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Patterns of nucleotide variation in homoeologous regulatory genes in the allotetraploid Hawaiian silversword alliance (Asteraceae)

AMY LAWTON-RAUH,* ROBERT H. ROBICHAUX† and MICHAEL D. PURUGGANAN*
*Department of Genetics, Box 7614, North Carolina State University, Raleigh, NC 27695 USA, †Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85721, USA

Abstract

Genome-wide duplication (polyploidization) is prevalent in a large number of eukaryotic organisms and is particularly widespread in flowering plants. Polyploid species appear to vary from their diploid progenitors in a variety of ecologically important traits, suggesting that genome duplications provide a mechanism for ecological diversification. Studies of nucleotide variation at duplicate genes that arise via polyploidization allow us to infer the evolutionary forces that act on these polyploid loci. In an effort to examine the evolutionary dynamics of homoeologous loci, molecular population genetic analyses were undertaken for duplicate regulatory genes in the allopolyploid Hawaiian silversword alliance, a premier example of adaptive radiation. The levels and patterns of nucleotide variation for the floral homeotic genes *ASAPETALA1 (ASAP1)* and *ASAPETALA3/TM6 (ASAP3/TM6)* were studied in two species representing different lineages within the Hawaiian silversword alliance: *Argyroxiphium sandwicense* ssp. *macrocephalum* and *Dubautia ciliolata* ssp. *glutinosa*. Homoeologueous copies of *ASAP1* and *ASAP3/TM6* show differing levels and patterns of nucleotide polymorphism. Duplicate *ASAP1* copies have similar levels of nucleotide diversity and haplotype structure in both species; by contrast, duplicate *ASAP3/TM6* genes display different levels and patterns of variation in *D. ciliolata* ssp. *glutinosa*. Additionally, *D. ciliolata* ssp. *glutinosa* appears to be segregating for a moderate frequency null allele in one *ASAP3/TM6* homoeologue. These results suggest that differing evolutionary forces can affect duplicate loci arising from allopolyploidization.

Keywords: adaptive radiation, allopolyploidy, floral homeotic genes, gene duplication, MADS-box, polyploidy

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Introduction

Gene duplication may arise from tandem repetition, retrotransposon activity, segmental duplication, or genome-wide duplication (Paterson *et al*. 2000; Bennetzen 2002; Schmidt 2002). The evolutionary pathways of duplicate gene copies can result in the origin and diversification of gene families and appear to play an important role in organismal complexity and evolution (Ohno 1970; Wendel 2000). Genome duplication (polyploidization) can occur either within a species (autopolyploidization) or as a result of an unreduced interspecific hybridization between two species (allopolyploidization). Polyploidization has been shown to occur across several eukaryotic groups, including yeast (Coissac *et al*. 1997; Mewes *et al*. 1997; Wolfe & Shields 1997), invertebrates (Foighil & Thiriot-Quievreux 1999) and vertebrates (Spring 1997; Pebusque *et al*. 1998; McLysaght *et al*. 2002).

Polyploidization is particularly common in plants, with estimates suggesting that 40–70% of land plants have evolutionary histories that include at least one episode of genome doubling (Stebbins 1971; Masterson 1994; Leitch & Bennett 1997). Additional studies indicate that genome duplication continues to occur in plants (Grant 1981; Masterson 1994). Furthermore, calculations suggest that

Correspondence: Michael Purugganan. Fax: 919 5153355; E-mail: michaelp@unity.ncsu.edu

~2–4% of angiosperm speciation events involve polyploidization, suggesting that genome duplication may be associated with species diversification (Otto & Whitton 2000). Several studies reveal that multiple, nested episodes of polyploidy occurred across modern angiosperms, thus the majority of flowering plants harbour remnants of genome-wide duplications of varying ages (Schmidt *et al*. 2001; Zhang *et al*. 2001; Mitchell-Olds & Clauss 2002). In the model plant *Arabidopsis thaliana*, for instance, there appear to have been at least four separate large-scale genome-wide duplications (Vision *et al*. 2000; Mitchell-Olds & Clauss 2002). Several of these duplication events occurred 100–200 million years ago, coincident with the Mesozoic era and possibly near the origin and expansion of early angiosperms (Vision *et al*. 2000).

Several important phenotypic traits differ markedly between polyploid plant taxa and their diploid progenitors. Examples of reproductive traits that may vary because of changes in ploidy level include the duration and initiation of flowering time, fertility, self-compatibility, apomixis, germination and organ size (Jackson 1976; Lewis 1980; Lumaret 1988; Segraves & Thompson 1999; Cook & Soltis 2000; Schranz & Osborn 2000). Changes in vegetative traits such as growth rate and drought tolerance, as well as susceptibilities to arthropod infestation and fungal diseases, have also been documented (Jackson 1976; Lewis 1980; Lumaret 1988).

Recent studies regarding the immediate effects of genome duplication have shed light on the structural events and expression changes that take place following polyploidization. Significant genome restructuring and gene silencing can occur quite rapidly following polyploidization, as found in *A. thaliana* allotetraploids (Comai *et al*. 2000), cotton (Zhao *et al*. 1998) and synthetic polyploids in *Brassica* (Song *et al*. 1995; Leitch & Bennett 1997) and in wheat (Feuillet *et al*. 2001; Ozkan *et al*. 2001; Shaked *et al*. 2001). Evolutionary genetic models have explored alternative fates of duplicate loci, including neo- and subfunctionalization, redundancy and pseudogene formation (Ohno 1970; Walsh 1995; Force *et al*. 1999; Lynch & Conery 2000). Moreover, a recent study in allopolyploid cotton indicates that duplicate gene copies can accumulate different levels of nucleotide variation, and that differential selection with respect to genomic origin of each gene duplicate is possible (Small & Wendel 2002). The above examples suggest significant short-term and long-term genetic consequences following gene duplication. The dramatic effects of gene duplication by polyploidization on phenotypic and molecular evolution emphasize the need for further studies in different organismal and ecological contexts.

Floral regulatory genes

The molecular genetics of floral and inflorescence development has been the subject of intense study (Weigel 1995; Yanofsky 1995; Liljegren & Yanofsky 1996). Much of this work has focused on *A. thaliana* (Brassicaceae), where several genes controlling various aspects of flower development have been identified. Mutational lesions in these genes result in the formation of aberrant floral organ types; thus these loci are referred to as floral homeotic genes (Bowman *et al*. 1991). Among the best characterized floral homeotic genes are the floral meristem identity gene *APETALA1* (*AP1*) (Mandel *et al*. 1992; Bowman *et al*. 1993; Gustafson-Brown *et al*. 1994; Irish 1998) and the floral organ identity gene *APETALA3* (*AP3*) (Bowman *et al*. 1991; Jack *et al*. 1992; Irish & Yamamoto 1995). Both *AP1* and *AP3* are transcription factors and are members of the extensive MADS-box gene family (Riechmann & Meyerowitz 1997; Alvarez-Buylla *et al*. 2000).

