Review

***Aquilegia* as a model system for the evolution and ecology of petals**

Elena M. Kramer^{1,*} and Scott A. Hodges²

¹*Department of Organismic and Evolutionary Biology, Harvard University, Cambridge, MA 02138, USA*

²*Department of Ecology, Evolution and Marine Biology, University of California, Santa Barbara, CA 93106, USA*

The ranunculid genus *Aquilegia* holds extraordinary promise as a model system for investigating a wide range of questions relating to the evolution and ecology of petals. New genetic and genomic resources, including an extensive EST database, BAC libraries and physical maps, as well as virus-induced gene silencing are facilitating this research on multiple fronts. At the developmental genetic level, *Aquilegia* has been important for elucidating the developmental programme for specifying petals and petaloid characteristics. Data suggest that duplication events among the petal and stamen identity genes have resulted in sub- and neofunctionalization. This expansion of gene function does not include the petaloidy of *Aquilegia* sepals, however, which does not depend on the same loci that control identity of the second whorl petals. Of special interest is the elaboration of the petal into a nectar spur, a major innovation for the genus. Intra- and interspecific variation in the shape and colour of petals, especially the spurs, has been shown to be adaptive for different pollinators. Thus, understanding the genetic basis of these traits will help us connect the ecological interactions driving speciation with the genetic changes responsible for remodelling morphology. Progress in this area has focused on the multiple, parallel transitions in flower colour and nectar spur length across the genus. For flower colour, upstream transcription factors appear to be primarily targets of natural selection. Thus research in *Aquilegia* spans the initial evolution of petals and petaloidy to the diversification of petal morphology to the ecological basis of petal form, thereby providing a comprehensive picture of the evolutionary biology of this critical angiosperm feature.

Keywords: *Aquilegia*; Ranunculaceae; petal evolution; adaptation; speciation; nectar spur

1. INTRODUCTION

The evolution of petaloid organs in the reproductive axis is a notable innovation of the angiosperms that has clearly played a major role in their diversification, especially in the context of attracting pollinators (Endress 1994). What makes petaloid features so intriguing is that they appear to be a true innovation with no obvious precursor in extant gymnosperms. Then, once petals evolved, more subtle changes to their shape and colour have been major factors allowing species to attract and become specialized on different pollinators. From a genetic standpoint, there are many aspects of petaloid organs that we would like to understand in terms of both macroevolutionary and microevolutionary processes. How many times have petaloid organs evolved? Is their developmental genetic basis the same when petaloid features are produced in different positions? How is the elaboration of petals controlled and how are these genetic pathways altered in the context of pollinator shifts? Do independent shifts to similar pollinators involve similar genetic changes to petals? These types of questions cover a

multitude of different genetic pathways and can only be answered through analysis of a similarly diverse set of genetic models. In this review, we discuss the new model system *Aquilegia* and how it is contributing to our understanding of the evolution of petaloid organs.

Flowers typically have two types of organs—reproductive and sterile. While the reproductive organs are divided into stamens (androecium) and carpels (gynoecium), the sterile organs are collectively termed the perianth. The phenomenon of petaloid organs is challenging because it can occur in any of these organs, as well as in extra-floral leaf-like organs termed bracts. If we restrict our consideration to the perianth, there are further distinctions. In some taxa, such as *Magnolia* or *Tulipa*, perianth parts are relatively similar in appearance (whether petaloid or not), in which case they are termed tepals. More commonly, the perianth is bipartite with two or more distinct organ types (in contrast to the unipartite condition of tepals). In these cases, the outer organs, called sepals, are often adapted for protective functions, while the inner organs, called petals, are more specialized for a role in pollinator attraction. Thus, classically, petals have a fixed position on the floral axis—in the second whorl between the sepals and androecium. However, as mentioned above, petaloid features can

* Author for correspondence (ekramer@oeb.harvard.edu).

One contribution of 16 to a Discussion Meeting Issue 'Darwin and the evolution of flowers'.

occur in either of the perianth whorls and in the case of the family Ranunculaceae, of which *Aquilegia* is a member, often occur in both. How then should we define petaloid features? While there is no clear set of criteria (Endress 1994), petaloidy can be defined as being conspicuous and brightly coloured or patterned as opposed to green or leaf-like. It is important to note that in some taxa, the true petals themselves are quite small, meaning that not all petals are dramatically petaloid.

Eames (1961) wrote that 'Theories of the nature and development of the perianth are closely bound up with theories of the origin of the flower', by which he was referring to two ideas about where perianth organs came from. According to the first idea, originally espoused by Goethe (1790), the perianth parts, and the petals in particular, are modified stamens termed andropetals (Takhtajan 1991). The alternative hypothesis is that perianth organs were derived from pre-existing sterile bracts that were associated with the reproductive axis, giving rise to bracteopetals (Takhtajan 1991). These two hypotheses may not be mutually exclusive. Some taxa may have perianth organs derived from both bracts and stamens while others may have only bracteopetals or andropetals. The criteria used to distinguish between andro- and bracteopetals are largely developmental and morphological, relating to vasculature, developmental kinetics, phyllotaxy and morphological similarities (Smith 1928; Eames 1961; Takhtajan 1991). However, beyond the details, the fundamental point is that the perianth in general and petals in particular have evolved many times independently within the angiosperms, with different precursor organs giving rise to sterile, attractive structures in different lineages (Eames 1961; Bierhorst 1971; Takhtajan 1991). Reconstruction of perianth evolution in the context of modern molecular phylogenies confirms that the bipartite perianth has evolved many different times independently (Zanis *et al.* 2003; Hileman & Irish 2009), as have novel types of petaloid features in various positions both inside and outside the flower (Walker-Larsen & Harder 2000; Jaramillo & Kramer 2007).

Our understanding of the developmental genetic basis of floral organ development has grown exponentially over the past 20 years, with considerable impact on our understanding and interpretation of floral evolution. Especially notable is the elucidation of the ABC model for floral organ identity (Coen & Meyerowitz 1991). This genetic model describes three classes of gene activity, expressed in overlapping domains of the floral meristem, thereby creating a combinatorial code. Each whorl of the flower has a distinct set of gene activities: A alone encodes sepals; A+B, petals; B+C, stamens and C alone, carpels (figure 1). The model has been modified over time by the addition of two other gene activities (D, for ovules and E, as a broad facilitator of ABC gene function) and substantial reconsideration of the nature of A function (Davies *et al.* 2006; Litt 2007). Most of the genes associated with the ABC model are members of the large type II class of MADS box containing transcription factors, which has facilitated their identification across a broad range of seed plants

Figure 1. (a) The classic ABC model with the addition of the E function. (b) The corresponding ABCE genes from *Arabidopsis*. The A class genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*) specify sepals (SEP) and with the B class genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), specify petals (PET). The B class genes with the C class gene *AGAMOUS* (*AG*) specify stamens (STA) and the C class gene alone specifies carpels (CAR). (c,d) The modified ABC model of *Aquilegia* based on expression studies of the B gene homologues: (c) corresponds to early developmental stages while (d) reflects expression after carpel initiation.

