peng et al., 1999). These genes are a subset of the GRAS family of plant transcriptional activators (Pysh et al., 1999), and mutants are known to cause dwarfing in both monocots and dicots (Peng et al., 1999).

As candidate genes possibly involved in regulating vegetative development in the silversword alliance, *GAI* homologues (designated as *DaGAI*) have been isolated from leaves of the Hawaiian species (Remington & Purugganan, 2002). The *DaGAI* gene, like the *ASAP1* and *ASAP3/TM6* genes, is present in duplicate copies in the tetraploid Hawaiian species. The duplicate copies share conserved features that have been reported previously for the gene subfamily containing *GAI* and *RGA* from *Arabidopsis*, *d8* from *Zea*, and *SLR1* from *Oryza* L., including a conserved N-terminal DELLA domain (Peng et ·al., 1999; Remington & Purugganan, 2002).

Molecular evolutionary analyses indicate strong selective constraint in the coding regions of *DaGAI* in the Hawaiian species, although the constraint is somewhat relaxed in one of the two copies relative to *DaGAI* in North American tarweed species (Remington & Purugganan, 2002). There is also no strong evidence for positive selection on individual codons in *DaGAI*. Substantial variation exists, however, in the upstream flanking regions of *DaGAI* (Remington & Purugganan, 2002), including an elevated rate of nucleotide substitution in the middle upstream region. In addition, rates of nucleotide substitution and insertion/deletion (indel) appear to be decoupled to a large degree, as evidenced by comparison of the proximal and distal upstream regions. An overall decoupling is also apparent when the upstream flanking regions of the duplicate *A* and *B* copies of *DaGAI* in the Hawaiian species are compared, with the *B* copy having a similar number of nucleotide substitutions to the *A* copy but an elevated number of indels.

The results suggest that for *DaGAI* loci in the silversword alliance, positive selection may exert itself in the *cis*-regulatory promoter regions rather than in the protein-coding regions (Remington & Purugganan, 2002). Doebley and Lukens (1998) have proposed that variation in *cis*-regulatory regions of transcription factors may be a predominant mechanism by which novel variation in morphology arises, because transcription factors are often involved in coordinated regulation of specific developmental processes. Though no lines of evidence demonstrate positive selection to the exclusion of other possibilities for the Hawaiian *DaGAI* loci, the possibility of divergent selection in the upstream flanking regions is intriguing. In particular, some of the larger indels in the upstream flanking regions co-occur with major evolutionary transitions in growth form within the silversword alliance (Remington & Purugganan, 2002).

## EPILOGUE

Regulatory gene evolution may thus play a key role in the adaptive radiation of the Hawaiian silversword alliance. In particular, the rapid and extensive diversification in reproductive and vegetative form may well be linked to alterations in the tempo and mode of evolution of the genes that control developmental processes. Additional analyses at macroevolutionary and microevolutionary scales in the silversword alliance, and in other lineages undergoing adaptive radiation, should provide deeper insight into the genetic basis of morphological diversification, especially in reference to regulatory genes.

A more detailed understanding of adaptive radiation thus offers a special window into the evolution of life's diversity. Yet peering through this window is becoming ever more difficult, as the lineages undergoing adaptive radiation, especially those on oceanic archipelagos, suffer the impacts of habitat modification and loss and of alien species (Robichaux et al., 1998). The nature of the challenge is well-illustrated by the silversword alliance. Eight taxa in the silversword alliance (*Argyroxiphium sandwicense* subsp. *sandwicense, A. kauense* (Rock & M. Neal) O. Deg. & I. Deg., *Dubautia herbstobatae* G. D. Carr, *D. kenwoodii* G. D. Carr, *D. latifolia, D. pauciflorula, D. plantaginea* Gaudich. subsp. *humilis* G. D. Carr, and *Wilkesia hobdyi* H. St. John) are critically endangered. Thirteen additional taxa are rare or vulnerable (Wagner et al., 1999). The main threats confronting the taxa are competition by alien plants, predation by alien ungulates, and predation of pollinators by alien insects (Robichaux et al., 1998; Friar & Robichaux, 2003). Without a serious and sustained effort to eliminate the threats, and to reverse declines in distribution and abundance, many of the extant taxa will likely be lost to extinction, as has already happened in one case (*A. virescens* Hillebr.). A similar challenge also faces other lineages in Hawaii, such as the endemic honeycreepers and drosophilids, and on other oceanic archipelagos.

Thus, taking action now to prevent extinction is key to further exploring adaptive radiation and its link to genome evolution. Yet adaptive radiation also carries an important message for conservation (Robichaux et al., 1998). It serves to highlight that the biotic landscapes of Hawaii and other oceanic archipelagos are filled with dynamic, actively evolving lineages of plants, animals, and other organisms. Incorporating a lineage-based view into conservation planning, including the goal of providing opportunities for continued lineage diversification, may prove critical to long-term success.

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