The *AP1* gene in *A. thaliana* controls floral meristem identity as well as sepal and petal organogenesis (Bowman *et al*. 1993; Irish 1998). Mutants at this locus display a loss of sepal and petal formation and a 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. *AP3* mutants display a homeotic transformation of these organs to sepaloid and carpelloid structures (Bowman *et al*. 1991). Homologues of *AP1* and *AP3* have been discovered in other plant taxa, indicating that these genes have relatively conserved functions among various angiosperm lineages. The function of the Solanaceae *AP3*-like paralogue *Lycopersicon TM6* gene is unknown, although its expression pattern is consistent with roles in petal and stamen development (Pnueli *et al*. 1991; Kramer *et al*. 1998). Orthologues of *AP1* and *AP3* from the Asteraceae have been isolated in *Gerbera hybrida* and appear to perform functions that are similar to their *Arabidopsis* counterparts (Yu *et al*. 1999).

The Hawaiian silversword alliance

The Hawaiian silversword alliance (Asteraceae, Heliantheae, Madiinae) is a premier example of plant adaptive radiation (Robichaux *et al*. 1990; Baldwin & Robichaux 1995; Baldwin 1997). The alliance includes 30 perennial species in three endemic genera: *Argyroxiphium*, *Dubautia* and *Wilkesia* (Carr 1985; Robichaux *et al*. 1990; Baldwin & Robichaux 1995; Baldwin 1997). These 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 occurring as single-island endemics. Members of the alliance also display a wide array of habitat preferences and morphological growth forms, including a striking diversity in inflorescence and floral architectures (Carr 1985). The periodic availability of new habitats (as a result of close proximity, formation and weathering of the Hawaiian Islands) and extensive chromosomal evolution are believed to have contributed to the adaptive radiation

of these taxa (Robichaux *et al*. 1990; Baldwin & Robichaux 1995).

The Hawaiian silversword alliance is a monophyletic group that descended from the *Madia* lineage of the North American tarweeds (Asteraceae, Heliantheae, Madiinae) (Baldwin *et al*. 1991; Baldwin & Robichaux 1995; Baldwin 1996, 1997). The onset of diversification in this adaptive radiation occurred within the last 5.2 ± 0.8 million years, based on a calibrated, rate-constant internal transcribed spacer (ITS) tree (Baldwin & Sanderson 1998). Evolutionary analysis of the Hawaiian silversword alliance indicates that the species are allotetraploids that originated from an interspecific hybridization event between two diploid North American tarweed species (Barrier *et al*. 1999).

Orthologues of several *A. thaliana* floral developmental loci have been isolated in the Hawaiian silversword alliance, including orthologues of the *A. thaliana* genes *AP1* (*ASAP1*) and *AP3* (*ASAP3/TM6*) genes (Barrier *et al*. 1999). Phylogenetic analyses of *ASAP1* and *ASAP3/TM6* indicate that both genes are present as two copies (homoeologues) deriving from separate North American tarweed lineages. The *A* homoeologue appears to derive from the ancestral lineage of *Anisocarpus scabridus* and the *B* homoeologue appears to derive from an ancestral lineage including *Carlquistia muirii* (Barrier *et al*. 1999). Both homoeologues of each isolated floral regulatory gene *(ASAP1-A*, *ASAP1-B*, *ASAP3/TM6-A*, and *ASAP3/TM6-B)* are expressed in the Hawaiian species (Barrier *et al*. 2001). Additionally, molecular evolutionary analysis of these homoeologous regulatory genes suggests an increase in the rate of protein evolution in the Hawaiian silversword alliance species compared to the North American tarweed species (Barrier *et al*. 2001).

In this study, we examined the effects of polyploidy on regulatory gene evolution in a plant adaptive radiation by investigating the molecular population genetics of homoeologous floral regulatory genes in two species of the Hawaiian silversword alliance [(*Argyroxiphium sandwicense* ssp. *macrocephalum* (A. Gray) Meyrat (Meyrat *et al*. 1983) and *Dubautia ciliolata* ssp. *glutinosa* G. Carr (Carr 1985)]. This study addresses the following questions. (i) Do homoeologous regulatory genes harbour different levels and patterns of nucleotide variation? (ii) Is an excess of replacement substitutions in regulatory genes across the Hawaiian silversword alliance reflected in a non-neutral pattern of within- and between-species protein evolution? (iii) Is there a progenitor genome-specific pattern of sequence variation in homoeologous regulatory genes?

We analysed levels and patterns of nucleotide variation for the *ASAP1* and *ASAP3/TM6* regulatory genes in *A. sandwicense* ssp. *macrocephalum* and *D. ciliolata* ssp. *glutinosa*. Our results suggest that evolutionary forces are similar among the *ASAP1* homoeologues in both species. By contrast, the *ASAP3/TM6* homoeologues in *D. ciliolata* ssp. *glutinosa* appear to have different levels of nucleotide diversity and haplotype structure, suggesting that evolutionary forces have acted differently between the duplicate *ASAP3/TM6* copies in this species. These results demonstrate that differential evolution between homoeologous copies may occur in plant species undergoing adaptive radiation.

Materials and methods

Study species

Argyroxiphium sandwicense ssp. *macrocephalum* and *D. ciliolata* ssp. *glutinosa* belong to different lineages within the Hawaiian silversword alliance, with the lineages having diverged early during their adaptive radiation (Baldwin & Robichaux 1995). The two taxa differ greatly in reproductive mode and in inflorescence and floral characters. *Argyroxiphium sandwicense* ssp. *macrocephalum* is a monocarpic rosette shrub, and produces a single large compound inflorescence containing 50–600 capitula (or heads), each with 11–42 ray and 120–600 disk flowers (Carr 1985). By contrast, *D. ciliolata* ssp. *glutinosa* is a polycarpic shrub, and produces many smaller compound inflorescences, each containing one to seven capitula, with the capitula having 5–11 disk flowers (unpublished observations). Both taxa grow in dry, high-elevation habitats, and both have large population sizes. *Argyroxiphium sandwicense* ssp. *macrocephalum* is endemic to the Island of Maui, and *D. ciliolata* ssp. *glutinosa* is endemic to the Island of Hawaii. For simplicity, the taxa are referred to without their subspecific designations for the remainder of this paper.

Sample collections and DNA extraction

Leaf tissue samples of *D. ciliolata* were collected from 10 to 24 haphazardly selected individuals from three localities on Mauna Kea, Hawaii: Puu Kanakaleonui, Puu Kawiiwi, and near Waipahoehoe gulch (Table 1). Leaf tissue samples of *A. sandwicense* were haphazardly collected from 15 to 16 individuals from four localities on Haleakala, Maui: Silversword Loop, Puu o Pele, Ka moa o Pele, and Puu Naue (Table 1).