(Becker & Theissen 2003). Overall, it appears that while A function is not conserved, the functions of the B and C class gene homologues are largely the same throughout the angiosperms, particularly in terms of stamen and carpel identity (Kramer *et al.* 2004; Zahn *et al.* 2005b; Kramer 2006).

This leads to the question of petal identity—is there a deeply conserved petal identity programme or, in keeping with the idea of multiple independent derivations, are there many genetically different ways to produce a petal? Answering this question has proved difficult, for a number of reasons. First, the B class genes, represented in *Arabidopsis* by the MADS box genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*; Jack *et al.* 1992; Goto & Meyerowitz 1994), have complex evolutionary histories (Kramer *et al.* 1998; Kramer & Irish 2000; Stellari *et al.* 2004) and have experienced gene duplication at every phylogenetic level. Most notably, the *AP3* lineage was duplicated at the base of the core eudicots to give rise to two distinct paralogues termed *euAP3* and *TM6* (Kramer *et al.* 1998). This is of interest because the *euAP3* lineage experienced a distinct sequence divergence at its C terminal end as the result of a frameshift mutation, which converted the otherwise deeply conserved paleoAP3 motif into the core eudicot-specific *euAP3* motif (Vandenbussche *et al.* 2003; Kramer *et al.* 2006). The biochemical implications of this event are still a matter of debate (Lamb & Irish 2003; Piwarzyk *et al.* 2007), but the fact remains that the defining B class genes, *AP3* from *Arabidopsis* and its orthologue *DEFICIENS* (*DEF*) from *Antirrhinum*, are members of a core eudicot-specific lineage and therefore do not have simple orthologues outside this clade (Kramer *et al.* 1998; Kramer & Zimmer 2006). Another complicating

factor for understanding the conservation of the petal identity programme is the potential for convergence at the genetic level. This phenomenon has frequently been observed among both plants and animals, and can result in non-homologous structures expressing homologous genes (Abouheif *et al.* 1997; Jaramillo & Kramer 2007). Convergence is particularly probable in the case of andropetals where independent sterilization of stamens could have repeatedly involved the recruitment of B gene homologues to control andropetal identity (Irish 2009). Even for bracteopetals, the transformation of bracts into petaloid organs might have occurred via outward expansion of the B gene domain from the stamens (Baum & Whitlock 1999; Irish 2009). How then should we interpret the finding that B gene homologues are almost always expressed in true petals or petaloid tepals (reviewed in Kim *et al.* 2004; Kramer & Jaramillo 2005; Zahn *et al.* 2005b)? Some have interpreted these data as evidence for a deeply conserved petal identity programme, suggesting that petal identity may have evolved once and just been redeployed to different positions in the flower (Bowman 1997). Others have held to the convergence model, that petals evolved many times but recruited similar genes to control their development in each case (Irish 2009). Discriminating among these alternatives may never be entirely possible, but growing evidence seems to support a mixed model—the identity of true petals in the second whorl may be controlled by a commonly inherited genetic programme in many cases but, at the same time, there are definitely genetic mechanisms that do not use the B gene programme for promoting petaloid features in other positions of the flower (Rasmussen *et al.* 2009; and see also below).

Of course, understanding petal diversity only starts with questions related to organ identity. Across taxa, petals differ in every aspect of their morphology, including colour, symmetry and other aspects of shape, and presence or absence of nectaries. Our knowledge of the genetic control of these aspects is very uneven. Few direct targets of the B class genes have been identified (Sablowski & Meyerowitz 1998; Mara & Irish 2008), but several loci have been implicated in the sculpting of petals, particularly factors controlling the balance between cell division and cell expansion (reviewed by Irish 2008). Among these is *APETALA2* (*AP2*), which was originally considered an A class gene but has subsequently been found to primarily affect petal development rather than identity (Drews *et al.* 1991; Litt 2007). Unlike the other genes of the ABC model, *AP2* is a member of the AP2/EREBP family of transcription factors (Weigel 1995; Okamura *et al.* 1997). Together with a related family member, *AINTEGUMENTA* (*ANT*), *AP2* contributes to petal initiation and size (Krizek *et al.* 2000; Keck *et al.* 2003).

Another major influence on petal shape is whether or not the flower is bilaterally or radially symmetric. In the case of the former, different petal morphologies are produced in different positions in the same whorl of the flower. Studies of the genetic control of bilateral symmetry in *Antirrhinum* (snapdragon) indicate that fine control of cell division patterns is also critical to

this type of differentiation (Gaudin *et al.* 2000). Studies of other bilaterally symmetric species, particularly legumes, have provided evidence that very similar genetic pathways have evolved by co-opting homologues of the same genes (reviewed by Preston & Hileman 2009; Rosin & Kramer 2009). At the same time, studies in legumes have demonstrated the potential to uncover novel factors that may be influencing the internal symmetry of individual petals (Wang *et al.* 2008).

Further elaborations of petal morphology take a variety of forms, but one component that appears to be a critical aspect of diversification is the presence of nectaries (Hodges & Arnold 1995; Hodges 1997a). These structures, which produce sugar-rich liquid that serves as rewards for visiting pollinators, are not restricted to petals and can be present on any organ in the flower. Although nectaries have evolved many times independently (Bernardello 2007), a study of the core eudicots surprisingly found that orthologues of the same gene, the YABBY transcription factor *CRABS CLAW* (*CRC*), are associated with nectaries from diverse taxa (Lee *et al.* 2005). However, this correlation between *CRC* and nectaries does not appear to extend outside the core eudicot clade. When nectaries occur on the perianth organs, they are often associated with the development of spurs that show a close correlation between their morphology and the feeding structures of the flower's pollinator. Currently, no candidate loci have been associated with the development of naturally occurring spurs, but an intriguing mutant in *Antirrhinum* may provide some insight. Transposon insertions near two *KNOX* transcription factors result in ectopic gene expression that appears to promote the development of spur-like structures (Golz *et al.* 2002). It will be very interesting to see whether *KNOX* gene expression is actually responsible for the normal development of spurs.

Aside from nectaries, petals use other morphological features to attract pollinators, particularly the interplay of colour and iridescence. The latter is usually produced by the presence of conical or papillated epidermal cells. In *Antirrhinum*, an MYB transcription factor, *MIXTA* (*MIX*), is critical to the formation of these cells as well as proper pollinator attraction (Glover *et al.* 1998; Martin *et al.* 2002; Whitney *et al.* 2009). *Antirrhinum* has also been an important model for the genetic dissection of colour production, particularly in regards to the upstream regulators, which are also members of the MYB gene family (Noda *et al.* 1994; Schwinn *et al.* 2006). Natural variation among these paralogues has been shown to control spatial patterns of colour production and colour differences (Schwinn *et al.* 2006; Whibley *et al.* 2006). The enzymatic pathways responsible for colour production, which are genetically downstream of the MYBs, have been well characterized in a number of systems including *Antirrhinum*, *Ipomoea* and, recently, *Aquilegia* (Martin & Gerats 1993; Whittall *et al.* 2006a,b; Rausher 2008; Hodges & Derieg 2009).