Genomic DNA was extracted from leaf tissue using a modified plant CTAB protocol (Saghai-Maroof *et al*. 1984). Genomic DNA extractions were purified with an EluQuick glass bead purification procedure (Schleichter and Schuell) to reduce the amount of pectin and secondary-product contamination.

Polymerase chain reaction (PCR) amplifications and sequencing

DNA fragments for both copies (homoeologues) of *ASAP1*

Table 1 Molecular variation in *ASAPETALA1* and *ASAPETALA3/TM6* homoeologues (A and B) from *Dubautia ciliolata* ssp. *glutinosa* and *Argyroxiphium sandwicense* ssp. *macrocephalum*

Gene	Species	Length (bp)	*n*	$n_{locality}$	N_{hap}	S	π_{silent}	π_{intron}	π_{synon}	π nonsyn	θ_W (95% C.I.)	Tajima's D
ASAP1-A	*D. ciliolata*	1661	20	8/6/6	11	27	0.0035	0.0037	0.0000	0.0053	0.0046 (0.0019, 0.0085)	–0.649 ($P > 0.10$)
	A. sandwicense	1696	15	8/3/1/3	9	21	0.0044	0.0043	0.0066	0.0005	0.0038 (0.0015, 0.0073)	–0.110 ($P > 0.10$)
ASAP1-B	*D. ciliolata*	1736	24	7/12/5	9	13	0.0019	0.0020	0.0000	0.0003	0.0020 (0.0006, 0.0041)	–0.617 ($P > 0.10$)
	A. sandwicense	1709	15	8/2/2/3	11	18	0.0029	0.0031	0.0000	0.0010	0.0032 (0.0011, 0.0068)	–0.777 ($P > 0.10$)
ASAP3/	*D. ciliolata*	949	21	8/8/5	13	22	0.0052	0.0046	0.0133	0.0006	0.0064 (0.0032, 0.0103)	–1.257 ($P < 0.05$)
TM6-A	*A. sandwicense*	1012	16	8/4/2/2	8	12	0.0036	0.0038	0.0000	0.0008	0.0037 (0.0015, 0.0063)	–0.578 ($P > 0.10$)
ASAP3/	*D. ciliolata*	1247	10	4/4/2	3	2	0.0005	0.0005	0.0000	0.0000	0.0006 (0.0000, 0.0017)	–0.691 ($P > 0.10$)
TM6-B	*A. sandwicense*	1230	16	8/3/2/3	5	5	0.0014	0.0015	0.0000	0.0021	0.0012 (0.0002, 0.0029)	0.823 ($P > 0.10$)

n is the number of individuals/alleles sampled. $n_{locality}$ is the distribution of individual/allele sampling by locality for *D. ciliolata* samples: Puu \Kanakaleonui/Puu Kawiiwi/Waipahoehoe gulch for *A. sandwicense* samples: Silversword Loop/Puu o Pele/Ka moa o Pele/Puu Naue. N_{hap} is the number of observed haplotypes. S is the number of observed segregating sites. π_{silent} is nucleotide diversity at silent sites. π_{intron} is nucleotide diversity at intron sites. π_{synon} is nucleotide diversity at synonymous sites. π_{nonsyn} is nucleotide diversity at nonsynonymous sites. θ_W (C.I.) is Watterson's estimate of θ with 95% confidence interval (95% C.I.).

and *ASAP3/TM6* were obtained via PCR amplification with the error-correcting *Pwo* polymerase (Roche, Indianapolis). The error-correcting *Pwo* polymerase was utilized to minimize PCR errors because of nucleotide misincorporation. Data from multiple, independent amplifications and resequencing of several genes indicates an error rate of less than one error in 7–10 kilobases (kb; unpublished observations).

Gene-specific primers for *ASAP1* and *ASAP3/TM6* were designed based on cDNA sequences as described in Barrier *et al*. (1999). Homoeologous copies of *ASAP1* (*ASAP1-A* and *ASAP1-B*) spanning exons 3–8 were amplified using primers AP1–3X (5′-CTGGACCATGGAGTACAACAAAC-3′) and AP1–8XR2 (5′-ATCGGCTGCAGACTCAGGTC-3′) (Fig. 1). The PCR conditions were generally as follows: 94 °C for a 5-min hot-start, followed by 10 cycles of 94 °C for 30 s, 52 °C for 30 s, 68 °C for 2 min, then 20 cycles of 94 °C for 30 s, 52 °C for 30 s, and 68 °C for 2 min with a 20-s per cycle auto-incrementation, and a final 7 min extension step at 68 °C. The ~1.2 kb PCR products were purified using either Spin-X and Ultrafree-MC filter purification (Millipore) or a QiaQuick extraction from agarose gels (Qiagen). Amplified product bands were cloned using either TA cloning or Zero Blunt TOPO TA cloning kits (Invitrogen). Restriction enzyme digests on cloned *ASAP1* gene fragments were utilized to screen for each homoeologous copy (*ASAP1-A* vs. *ASAP1-B*) from multiple clones per individual.

For the *ASAP3/TM6-A* and *-B* copies, primers APETALA3–3 (5′-TACAAACAGGCAGGTGACATACTC-3′) and ASAP3–2R4 (5′-CTGCTGCTCGAGAATGGTTA-GATC-3′) were used to amplify simultaneously a region spanning exons 1–4 from both homoeologues (Fig. 2). The following general PCR amplification conditions were utilized: 94 °C for a 5-min hot-start followed by 10 cycles of 94 °C for 15 s, 55 °C for 30 s, 72 °C for 1 min, then 20 cycles of 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 1 min with a 5-s per cycle auto-incrementation, followed by a final 7-min extension step at 72 °C. Amplified PCR products were isolated and cloned using the same protocol reported above for the *ASAP1* homoeologues. The homoeologous *ASAP3/TM6* copies are significantly different in size (1.1 kb vs. 1.4 kb for *ASAP3/TM6-A* and *-B*, respectively), and thus can be readily differentiated by fractionation in a 1.5% agarose gel.

All genes were sequenced with automated DNA sequencers at various facilities (NCSU DNA Sequencing Facility, Iowa State University Sequencing Facility, NCSU Genome Research Laboratory) using a nested primer series designed in both directions. Sequences were aligned and visually refined. Polymorphic sites were confirmed by visual inspection of chromatograms. The DNA sequences are available from GenBank (accession numbers AY259925–AY259993).