This leads to the question of *why* petals are so diverse. Over a century of research, including that of Darwin, has shown that pollinator interactions play a

Figure 2. Floral variation across natural species and one cultivar of *Aquilegia*. (a) *A. caerulea*. (b) *A. shockleyi*. (c) *A. pubescens*. (d) *A. chrysantha*. (e) *A. caerulea* var. *daileyae*, which lacks spurs. (f) *A. vulgaris* 'Black Tower', which has stamens transformed into petals. Photos: (a) Nathan Derieg; (b–e) Scott Hodges and (f) Elena Kramer.

major role in shaping petal characteristics. As we review below, research in *Aquilegia* has shown that much of the intra- and interspecific variation in petals is intimately associated with variation in primary pollinators. These associations are especially true for variation in nectar spur length and colour (figure 2a–d). Because specialization to different pollinators causes reproductive isolation, petals represent important adaptive features in *Aquilegia* that may play direct roles in speciation processes. Thus, dissecting the genetic basis of these traits will provide an understanding of how adaptation and speciation proceed.

2. MORPHOLOGY AND EVOLUTIONARY HISTORY OF *AQUILEGIA*

Aquilegia is a member of the family Ranunculaceae, which falls within the eudicot order Ranunculales (APG 2003; figure 3). This order is sister to the rest of the core eudicots (Hoot *et al.* 1999). *Aquilegia*, therefore, adds an important third data point to deep evolutionary comparisons between the monocot grasses and core eudicot models such as *Arabidopsis*, *Petunia* and *Antirrhinum*. *Aquilegia* flowers are unique among these model genetic systems in that they possess five types of floral organs instead of the typical four: petaloid sepals in the first whorl, petals with

Figure 3. Simplified phylogeny of the angiosperms based on Moore *et al.* (2007) showing the position of *Aquilegia* relative to other major model systems.

nectar spurs in the second whorl, four to seven whorls of stamens, one whorl of staminodia, and an innermost whorl of carpels (Kramer 2009). The development of these organs has been studied in detail, confirming close affinities between the stamens and staminodia (Tucker & Hodges 2005). Some of the floral features of *Aquilegia* are found across the Ranunculaceae, particularly the petaloid sepals and petal nectaries, while the petal spurs and staminodia are recently evolved (Hodges & Arnold 1994a; Ro & McPherson 1997; Tucker & Hodges 2005). The petal spurs, in particular, have been the subject of considerable research due to their critical role in pollinator interactions (Hodges & Arnold 1994a; Hodges 1997b; Hodges *et al.* 2004). The spurs initiate relatively late in floral development, after the stamens have differentiated into filaments and anthers (Tucker & Hodges 2005). Beginning as an outpocketing close to the base of the concave petal, the spur does not elongate substantially until the last phases of floral development, reaching a final length of anywhere from 1–2 mm to 10–12 cm (Hodges & Arnold 1995; Tucker & Hodges 2005). The nectary develops inside the distal tip of the spur. It appears that diversity in floral spurs, along with other aspects of floral morphology such as orientation and colour, has facilitated the rapid radiation of *Aquilegia* species in less than 2 Myr (Hodges 1997b; Kay *et al.* 2006a,b; Whittall & Hodges 2007). This recent evolutionary history has made the genus an excellent model for understanding speciation via pollinator diversification (Hodges *et al.* 2004). Other interesting morphological and physiological features of *Aquilegia* include compound leaves, a perennial life cycle, vernalization-based control of flowering time, and adaptations to a variety of environments including alpine and desert.

3. GENETIC AND GENOMIC RESOURCES FOR *AQUILEGIA*

To facilitate the genetic dissection of traits in *Aquilegia*, genomic resources have been, and are being, developed for the genus. One consequence of the recent and rapid divergence of *Aquilegia* species is extreme similarity at the DNA level among species (Hodges & Arnold 1994a; Kay *et al.* 2006a,b; Whittall *et al.* 2006a,b; Whittall & Hodges 2007). Thus, resources developed from one species are often readily transferable to most other species of the genus (Yang *et al.* 2005; Whittall *et al.* 2006a,b). This transferability across species that vary markedly in ecology and morphology is a great advantage to *Aquilegia* as a model system.

At the core of the development of new model species is the creation of an EST database (Abzhanov *et al.* 2008). An EST database is ideal for the identification of candidate genes (e.g. Hodges & Derieg 2009), the development of expression arrays, and can be used for phylogenomics (Abzhanov *et al.* 2008). Such a database becomes increasingly useful as the full transcriptome becomes represented. For the *Aquilegia* EST database (the *Aquilegia* Gene Index, AqGI, <http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=aquilegia>), mRNA was isolated from a broad range of tissues and developmental stages including vegetative and floral apical meristems, flowers from early buds through anthesis, as well as leaves and roots from hybrids of *Aquilegia formosa* × *Aquilegia pubescens*. This diversity and emphasis on flowers was designed to capture a large portion of the transcriptome and those genes involved with floral development in particular. In addition, the mRNA was selected for full-length transcripts and normalized in order to obtain as many transcripts with their entire, or nearly entire, sequence.

The current version of the AqGI (release 2.1) contains over 85 000 sequences that assemble into 13 556 tentative consensus (TC) sequences and 7278 singletons. This is likely to represent a high percentage of *Aquilegia* genes, given that *Arabidopsis* currently has just over 27 000 protein-coding genes (TAIR9). The *Aquilegia* TCs average 1293 bp in length (figure 4), close to the mean length found for eukaryotic genes (1346 bp; Xu *et al.* 2006). This similarity suggests that most of the TCs in the AqGI represent near full-length transcripts. Many of the genes have been classified using GO vocabularies to various molecular functions, biological processes and cellular components (figure 5a–c). As expected, general categories such as ‘catalytic activity’ and ‘cellular processes’ are highly represented, but there is also a broad range of classifications highlighting the diversity of genes represented. Thus, the AqGI provides an excellent resource for searching for homologues of specific genes and also for transcriptome studies using either oligonucleotide arrays or next generation sequencing.

In addition to the AqGI, other genetic and genomic tools are being developed for *Aquilegia*. Currently there are three BAC libraries, two for *A. formosa* and one for *Aquilegia caerulea* Goldsmith, an inbred horticultural line. The *A. formosa* BAC libraries have been fingerprinted and assembled into a physical map

Figure 4. Frequency distribution of the length of tentative consensus (TC) sequences in the AqGL.

(<https://www.genome.clemson.edu/activities/projects/aquilegia/pmap/>). These maps will greatly aid in the assembly of a complete genome sequence, which is currently in production at the DOE Joint Genome Institute. A complete genome sequence will be especially useful for identifying the genes underlying genetic variants.

While the above resources are ideal for identifying candidate genes, in order to test if they actually influence specific traits, a functional assay is essential (Abzhanov *et al.* 2008). Luckily, we have been able to adapt a reverse genetic tool, virus-induced gene silencing (VIGS), for use in *Aquilegia* (see Gould & Kramer 2007 for detailed protocol). This approach uses the tobacco rattle virus to induce RNAi-based silencing of targeted genes (Burch-Smith *et al.* 2004). This method is especially useful since the effect of repressing gene expression can be observed in a matter of weeks, but it also has a number of disadvantages (Gould & Kramer 2007). These include the fact that genes can only be downregulated, not over-expressed, and the fact that silencing often occurs in clonal sectors of varying size rather than constitutively in all tissues. In order to address these problems, we are working to develop stable transformation as a complement to VIGS. We believe that *Aquilegia* may be a good candidate for dip transformation due to the fact that its carpels do not close until quite late in development (Tucker & Hodges 2005), but we are also pursuing the possibility of tissue culture transformation.

4. DEVELOPMENTAL GENETICS

The diverse floral organ types of *Aquilegia* bring several evolutionary questions to mind. Are the B class genes responsible for petaloid aspects in the first whorl sepals? If they are, how is the identity of the second whorl petals distinguished from that of the petaloid sepals? What genetic pathways are responsible for the development of nectar spurs on the petals? How is the identity of the fifth organ type, the staminodia,

determined? Answering all of these questions starts with identifying homologues of the floral organ identity genes from *Aquilegia* as well as related taxa.

Multiple studies of the MADS box genes have found that they are highly prone to retain duplicate copies, which may lead to divergences in gene function (Litt & Irish 2003; Zahn *et al.* 2005a; Kramer & Zimmer 2006). For this reason, it is critical to first establish whether paralogues are present and, if so, when the duplication events occurred. In the case of *Aquilegia*, we have detected three *AP3* homologues and one *PI* (Kramer *et al.* 2003). The multiple *AP3* paralogues are derived from relatively ancient events that predated the diversification of the Ranunculaceae and, most probably, the bulk of the order Ranunculales (Kramer *et al.* 2003; Rasmussen *et al.* 2009). In keeping with these ancient paralogues, the expression patterns of the *AP3* loci, termed *AqAP3-1*, *AqAP3-2* and *AqAP3-3*, are quite different from one another (figure 1; Kramer *et al.* 2003, 2007; Rasmussen *et al.* 2009). *AqAP3-1* is broadly expressed in petal, stamen and staminodium primordia at very early stages but, as soon as the carpels initiate, this domain begins to contract until it only comprises the staminodia (Kramer *et al.* 2007). The second paralogue, *AqAP3-2*, is turned on slightly later in a region covering the stamens and staminodia but not the petals. At slightly later stages, it shifts to exclude the staminodia but comes on in the petals. Lastly, *AqAP3-3* is very specifically expressed in the petals throughout their development. The other B gene homologue, *AqPI*, is broadly expressed throughout the petals, stamens and staminodia. This is in keeping with the finding that the AqPI protein heterodimerizes specifically with all three AP3 proteins (Kramer *et al.* 2007). The spatially and temporally distinct expression patterns of each *AP3* paralogue are suggestive of subfunctionalization (Force *et al.* 1999).

Subfunctionalization is an evolutionary process whereby complex, ancestral functional repertoires become genetically parsed among duplicate gene copies. In the case of these B class gene homologues, it would appear that an ancestral expression domain encompassing the petals and stamens throughout development has become subdivided such that one copy is primarily expressed at early stages (*AqAP3-1*), another is primarily in stamens (*AqAP3-2*) and the third is limited to petals (*AqAP3-3*; Kramer *et al.* 2007). This situation is made more complex by the presence of the staminodia, which are a novel organ type that evolved very recently (Ro & McPherson 1997; Tucker & Hodges 2005). In these organs, we see specific late expression of the *AqAP3-1* paralogue to the exclusion of the other two (Kramer *et al.* 2007). This suggests an attractive hypothesis whereby the evolution of this new organ identity has been facilitated by the presence of B class gene paralogues—an example of neofunctionalization following gene duplication (Force *et al.* 1999).

Another important point is in regard to the petaloid sepals. The expression of the B gene homologues can be detected in these organs at later stages using RT-PCR, but *in situ* hybridization at early developmental stages does not detect *AP3* and *PI* expression (Kramer *et al.*

Figure 5. Classification of sequences from the AqGI to GO vocabularies. (a) Molecular function, (b) biological processes and (c) cellular component.

2003, 2007). Given that studies in core eudicot models indicate that expression is required from inception to determine proper organ identity (Bowman *et al.* 1989; Zachgo *et al.* 1995), these results indicate that the loci are not contributing to the identity of the petaloid sepals. The data do leave open the possibility that later stage expression could be important for promoting petaloid features, which actually appear fairly late in development (Kramer *et al.* 2007).

These hypotheses require rigorous functional testing in order to determine whether the loci are actually required for the identity of petals, stamens and staminodia as well as the late petaloidy of the sepals. Application of VIGS to the *AqPI* locus resulted in dramatic homeotic transformations of petals into sepals and both stamens and staminodia into carpels (Kramer *et al.* 2007). A strongly modified phenotype would be expected given the fact that AqPI is required for the function of all three AqAP3 paralogues due to their heterodimerization. These findings demonstrate that the B class genes are, in fact, essential to petal, stamen and staminodium identity. Interestingly, however, there was no effect on sepal identity, and the conical epidermal cells, which are among the hallmarks of petaloidy, were not affected. This leads us

to believe that the B gene homologues are not critical to either the identity of the sepals or their petaloidy. This finding is in keeping with studies of other taxa with petaloid sepals that have similarly found that B gene homologues are not expressed in these organs (Park *et al.* 2003; Jaramillo & Kramer 2004; Park *et al.* 2004; Geuten *et al.* 2006).