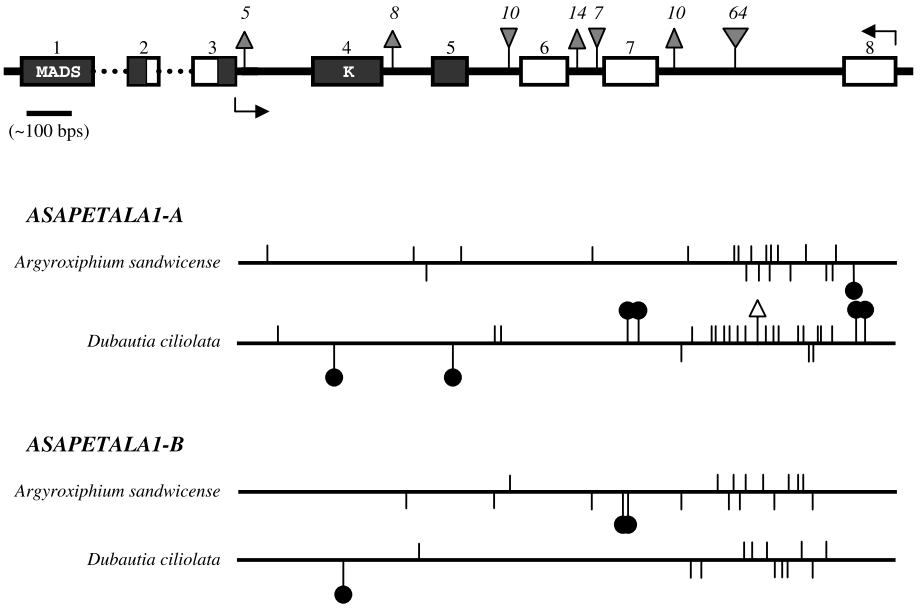

Fig. 1 Gene maps and summary figures of nucleotide variation in *ASAPETALA1* homoeologues from *Argyroxiphium sandwicense* ssp. *macrocephalum* and *Dubautia ciliolata* ssp. *glutinosa*. Exons are shown as numbered boxes. Bent arrows indicate positions of PCR primers used to amplify loci. Gray triangles above each gene map represent major insertions and deletions (indels) of *B* homoeologues relative to *A* homoeologues, with italicized numbers above each grey triangle indicating indel size. The MADS box and K-box regions are shown as darkened, labelled sections of exons. The scale bar represents approximately 100 bp. Dotted lines between exons indicate an extended length beyond the scale bar. In every summary figure, vertical bars above the line represent nonsingleton polymorphic sites and vertical bars below the line represent singleton sites. Each filled circle represents a replacement polymorphism and each triangle represents an indel polymorphism. All polymorphic sites indicated in the summary figures are general locations based on joint alignments including both homoeologues of each floral regulatory gene.

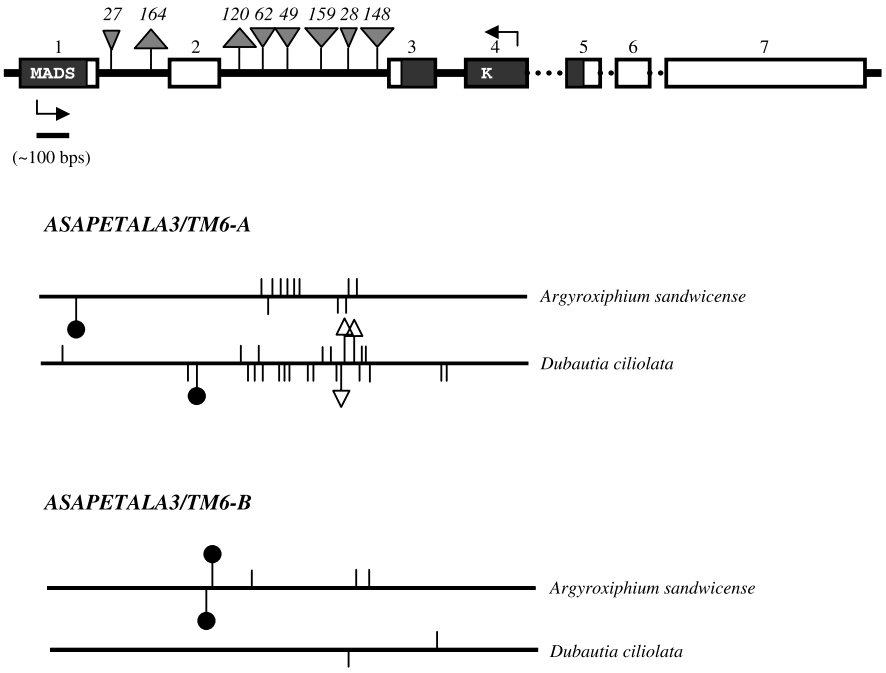

Fig. 2 Gene map and summary figure of nucleotide variation in *ASAPETALA3/TM6* homoeologues from *Argyroxiphium sandwicense* ssp. *macrocephalum* and *Dubautia ciliolata* ssp. *glutinosa*. Detailed description of figure as for Fig. 1

Sequence data analyses

Sequence variation was estimated using nucleotide diversity (π) (Nei 1987) and the population mutation parameter (θ_W) (Watterson 1975) in DNASP version 3.53 (Rozas & Rozas 1999). Coalescent simulations with 2000 replications were conducted to calculate the confidence interval of θ_W. Haplotype networks were constructed using the 95% statistical parsimony support criterion for inferred estimated gene genealogies (Templeton *et al.* 1992) as implemented in the TCS program (Clement *et al.* 2000). The Hudson–Kreitman–Aguade (HKA) test was performed using the number of segregating sites and average number of differences for each locus (Hudson *et al.* 1987).

Tajima's test (Tajima 1989) for selection was performed, and significance was assessed based on coalescent simulations of 10 000 runs using the number of segregating sites and the estimated recombination parameter. The population recombination parameter was calculated from the data using SITES (Hey & Wakeley 1997). The McDonald–Kreitman test (McDonald & Kreitman 1991) was performed to test for neutral evolution in protein-coding regions. For each locus in *D. ciliolata*, *A. sandwicense* was designated as the outgroup for interspecific divergence comparison. For each locus in *A. sandwicense*, *D. ciliolata* was designated as the outgroup for interspecific divergence.

Results

Nucleotide variation at homoeologous ASAPETALA1 *loci*

The levels and patterns of nucleotide variation provide information on the evolutionary forces that have acted on genes. The latter can be inferred from calculations of per-site nucleotide diversity (π, Nei 1987) and the population mutation parameter (θ_W, Watterson 1975). Alleles for homoeologues of *ASAP1* and *ASAP3/TM6* were isolated and sequenced from *Dubautia ciliolata* individuals sampled among three Mauna Kea, Hawaii localities and from *Argyroxiphium sandwicense* individuals sampled among four Haleakala, Maui localities (see $n_{locality}$, Table 1).