Several research directions immediately suggest themselves from these results. First, we would like to use VIGS to knock down each of the three *AP3* paralogues in turn. This will determine their degree of redundancy and whether they have specific functions in different whorls. One challenge will be the specific targeting of each paralogue separate from the others, which may not be possible using VIGS, although other studies indicate a high degree of specificity (Liu *et al.* 2004). Another matter of considerable interest is how petaloidy is actually promoted in the sepals, since this does not appear to involve B gene homologues. To pursue this question, we are taking a mixed approach of both forward and reverse genetics/genomics. In the context of reverse genetics, which relies on candidate genes, we will examine two loci called *AqSEP1* and *AqSEP2*. These genes are homologues of the *Arabidopsis* E class genes *SEPALLATA1/2/4*

Figure 6. Comparison of individual petals among species of *Aquilegia*. (a) *Aquilegia longissima*, (b) *Aquilegia pinetorum*, (c) *Aquilegia chrysantha*, (d,e) *A. formosa* and (f) *Aquilegia flabellata* (scale bar, 1 cm).

and show sepal-specific expression in *Aquilegia* (Kramer *et al.* 2007). When *AqPI* is silenced and the petals are transformed into sepals, the expression of these genes expands into the second whorl, suggesting that they are closely associated with sepal identity. We will also take a gene discovery approach that uses next generation sequencing and available horticultural homeotic mutants of *Aquilegia* (figure 2f). By comparing the floral transcriptome of flowers composed almost exclusively of sepals to that of flowers composed primarily of petals, we hope to be able to identify sepal-specific gene networks that are involved in promoting petaloidy. This experiment has the added benefit of identifying petal-specific pathways, which could include genes involved in nectary and spur development. As noted above, the *Aquilegia* CRC orthologue does not appear to be involved in nectary development (Lee *et al.* 2005) so we do not currently have candidate genes for the nectary.

For the spur itself, we are considering an array of candidate loci including KNOX genes and several genes controlling the balance between cell division and expansion such as *JAGGED*, *BIG PETAL* and *AINTEGUMENTA* (reviewed by Irish 2008). This line of research can be explored on an evolutionary scale as well. Since the nectar spur is specific to *Aquilegia*, candidate loci for its development can be studied comparatively relative to outgroup taxa in order to understand how these genetic modules were recruited to function in spur development. Within the genus, the critical question is how diversification of spur length and morphology is controlled at the genetic level. Candidate genes for spur development will, therefore, also become candidates for QTL studies of natural variation in spur morphology. Previous genetic studies

using interspecies hybrids have suggested that there may be relatively few loci controlling spur initiation with higher numbers of genes modulating length (Prazmo 1965).

5. PHYLOGENY, ECOLOGY AND POLLINATION BIOLOGY

While *Aquilegia* is ideal for dissecting the genetic basis of floral organ differentiation, the genus has also long been noted for the diversity of shape and colour of its petals in association with variation in pollinators. For example, just among North American species, spur length varies over a 16-fold range (Whittall & Hodges 2007). In addition, spurs can be straight or highly curled and the diameter of the tube varies, especially at the opening, from fairly broad to quite narrow (figure 6). Petal blades vary similarly in length (both absolute and relative to spur length), width and orientation to the floral axis (figures 2a–d and 6). Finally, the colour of the spurs varies tremendously including red, blue, purple, violet, yellow and white. Both the spur and the blade may be the same colour (figure 6a–c) or, often, the blade may be wholly or partially contrasting with the spur (figure 6d–f). Much of this variation in petals has been correlated with the types of pollinators visiting *Aquilegia*, thus establishing these traits as adaptations. The genetic dissection of this variation is therefore of particular interest as debate continues over the types of mutations and the genes involved in adaptation (e.g. Hoekstra & Coyne 2007).

Darwin himself was interested in the association of petal spurs with their pollinators and proposed a co-evolutionary model to account for the exceptionally

long spurs of *Angraecum sesquipedale* (Darwin 1862). He envisioned reciprocating evolution between a pollinator's tongue and the plant's spur; when the average tongue length is shorter than spur length, longer-tongued individuals have a fitness advantage by gaining more nectar rewards, and when the average spur length is shorter than tongue length, longer-spurred individuals have a fitness advantage by having more pollen dispersed and received. This reciprocating selection pressure would then lead to a gradual lengthening of both tongues and spurs. Alternatively however, evolution may be one-sided with floral-spurs evolving to fit the tongue-lengths of pollinators, which do not evolve in response to spur-length (Wasserthal 1997). This hypothesis suggests that increases in spur length will occur during transitions between primary pollinators with different tongue lengths (Wasserthal 1997). Recently these hypotheses were tested phylogenetically using the North American *Aquilegia* species (Whittall & Hodges 2007). Transitions between pollination classes were found to be directional (bee to hummingbird and hummingbird to hawkmoth), with concomitant increases in spur length. In addition, models of the pace of spur-length evolution strongly favoured those where evolution occurred primarily at the time of speciation. Thus, in *Aquilegia*, spur-length evolution has probably proceeded primarily due to rapid adaptation to the already established tongue-lengths of its primary pollinators rather than in a co-evolutionary race as envisioned by Darwin and others (Whittall & Hodges 2007; Hodges & Whittall 2008).

The match between spur and pollinator tongue length has been studied in depth in *A. formosa* and *A. pubescens* along with a number of other petal characteristics. Over 50 years ago, Verne Grant used these two species as the first example of how floral characters could be associated with specific pollinator visitation and thus affect the degree of reproductive isolation between species (Grant 1952). In particular, Grant noted the differences between the species in the colour and length of the petal spurs and blades. He found that hummingbirds visited *A. formosa* with short, red and yellow petals, while hawkmoths visited *A. pubescens* with long, white or pale-yellow petals. Given that these two species are highly intercompatible, Grant concluded that these petal traits were a major source of reproductive isolation between these species due to their affect on pollinator discrimination (Grant 1952, 1976). Chase & Raven (1975) challenged Grant's conclusion because they observed instances of visitation by hummingbirds and hawkmoths to both species. Grant (1976) then disputed Chase and Raven's findings, noting that their observations were not made when both species were blooming simultaneously, the situation most relevant to reproductive isolation. Subsequently, simultaneous observations of both species in natural stands and in experimental arrays showed that hummingbirds and hawkmoths do strongly differentiate between these species (Fulton & Hodges 1999; Hodges *et al.* 2002). Furthermore, manipulative experiments showed that spur length strongly affects pollen removal (and probable deposition on stigmas as well), while flower colour

and orientation strongly affect pollinator visitation (Fulton & Hodges 1999; Hodges *et al.* 2002) and these traits show steeper clines than neutral genetic markers (Hodges & Arnold 1994b). Thus both petal colour and spur-length can directly affect reproductive isolation and speciation in *Aquilegia*.

In addition to spur length, the colour of the petals has been strongly linked to different pollination syndromes in *Aquilegia* (Hodges *et al.* 2004; Whittall & Hodges 2007). Species with predominantly bee pollination tend to be blue-purple, while those with strong hummingbird visitation are red, and those with hawkmoth visitation tend to be white or yellow (Fulton & Hodges 1999; Hodges *et al.* 2004; Brunet 2009). The adaptive nature of these colour differences has been best studied with hawkmoths, which discriminate against red flowers in favour of white ones (Hodges *et al.* 2004). In *A. caerulea*, where flower colour can be polymorphic within populations (blue and white), populations with consistent hawkmoth visitation tend to have whiter flowers (Brunet 2009), and in populations with variable abundance of hawkmoths, white flowers set more seed when they are present (Miller 1981). Collectively these studies strongly support the evolution of light-coloured flowers as an adaptation to hawkmoth pollination.

Anthocyanin pigments produce the blue/purple and red pigments of *Aquilegia* flowers while yellow is produced by carotenoids (Taylor 1984). Thus a major transition in *Aquilegia* has been from flowers producing anthocyanins (hummingbird pollination) to those lacking these pigments (hawkmoth pollination; Whittall *et al.* 2006a,b). Given that petal colour has been a major focus of genetic studies since Mendel's original experiments with the garden pea, a great deal is known about the genes underlying the anthocyanin biochemical pathway (ABP), including the transcriptional regulators (reviewed in Hodges & Derieg 2009). The core pathway consists of only six enzymatic steps and these loci, along with their transcriptional regulators, have been the subject of evolutionary studies in a number of species (Martin & Gerats 1993; Rausher 2008; Streisfeld & Rausher 2009). However, in addition to these genes, a number of other enzymatic pathways (e.g. those producing flavones and flavonols) intersect with the core pathway and can cause flux away from anthocyanin production and thus may influence flower colour (see Hodges & Derieg 2009).

Phylogenetic analysis indicates multiple independent transitions to hawkmoth pollination in *Aquilegia*, all of which involve the loss of anthocyanins from petals. This distinctive pattern has allowed us to test whether convergence in phenotype is precipitated by convergence at the molecular level (Whittall *et al.* 2006a,b). Across multiple independent losses of anthocyanins, Whittall *et al.* (2006a,b) found that most involve down-regulation of multiple genes late in the core ABP. This finding, along with multiple studies suggesting that the losses of floral anthocyanins in *Aquilegia* is due to single QTL (Prazmo 1965; Taylor 1984; Hodges *et al.* 2002), point to mutations in translational regulators as common causes of these evolutionary transitions. However, ideally, all the

genes in the ABP, the pathways that intersect with the ABP, and their translational regulators should be evaluated. Using the AqGI and RT-PCR, Hodges & Derieg (2009) identified 34 candidate genes for the entire flavonoid pathway and its regulators. To monitor the expression of all of these genes with traditional methods such as RT-PCR would be a daunting task. However, with the advent of next-generation sequencing, it should be possible to monitor both the expression and sequence variation of all of these genes simultaneously by sequencing directly from mRNA. Another future direction will be to establish that the candidate genes, especially the translational regulators, function as predicted. This could be accomplished using VIGS to target these genes and determining if anthocyanin production is suppressed (Gould & Kramer 2007).

While the shape and colour of the petals of *Aquilegia* clearly have been central to adaptation to pollinators and speciation in the genus, the evolution of the nectar spur itself is especially intriguing. The origin of nectar spurs across numerous groups of flowering plants has been correlated with species diversification and termed a 'key innovation' (Hodges & Arnold 1995; Hodges 1997a,b; Kay *et al.* 2006a,b). Thus, as noted above, an important goal is to identify the genetic programme that generates the spur itself. Prazmo (1961) performed now classic experiments by crossing the spurless columbine, *Aquilegia ecalcarata*, and spurred species. When she classified F2 offspring from these crosses as spurred/non-spurred, Prazmo found simple Mendelian ratios, suggesting that only one or two QTL were responsible for the presence of a spur. Plants that did possess petal spurs had a broad range of spur length, suggesting a more complex and polygenic control of this aspect of spur development. If *A. ecalcarata* represents the ancestral character state of lacking spurs, then these experiments would indicate that spurs could evolve through one or two mutations (Gottlieb 1984; Orr & Coyne 1992). However, phylogenetic analysis suggests that *A. ecalcarata* is nested within *Aquilegia* and that it represents the loss of petal spurs rather than the ancestral state (Hodges & Arnold 1995). Thus the origin of nectar spurs may have required a more complex set of genetic changes. Regardless, comparisons of gene expression patterns between *A. ecalcarata* and spurred species, as well as between progeny of segregating populations will probably provide strong candidates for important genes involved with the development of this novel trait.

6. DEEPER QUESTIONS ABOUT THE ASSESSMENT OF HOMOLOGY IN PETALS

As discussed in §1, petals are thought to have evolved many times independently across the angiosperms. These ideas about independent derivations, particularly in regards to andropetals, have been greatly elaborated in the family Ranunculaceae. Starting with the earliest morphological studies, the second whorl organs of many members of the family were set apart as 'nectar leaves' or, more poetically, 'honey leaves' (Prantl 1887). Although these organs are sterile

and positioned in the second whorl of the flower, thereby fitting the broader definition of petals, they were considered to have evolved completely independently on many occasions, each case resulting from a sterilization of outer stamens (Prantl 1887; Worsdell 1903; Hiepko 1965; Tamura 1965). Evidence for these derivations is drawn from vascular patterns, developmental and morphological similarities, and homeotic interconversions. Such explicit hypotheses make the Ranunculaceae a very attractive group for investigating the genetic evidence for independent petal derivations. One caveat, however, is the problem with interpreting expression of B gene homologues—does 'conserved' expression support a deeply conserved petal identity programme or does it simply indicate independent recruitment of homologous genes? In the case of *Aquilegia*, there are two important factors. First, we have moved beyond mere expression data to demonstrate that the B gene homologues are essential to petal identity (Kramer *et al.* 2007). Second, the *AP3* duplications provide us with a unique and fortuitous marker for petal identity: the *AqAP3-3* orthologue is petal specific in its expression. This condition could have arisen in several different ways, however. If the petals of *Aquilegia* evolved independently relative to those of other Ranunculaceae, then *AqAP3-3* could have been recruited to its petal-specific domain quite recently. Under this model, we would not expect orthologues of *AqAP3-3* to have similar expression patterns in other genera. On the other hand, if a commonly inherited genetic programme controls petals in the Ranunculaceae, then other genera could show the same petal-specific expression.

We used RT-PCR to survey gene expression patterns among orthologues of *AqAP3-1*, *-2* and *-3* across 13 members of three families of the Ranunculales, which was combined with previous studies of seven genera from additional ranunculid families. We found that among taxa bearing petals, seven of eight genera of the Ranunculaceae and both of the surveyed Berberidaceae genera have *AqAP3-3* orthologues that are specifically expressed in the petals (Rasmussen *et al.* 2009). Furthermore, members of this gene lineage tend not to be expressed in flowers that lack petals, such as those of *Caltha* and *Thalictrum*. We believe that the most parsimonious explanation for this pattern is that a commonly inherited petal identity programme is functioning across the Ranunculaceae and Berberidaceae families. Under this model, the apetalous taxa of the two families probably represent petal loss events rather than an ancestral condition. One important caveat, however, is that there is at least one case where petals have clearly reappeared from an apetalous ancestor, that being the *Atragene* and *Naraveliopsis* sections of the genus *Clematis* (Miikeda *et al.* 2006).