Molecular variation was estimated for a region spanning exons 3–8 (and the intervening five introns) of each *ASAP1* homoeologue (Fig. 1). The amplified portions of both homoeologues include the coding sequences for the K-box and C-terminal domains of the encoded MADS-box transcription activator. Sequence analysis indicates that the *D. ciliolata ASAP1-A* alleles from 20 individuals form 11 haplotypes with 27 segregating sites and one 1-bp insertion/deletion (indel) polymorphism (Table 1 and Fig. 1). Most polymorphic sites are in intron 7. Among the polymorphic sites in exons, six are replacement mutations and none is a synonymous mutation. Silent-site nucleotide diversity (π_{silent}) at this locus is 0.0035, which is comparable to the nucleotide diversity levels reported for the orthologous *AP1* gene in *A. thaliana* (π_{silent} = 0.004; Olsen *et al.* 2002) and similar to the estimated population mutation parameter (θ_W = 0.0046). The *A. sandwicense ASAP1-A* alleles from 15 individuals fall into nine haplotypes and have 21 segregating sites (Table 1 and Fig. 1). There is one replacement and one synonymous polymorphism in the coding region of this gene, and most polymorphic sites are in intron 7. Silent site nucleotide diversity (π_{silent}) at this locus is 0.0044, comparable to the estimate for this gene in *D. ciliolata* as well as the population mutation parameter (θ_W = 0.0038).

The levels of nucleotide variation appear to be lower for the *B* homoeologue of the *ASAP1* gene than for the *A* homoeologue. Sequence analysis of *ASAP1-B* alleles from the 24 *D. ciliolata* individuals indicates that this locus has nine haplotypes and 13 segregating sites (Table 1 and Fig. 1). The protein coding region of *ASAP1-B* has one replacement and no synonymous polymorphisms. There are no insertion/deletion polymorphisms and most polymorphic sites are in intron 7. Silent site nucleotide diversity (π_{silent}) at this locus is 0.0019, which is similar to the population mutation parameter (θ_W = 0.0020) and is about half the value of θ_{silent} for *ASAP1-A* in this species. The *A. sandwicense ASAP1-B* allele dataset from 15 sampled individuals includes 11 haplotypes and 18 segregating sites (Table 1 and Fig. 1). Among protein coding region polymorphic sites, two are replacement changes and none is a synonymous change. Most polymorphic sites are in intron 7. Silent site nucleotide diversity (π_{silent}) at this locus is 0.0029, which is similar to the population mutation parameter (θ_W = 0.0032) and two-thirds the value of π_{silent} for *ASAP1-A* in this species.

Nucleotide variation at homoeologous ASAPETALA3/TM6 *loci*

Nucleotide variation was examined in a region spanning exons 1–4 of each *ASAP3/TM6* homoeologue. The amplified portions of both homoeologues include the coding sequences for the DNA-binding MADS-box domain of this transcriptional activator and part of the K-box domain (Fig. 2). *ASAP3/TM6-A* alleles were sequenced from 21 individuals of *D. ciliolata* and from 16 individuals of *A. sandwicense* (Table 1). Nucleotide sequence analysis of the *D. ciliolata ASAP3/TM6-A* alleles reveals 13 haplotypes and 22 segregating sites, with three 1-bp indels (Table 1 and Fig. 2). There are one replacement and two synonymous polymorphisms in the protein coding region of this gene and most polymorphic sites are in intron 2. Silent site nucleotide diversity (π_{silent}) at this locus is 0.0052, which is similar in magnitude to the estimated population mutation parameter for this gene (θ_W = 0.0064) and lower than the value reported for *AP3* in *A. thaliana* (π_{silent} = 0.0076; Olsen *et al.* 2002). The *A. sandwicense ASAP/TM6-A*

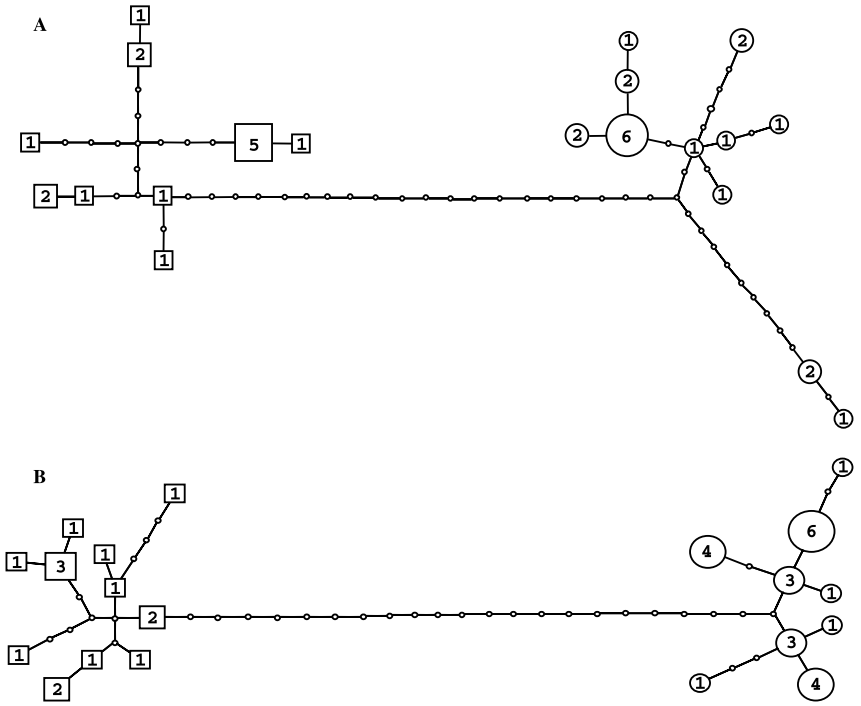

Fig. 3 Statistical parsimony haplotype networks of *ASAPETALA1* homoeologues in *Dubautia ciliolata* ssp. *glutinosa* and *Argyroxiphium sandwicense* ssp. *macrocephalum*. (A) is the *ASAP1-A* homoeologue haplotype network and (B) is the *ASAP1-B* homoeologue haplotype network. Circles represent *D. ciliolata* haplotypes and squares represent *A. sandwicense* haplotypes. Numbers within and relative sizes of haplotype shapes indicate the number of sampled alleles of specific haplotypes. Small circles represent missing haplotypes with > 95% statistical parsimony support.

allele sequences are distributed among eight haplotypes and have 12 segregating sites (Table 1 and Fig. 2). There are one replacement and no synonymous mutations in the exons of this gene and most polymorphic sites are in intron 2. Silent site nucleotide diversity (π_{silent}) at this locus is 0.0036, which is similar to the population mutation parameter ($\theta_W = 0.0037$).

Alleles from the homoeologous *ASAP3/TM6-B* gene were sequenced from 10 individuals of *D. ciliolata* and 16 individuals of *A. sandwicense*. We were unable to amplify the *B* homoeologue of *ASAP3/TM6* from several additional *D. ciliolata* individuals. In an effort to determine if this copy was present, we utilized a saturated PCR approach, using 41 primer combinations throughout the gene. This PCR-based approach to determine the presence of this copy was necessitated by the difficulties in conducting Southern blot analyses within Madiinae species, which have large genomes and high DNA methylation levels (Barrier *et al*. 1999). The approach utilized in this study was used to successfully co-amplify both *ASAP3/TM6* homoeologues (*ASAP3/TM6-A* and *-B*) in many other species across the Hawaiian silversword alliance (Barrier *et al*. 1999). This co-amplification strategy was not consistently successful, however, for amplification of both *ASAP3/TM6* homoeologues in *D. ciliolata*. Whereas *ASAP3/TM6-A* was successfully amplified using most of the 41 primer combinations in several samples, amplification of *ASAP3/TM6-B* from 14 out of 24 individuals (42% success rate) in *D. ciliolata* was not successful. Taken together, this suggests that *ASAP3/TM6-B* is deleted in the plants from which we were unable to amplify both *ASAP3/TM6* homoeologues.

The *D. ciliolata ASAP3/TM6-B* allele sequences that were obtained have the lowest diversity of the genes in this study, with only three haplotypes and two segregating sites, of which one segregating site is a singleton (Table 1 and Fig. 2). The two polymorphisms in *D. ciliolata ASAP3/TM6-B* are in introns 2 and 3 (Fig. 2). Silent site nucleotide diversity (π_{silent}) at this locus is 0.0005, which is 4–10-fold lower than calculations in other currently sequenced genes in this species. This pattern in π_{silent} is further reflected in calculated θ_W values, where the 95% confidence intervals do not overlap between *ASAP3/TM6-A* and *ASAP3/TM6-B*-values for *D. ciliolata*.

Silent site nucleotide diversity at *A. sandwicense ASAP3/TM6-B* is also low, but not nearly as low as the estimate for this gene in *D. ciliolata*. In the *A. sandwicense ASAP3/TM6-B* allele sample, there are five haplotypes and five segregating sites (Table 1 and Fig. 2). Among protein coding region polymorphic sites, there are two replacement polymorphisms in exon 2, with no observed synonymous variation. Silent site nucleotide diversity (π_{silent}) at this locus is 0.0014, which is comparable to the population mutation parameter ($\theta_W = 0.0012$; Table 1).

Haplotype networks of floral regulatory genes

The statistical parsimony-based haplotype networks of *D. ciliolata* and *A. sandwicense* for each homoeologous gene pair are displayed in Figs 3 and 4. The *ASAP1-A* and *ASAP1-B* haplotype networks reveal separate clades for alleles in *D. ciliolata* and *A. sandwicense*, indicating several fixed differences between species haplotypes and the lack

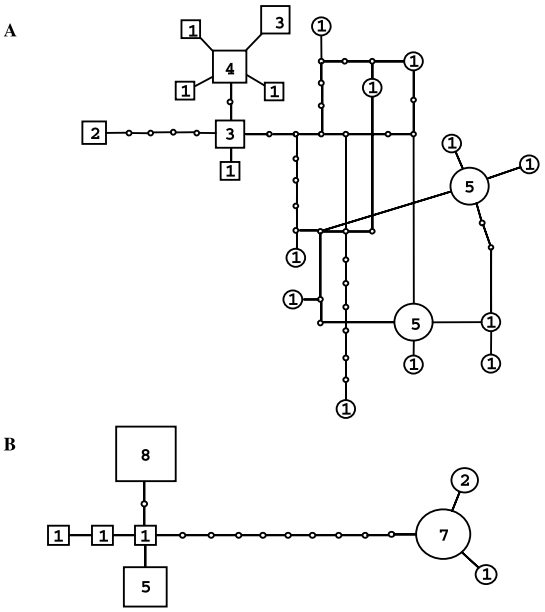

Fig. 4 Statistical parsimony haplotype networks of *ASAPETALA3/TM6* homoeologues in *Dubautia ciliolata* ssp. *glutinosa* and *Argyroxiphium sandwicense* ssp. *macrocephalum*. (A) is the *ASAP3/TM6-A* homoeologue haplotype network and (B) is the *ASAP3/TM6-B* homoeologue haplotype network. Circles represent *D. ciliolata* haplotypes and squares represent *A. sandwicense* haplotypes. Numbers within and relative sizes of haplotype shapes indicate the number of sampled alleles of specific haplotypes. Small circles represent missing haplotypes with > 95% statistical parsimony support.

of shared polymorphisms (Fig. 3). The inferred haplotype networks of *ASAP3/TM6* homoeologues also show reciprocal monophyly between species, with no shared haplotypes between the two taxa (Fig. 4). The *ASAP3/TM6-A* haplotype network shows nonlinear connections among several *D. ciliolata* haplotypes, reflecting the moderate level of recombination at this locus that was also detected in our analyses of recombination in this species (results not shown).

Reduced variation in the ASAPETALA3/TM6-B *gene*

The HKA test compares levels of intraspecific polymorphism and interspecific divergence among loci to determine if the loci have significantly different levels of nucleotide variation (Hudson *et al*. 1987). This test can be applied to homoeologous gene copies to examine whether loci that arise from allopolyploidization are subject to contrasting evolutionary forces. The HKA test results in this study are displayed in Table 2.

Silent site diversity (π_{silent}) is almost two-fold higher for *ASAP1-A* compared to *ASAP1-B* in *D. ciliolata* (Table 1). Based on HKA test results, however, this difference in diversity is not significant for this homoeologous gene pair ($P > 0.10$, Table 2). A similar trend is observed between the homoeologous *ASAP1* gene copies in *A. sandwicense*. Silent site nucleotide diversity (π_{silent}) is approximately 1.5 times higher in *ASAP1-A* compared to *ASAP1-B* (Table 1) but this difference is not significant in an HKA test ($P > 0.10$, data not shown).

Table 2 Hudson–Kreitman–Aguade contingency test values for *ASAPETALA1* and *ASAPETALA3/TM6* homoeologues (*A* and *B*) using intraspecific data from *Dubautia ciliolata* ssp. *glutinosa* and interspecific divergence between *Dubautia ciliolata* ssp. *glutinosa* and *Argyroxiphium sandwicence* ssp. *macrocephalum*

	ASAP1-A	*ASAP1-B*	*ASAP3/TM6-A*	*ASAP3/TM6-B*
Intraspecific polymorphism				
Observed	20	9	11	2
Expected	16.67	12.33	7.64	5.36
Interspecific divergence				
Observed	22.68	22.57	8.50	11.70
Expected	26.01	19.24	11.86	8.34
No. of silent sites	1380.54	1427.86	770.00	1057.82
χ^2 value	1.027		4.485	
P-value	0.311		0.034*	

*Significant at the $\alpha = 0.05$ level.