This hypothesis regarding process homology among Ranunculaceae petals contradicts over a century of botanical theory and, therefore, must be thoroughly evaluated. Functional tests on *Aquilegia AqAP3-3* as well as its orthologues in several other genera would be ideal. At the same time, it is equally important to reconsider certain typologies regarding the 'nectar leaves' of the Ranunculaceae. For example, the

common presence of nectaries on Ranunculaceae petals has been considered a feature that associates the petals with the stamens (Prantl 1887; Worsdell 1903). However, nectaries are never present on the stamens of flowers that lack petals (Tamura 1993), which would seem to suggest that their common presence on petals is a trait that associates the petals with each other rather than the petals with stamens. Given our already detailed understanding of plant developmental genetics, it is a timely endeavour to re-evaluate such long-held concepts, which were largely developed to provide structure to a field that had few consistent characters to use for assessments of the homology and evolutionary derivation of floral organs.

7. CONCLUDING REMARKS

Aquilegia is not simply a model species but a model genus, with up to 70 different species that can be used to address a wide range of biological questions. We have focused on the evolution and ecology of petals and petaloid organs since they offer an opportunity to explore the full spectrum of genetics to morphology to evolutionary diversification to ecology of biotic interactions. However, future research into the evolutionary genetics of nectar spurs and floral colour promises even more exciting results. Of course, there is more to *Aquilegia* than just petals. In addition to the novel staminodia, their compound leaves, cymose inflorescences, vernalization response, soil adaptations and perenniality are all worthy of investigation. The development of genomic data for a wide range of *Aquilegia* species will allow these characteristics to be fully explored by a growing community of researchers.

We thank the anonymous reviewers for comments on the manuscript.

Funding. Work described here was funded by NSF-EF grant 0412727 to S.H. and E.M.K., DEB grant 0309378 to S.H. and NSF-IBN grants 0319103 and 0720240 to E.M.K.

REFERENCES

- Abouheif, E., Akam, M., Dickinson, W. J., Holland, P. W. H., Meyer, A., Patel, N. H., Raff, R. A., Roth, V. L. & Wray, G. A. 1997 Homology and developmental genes. *Trends Genet.* **13**, 432–433. (doi:10.1016/S0168-9525(97)01271-7)
- Abzhanov, A., Extavour, C. G., Groover, A., Hodges, S. A., Hoekstra, H. E., Kramer, E. M. & Monteiro, A. 2008 Are we there yet? Tracking the development of new model systems. *Trends Genet.* **24**, 353–360. (doi:10.1016/j.tig.2008.04.002)
- APG 2003 An update of the Angiosperm Phylogeny Group classification for the orders and families of flowering plants: APG II. *Bot. J. Linn. Soc.* **141**, 399–436. (doi:10.1046/j.1095-8339.2003.t01-1-00158.x)
- Baum, D. A. & Whitlock, B. A. 1999 Genetic clues to petal evolution. *Curr. Biol.* **9**, 525–527.
- Becker, A. & Theissen, G. 2003 The major clades of MADS-box genes and their role in the development and evolution of flowering plants. *Mol. Phylogenet. Evol.* **29**, 464–489. (doi:10.1016/S1055-7903(03)00207-0)
- Bernardello, G. 2007 A systematic survey of floral nectaries. In *Nectaries and nectar* (eds S. W. Nicolson, M. Nepi & E. Pacini), pp. 19–128. Dordrecht, The Netherlands: Springer.
- Bierhorst, D. W. 1971 *Morphology of vascular plants*. New York, NY: Macmillan.
- Bowman, J. L. 1997 Evolutionary conservation of angiosperm flower development at the molecular and genetic levels. *J. Biosci.* **22**, 515–527. (doi:10.1007/BF02703197)
- Bowman, J. L., Smyth, D. R. & Meyerowitz, E. M. 1989 Genes directing flower development in *Arabidopsis*. *Plant Cell* **1**, 37–52. (doi:10.1105/tpc.1.1.37)
- Brunet, J. 2009 Pollinators of the Rocky Mountain columbine: temporal variation, functional groups and associations with floral traits. *Ann. Bot.* **103**, 1567–1578. (doi:10.1093/aob/mcp096)
- Burch-Smith, T. M., Anderson, J. C., Martin, G. B. & Dinesh-Kumar, S. P. 2004 Applications and advantages of virus-induced gene silencing for gene function studies in plants. *Plant J.* **39**, 734–746. (doi:10.1111/j.1365-313X.2004.02158.x)
- Chase, V. C. & Raven, P. H. 1975 Evolutionary and ecological relationships between *Aquilegia formosa* and *Aquilegia pubescens* (Ranunculaceae), 2 perennial plants. *Evolution* **29**, 474–486. (doi:10.2307/2407260)
- Coen, E. S. & Meyerowitz, E. M. 1991 The war of the whorls: genetic interactions controlling flower development. *Nature* **353**, 31–37. (doi:10.1038/353031a0)
- Darwin, C. 1862 *The various contrivances by which orchids are fertilized by insects*. London, UK: John Murray.
- Davies, B., Cartolano, M. & Schwarz-Sommer, Z. 2006 Flower development: the *Antirrhinum* perspective. *Adv. Bot. Res.* **44**, 279–321. (doi:10.1016/S0065-2296(06)44007-6)
- Drews, G. N., Bowman, J. L. & Meyerowitz, E. M. 1991 Negative regulation of the *Arabidopsis* homeotic gene *AGAMOUS* by the *APETELA2* product. *Cell* **65**, 991–1002. (doi:10.1016/0092-8674(91)90551-9)
- Eames, A. J. 1961 *Morphology of the angiosperms*. New York, NY: McGraw-Hill.
- Endress, P. K. 1994 *Diversity and evolutionary biology of tropical flowers*. Cambridge, UK: Cambridge University Press.
- Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y.-L. & Postlethwait, J. 1999 Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* **151**, 1531–1545.
- Fulton, M. & Hodges, S. A. 1999 Floral isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proc. R. Soc. Lond. B* **266**, 2247–2252. (doi:10.1098/rspb.1999.0915)
- Gaudin, V., Lunness, P. A., Fobert, P. R., Towers, M., Riou-Khamlichi, C., Murray, J. A. H., Coen, E. & Doonan, J. H. 2000 The expression of D-cyclin genes defines distinct development zones in snapdragon apical meristems and is locally regulated by the *Cycliadea* gene. *Plant Phys.* **122**, 1137–1148. (doi:10.1104/pp.122.4.1137)
- Geuten, K., Becker, A., Kaufmann, K., Caris, P., Janssens, S., Viaene, T., Theissen, G. & Smets, E. 2006 Petaloidy and petal identity MADS-box genes in the balsaminoid genera *Impatiens* and *Marcgravia*. *Plant J.* **47**, 501–518.
- Glover, B., Perez-Rodriguez, M. & Martin, C. 1998 Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor. *Development* **125**, 3497–3508.
- Goethe, J. W. V. 1790 *Versuch die Metamorphose der Pflanzen zu erklären*. Gotha, Germany: C. W. Ettinger.
- Golz, J. F., Keck, E. J. & Hudson, A. 2002 Spontaneous mutations in KNOX genes give rise to a novel floral structure in *Antirrhinum*. *Curr. Biol.* **12**, 515–522. (doi:10.1016/S0960-9822(02)00721-2)
- Goto, K. & Meyerowitz, E. M. 1994 Function and regulation of the *Arabidopsis* floral homeotic gene