The results for *ASAP1* in both species are in contrast to the results for *ASAP3/TM6* loci. Sequence analysis reveals a difference in silent site nucleotide diversity levels between the *ASAP3/TM6* homoeologues obtained from *D. ciliolata*, with the *ASAP3/TM6-B* homoeologue having an apparent 10-fold lower nucleotide diversity compared to the *ASAP3/TM6-A* homoeologue (Table 1). This pattern is further reflected in θ_W values of *ASAP3/TM6-A* and *ASAP3/TM6-B*, which differ by an order of magnitude and have nonoverlapping 95% confidence intervals (Table 1). The HKA test between *ASAP3/TM6-A* and *-B* genes in *D. ciliolata* indicates a significant deviation in sequence variation between these two loci ($P < 0.05$, Table 2). This significant difference appears to result in part from the observed number of polymorphisms being 144% of the expected value in *ASAP3/TM6-A* and 37% of the expected value in *ASAP3/TM6-B* (Table 2).

As in *D. ciliolata*, the *ASAP3/TM6* gene pair also shows a difference in silent site nucleotide diversity (π_{silent}) in *A. sandwicense*. The *ASAP3/TM6-B* homoeologue is approximately 2.6-fold lower in diversity compared to the *ASAP3/TM6-A* homoeologue (Table 1). Unlike in *D. ciliolata*, however, the HKA test for these two loci using *A. sandwicense* intraspecific polymorphism is not significant ($P > 0.10$, data not shown).

Nucleotide diversity at intron, synonymous, and nonsynonymous sites

In most loci, the majority of silent site nucleotide diversity (π_{silent}) is the result of mutations in introns (π_{intron}) rather

than at synonymous sites (π_{synon}; Table 1). The increased variation at intron sites rather than at synonymous sites can be attributed to polymorphisms in intron 7 in both copies of *ASAP1 and* intron 2 of *ASAP3/TM6-A* in both *A. sandwicense* and *D. ciliolata* (Figs 1 and 2). The increased diversity at these specific introns across different species suggests elevated neutral mutation rates in these localized regions. Moreover, there is greater diversity at nonsynonymous sites (π_{nonsyn}) than at synonymous sites (π_{synon}) in most loci (Table 1), a pattern consistent with the possibility of positive selection on protein structure.

Excess low-frequency polymorphisms in floral regulatory genes

The neutral-equilibrium model predicts the expected frequency of polymorphisms in an allelic sample and Tajima's test of selection examines deviations from this neutral expectation (Tajima 1989). This test of selection calculates the statistic *D*; loci evolving neutrally have *D*-values equal to zero, whereas positive *D*-values suggest an excess of intermediate frequency polymorphisms. Negative values of Tajima's *D* indicate an excess of low-frequency polymorphisms, which may arise from a recent selective sweep or demographic factors such as population expansion.

Almost all genes examined in this study, with the exception of *A. sandwicense ASAP3/TM6-B*, have negative Tajima's *D*-values. Observed Tajima's *D*-values range from −0.777 to −0.110 for the *ASAP1* homoeologues (Table 1). Negative values are also observed for *ASAP3/TM6-A* in *D. ciliolata* (–1.257) and *A. sandwicense* (–0.578), and for *ASAP3/TM6-B* in *D. ciliolata* (–0.691; Table 1). The only observed positive Tajima's *D*-value among all loci examined in both of these species is the one estimated for the *A. sandwicense ASAP3/TM6-B* homoeologue. Of all genes in this study, however, only the *ASAP3/TM6-A* gene in *D. ciliolata* has an excess of low-frequency variants that is significantly different from the neutral-equilibrium model expectations ($P < 0.05$). Although not significantly different from neutral expectations, negative Tajima's *D*-values in all but one of the floral regulatory genes in these two species suggest a trend of excess low-frequency polymorphisms at most loci. (It should be noted that the limited number of polymorphisms in some samples, e.g. *D. ciliolata ASAP3/TM6-B*, significantly reduces the power of this test to reject neutral-equilibrium expectations.)

Evolution of protein coding regions

Barrier *et al*. (2001) found higher ratios of nonsynonymous (Ka) to synonymous (Ks) mutations in the coding regions of the floral regulatory genes in Hawaiian silversword alliance species than in North American tarweed species. Among the Hawaiian species, many of the pairwise interspecific Ka/Ks values for the *ASAP1* and *ASAP3/TM6* loci were greater than 1, which suggests that selection and adaptive divergence may have operated to shape the structure of these loci. Selection in protein-coding regions can also be inferred with population-level sequence data using the McDonald–Kreitman test, which examines the relative levels of within- and between-species nonsynonymous to synonymous changes (McDonald & Kreitman 1991). None of the McDonald–Kreitman tests was significant (*G*-tests, $P = 0.16–1.00$).

Discussion

It has been proposed that the increased number of possible gene products encoded by duplicate gene copies may lead to potential increases in genetic adaptability in polyploid species, particularly allopolyploid taxa, relative to their diploid progenitors (Leitch & Bennett 1997; Wendel 2000). Polyploidization has been postulated as playing a significant role in diversification, including the evolution of *HOX* clusters in early vertebrates (Malago-Trillo & Meyer 2001), the addition to multigene families such as the *rbcS* family (Sasanuma 2001), and the radiation of early vertebrates during the Cambrian explosion (Spring 1997; Valentine *et al*. 1999; Miyata & Suga 2001). Furthermore, regulatory and structural gene evolution may be quickly uncoupled in polyploid taxa because of an accelerated rate of mutational accumulation at duplicate regulatory genes relative to duplicate structural genes (Ferris & Whitt 1979). Taken together, the increased availability of unique gene product combinations and an accelerated rate of evolution in duplicated regulatory genes may play significant roles in the adaptive diversification of naturally occurring polyploids.

We examined patterns of variation in homoeologous copies of the floral regulatory genes *ASAP1* and *ASAP3/TM6* in two species of the allopolypoid Hawaiian silversword alliance, *Dubautia ciliolata* and *Argyroxiphium sandwicense*. Orthologues to these genes in other dicotyledonous species suggest that these loci control inflorescence and floral development (Ng & Yanofsky 2001). Species in the Hawaiian silversword alliance differ greatly in inflorescence and floral morphologies (Carr 1985), suggesting that floral regulatory gene evolution may underlie the diversification of reproductive morphologies in this group. Indeed, a previous study indicates accelerated protein evolution at these regulatory loci within the Hawaiian silversword alliance (Barrier *et al*. 2001).

The range of morphological traits found in the Hawaiian silversword alliance is remarkable given the relatively low genetic distances among these taxa. Population studies, for example, indicate very little genetic differentiation in 10 allozyme loci between morphologically distinct species in the Hawaiian silversword alliance (Witter & Carr 1988). This suggests that relatively few genetic changes of large

phenotypic effect, possibly at regulatory loci, may be responsible for the wide phenotypic range in these allopolyploid taxa. Such a pattern would be consistent with an extensive analysis of multilocus isozymes in several polyploid catostomid fish species indicating that polyploidization may be followed by an accelerated rate of mutational accumulation in regulatory genes (Ferris & Whitt 1979).

The type and distribution of polymorphisms within coding regions of the *ASAP1 and ASAP3/TM6* loci in *A. sandwicense* and *D. ciliolata* suggest a signature consistent with non-neutral protein evolution. Though polymorphism levels are low in the coding regions of the loci, most coding region diversity, at least for five of the eight loci, occurs as replacement polymorphisms (Table 1, Figs 1 and 2). This trend is consistent with the results of Barrier *et al*. (2001), which found higher pairwise interspecific Ka/Ks ratios in the *ASAP1 and ASAP3/TM6* loci among the Hawaiian silversword alliance species than among the North American tarweed species.

Although the patterning of replacement polymorphisms in the coding regions of most of the loci suggests the action of positive selection, most of the statistical tests for selection cannot reject the possibility that the loci are evolving neutrally. Given the limited number of variable sites, especially in coding regions, the lack of detectable deviation from neutral-equilibrium expectations may not be surprising. With such a limited number of variable sites, the McDonald–Kreitman and Tajima's tests have reduced power to reject neutral-equilibrium expectations.

The negative Tajima's *D*-values calculated for most loci, though significantly different from zero in only one case, suggest an excess of low-frequency polymorphisms. This trend toward an excess of low-frequency polymorphisms suggests a genome-wide signature consistent with the effects of demographic changes such as rapid population expansion. Rapid population expansion may have followed inter-island dispersal and/or colonization of new habitats. Forthcoming research with these species will further address this possibility.

The marked differences in levels of nucleotide diversity and in the number and distribution of haplotypes between *ASAP3/TM6* homoeologues in *D. ciliolata* suggest that the duplicate copies of this floral regulatory gene have been subject to different evolutionary forces (Table 2, Fig. 4). A deletion allele may be segregating at moderate frequency in *D. ciliolata*, leading to only one *ASAP3/TM6* copy in several individuals of this species. This segregation pattern does not appear to be confined to specific populations; the *ASAP3/TM6-B* alleles that were successfully amplified from *D. ciliolata* were found in all three sampling localities (see $n_{locality}$, Table 1). Additionally, microsatellite-based studies, including samples from the localities used in this study, reveal no evidence of population structure in *D. ciliolata* (E. Friar, personal communication).

In the extant *ASAP3/TM6-B* alleles of *D. ciliolata* there is a 10-fold reduction in the level of silent site nucleotide variation compared to *ASAP3/TM6-A* (π_{silent} = 0.0005 vs. 0.0052, respectively). Although the sample size of *D. ciliolata ASAP3/TM6-B* alleles is reduced as a result of the putative deletion allele, this should not significantly affect estimates of nucleotide variation levels because π_{silent} and θ_W estimates are corrected for sample size variation. The HKA test indicates that the observed reduction in nucleotide polymorphism levels for *ASAP3/TM6-B* compared to *ASAP3/TM6-A* in *D. ciliolata* is significant (Table 2). The difference in levels and patterns of nucleotide diversity between these two genes is also reflected in the haplotype networks, which display a reduced number and decreased diversity of *ASAP3/TM6-B* haplotypes relative to *ASAP3/TM6-A* haplotypes (Fig. 4). None of the other regulatory genes examined (*ASAP1* in *D. ciliolata* and *A. sandwicense*, and *ASAP3/TM6* in *A. sandwicense*) exhibits such large differences between homoeologues.

The reduced levels of variation observed for the *ASAP3/TM6-B* alleles segregating in *D. ciliolata* may result from positive selection (Fay & Wu 2000; Charlesworth *et al*. 2001). If extant *ASAP3/TM6-B* alleles were evolving neutrally, then these alleles would have variation levels comparable to other neutral loci in this species. It may be that the deletion allele for *ASAP3/TM6-B* is being selected against, and that the extant alleles are in the process of sweeping through *D. ciliolata*, resulting in decreased variation at *ASAP3/TM6-B* in this species. Physical mapping of the putative deletion and an investigation of the levels and patterns of variation in flanking sequences may help clarify the evolutionary dynamics of this locus in *D. ciliolata*.

The patterns of molecular evolution within these floral regulatory loci in allopolyploid Hawaiian silversword alliance species can be compared to patterns observed between homoeologous genes in allopolyploid cotton taxa. Molecular evolutionary studies of 16 homoeologous gene pairs in polyploid and diploid *Gossypium* species indicate that there are no significant differences in rates of evolution between homoeologous gene copies following allopolyploidization (Cronn *et al*. 1999). Molecular population genetic studies in these species, however, indicate that genome-specific differential evolution can occur between homoeologous genes (Small & Wendel 2002). Homoeologous copies of two *Adh* paralogues present in diploid species (*AdhA* and *AdhC*) were examined in two allopolyploid species that have homoeologous copies of both *AdhA* and *AdhC*. Nucleotide sequence analysis shows a consistent difference between substitution patterns of duplicate loci correlated with genome origin. Relative rate tests also suggest that *AdhC* is evolving more quickly than *AdhA* (Small & Wendel 2002). Additionally, a higher nonsynonymous diversity in

AdhC implies relaxed selection on the D-subgenome and purifying selection on the A-subgenome in *Gossypium hirsutum* and another allotetraploid *G. barbadense*.

In the Hawaiian silversword alliance species, differential evolution between homoeologues is not associated with the genome origins of the duplicate loci. The homoeologous copies of *ASAP1* within both *D. ciliolata* and *A. sandwicense* do not differ significantly in levels and patterns of nucleotide variation, suggesting that similar evolutionary forces have acted on both gene copies in each species. By contrast, *D. ciliolata ASAP3/TM6* homoeologues show evidence of differential evolution between gene copies. This pattern, however, is not observed for the *ASAP3/TM6* homoeologues in *A. sandwicense*, suggesting a species-specific effect associated with differential evolution.

It remains unclear to what extent genome restructuring as a result of polyploidization continues to occur in the Hawaiian silversword alliance species, given that the allopolyploidization event may have occurred more than 5.2 ± 0.8 million years ago (Baldwin & Sanderson 1998; Barrier *et al*. 1999). Cytogenetic studies suggest continuing chromosomal repatterning, with eight genomic rearrangements distinguished by reciprocal translocations and an aneuploid reduction found among the species (Carr & Kyhos 1981, 1986). More studies will be needed to unravel the precise relationship between polyploidization, the diversification of homoeologous regulatory gene copies, and the adaptive radiation of the Hawaiian silversword alliance.

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Amy Lawton-Rauh conducted this research as a graduate student in the Department of Genetics at North Carolina State University. She is currently a postdoctoral fellow in the Department of Genetics and Evolution at the Max Planck Institute of Chemical Ecology, where she is working in the laboratory of T. Mitchell-Olds. Robert H. Robichaux is a University Distinguished Professor in the Department of Ecology and Evolutionary Biology at the University of Arizona studying the ecology and evolution of the Hawaiian silversword alliance. Michael D. Purugganan is an Associate Professor in the Department of Genetics at North Carolina State University studying the molecular evolutionary, ecological and developmental genetics of plants.