- PISTILLATA*. *Genes Dev.* **8**, 1548–1560. (doi:10.1101/gad.8.13.1548)
- Gottlieb, L. D. 1984 Genetics and morphological evolution in plants. *Am. Nat.* **123**, 681–709.
- Gould, B. & Kramer, E. M. 2007 Virus-induced gene silencing as a tool for functional analyses in the emerging model plant *Aquilegia* (columbine, Ranunculaceae). *Plant Meth.* **3**, 6. (doi:10.1186/1746-4811-3-6)
- Grant, V. 1952 Isolation and hybridization between *Aquilegia formosa* and *A. pubescens*. *Aliso* **2**, 341–360.
- Grant, V. 1976 Isolation between *Aquilegia formosa* and *Aquilegia pubescens*—reply and reconsideration. *Evolution* **30**, 625–628. (doi:10.2307/2407586)
- Hieppo, P. 1965 Vergleichend-morphologische und entwicklungsgeschichtliche Untersuchungen über das Perianth bei den Polycarpiceae. *Bot. Jahrb. Syst.* **84**, 359–426.
- Hileman, L. C. & Irish, V. F. 2009 More is better: the uses of developmental genetic data to reconstruct perianth evolution. *Am. J. Bot.* **96**, 83–95. (doi:10.3732/ajb.0800066)
- Hodges, S. A. 1997a Floral nectar spurs and diversification. *Int. J. Plant Sci.* **158**, S81–S88. (doi:10.1086/297508)
- Hodges, S. A. 1997b Rapid radiation due to a key innovation in columbines. In *Molecular evolution and adaptive radiation* (eds T. J. Givnish & K. J. Sytsma), pp. 391–405. Cambridge, UK: Cambridge University Press.
- Hodges, S. A. & Arnold, M. L. 1994a Columbines—a geographically widespread species flock. *Proc. Natl Acad. Sci. USA* **91**, 5129–5132. (doi:10.1073/pnas.91.11.5129)
- Hodges, S. A. & Arnold, M. L. 1994b Floral and ecological isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Proc. Natl Acad. Sci. USA* **91**, 2493–2496. (doi:10.1073/pnas.91.7.2493)
- Hodges, S. A. & Arnold, M. L. 1995 Spurring plant diversification: are floral nectar spurs a key innovation? *Proc. R. Soc. Lond. B* **262**, 343–348. (doi:10.1098/rspb.1995.0215)
- Hodges, S. A. & Derieg, N. J. 2009 Adaptive radiations: from field to genomic studies. *Proc. Natl Acad. Sci. USA* **106**, 9947–9954. (doi:10.1073/pnas.0901594106)
- Hodges, S. A. & Whittall, J. B. 2008 One-sided evolution or two? A reply to Ennos. *Heredity* **100**, 541–542. (doi:10.1038/hdy.2008.12)
- Hodges, S. A., Whittall, J. B., Fulton, M. & Yang, J. Y. 2002 Genetics of floral traits influencing reproductive isolation between *Aquilegia formosa* and *Aquilegia pubescens*. *Am. Nat.* **159**, S51–S60.
- Hodges, S. A., Fulton, M., Yang, J. Y. & Whittall, J. B. 2004 Verne Grant and evolutionary studies of *Aquilegia*. *New Phytol.* **161**, 113–120. (doi:10.1046/j.1469-8137.2003.00950.x)
- Hoekstra, H. E. & Coyne, J. A. 2007 The locus of evolution: evo devo and the genetics of adaptation. *Evolution* **61**, 995–1016. (doi:10.1111/j.1558-5646.2007.00105.x)
- Hoot, S. B., Magallon, S. & Crane, P. R. 1999 Phylogeny of basal eudicots based on three molecular data sets: *atpB*, *rbcL* and 18S nuclear ribosomal DNA sequences. *Ann. Mo. Bot. Gard.* **86**, 1–32. (doi:10.2307/2666215)
- Irish, V. 2008 The *Arabidopsis* petal: a model for plant organogenesis. *Trends Plant Sci.* **13**, 430–436. (doi:10.1016/j.tplants.2008.05.006)
- Irish, V. 2009 Evolution of petal identity. *J. Exp. Bot.* **60**, 2517–2527. (doi:10.1093/jxb/erp159)
- Jack, T., Brockman, L. L. & Meyerowitz, E. M. 1992 The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell* **68**, 683–697. (doi:10.1016/0092-8674(92)90144-2)
- Jaramillo, M. A. & Kramer, E. M. 2004 *APETALA3* and *PISTILLATA* homologs exhibit novel expression patterns in the unique perianth in *Aristolochia* (Aristolochiaceae). *Evol. Dev.* **6**, 449–458. (doi:10.1111/j.1525-142X.2004.04053.x)
- Jaramillo, M. A. & Kramer, E. M. 2007 The role of developmental genetics in understanding homology and morphological evolution in plants. *Int. J. Plant Sci.* **168**, 61–72. (doi:10.1086/509078)
- Kay, K. M., Voelckel, C., Yang, J. Y., Hufford, K. M., Kaska, D. D. & Hodges, S. A. 2006a Floral characters and species diversification. In *Ecology and evolution of flowers* (eds L. Harder & C. H. Spencer), pp. 311–325. Oxford, UK: Oxford University Press.
- Kay, K. M., Whittall, J. B. & Hodges, S. A. 2006b A survey of nuclear ribosomal internal transcribed spacer substitution rates across angiosperms: an approximate molecular clock with life history effects. *BMC Evol. Biol.* **6**. (doi:10.1186/1471-2148-6-36)
- Keck, E., McSteen, P., Carpenter, R. & Coen, E. 2003 Separation of genetic functions controlling organ identity in flowers. *EMBO* **22**, 1058–1066.
- Kim, S., Yoo, M., Albert, V. A., Farris, J. S., Soltis, P. S. & Soltis, D. E. 2004 Phylogeny and diversification of B-function genes in angiosperms: evolutionary and functional implications of a 260-million year old duplication. *Am. J. Bot.* **91**, 2102–2118. (doi:10.3732/ajb.91.12.2102)
- Kramer, E. M. 2006 Floral patterning and control of floral organ formation. In *Flowering and its manipulation*, vol. 20 (ed. C. Ainsworth), pp. 49–70. Oxford, UK: Blackwell Publishing.
- Kramer, E. M. 2009 *Aquilegia*: a new model for plant development, ecology, and evolution. *Annu. Rev. Plant Biol.* **60**, 261–277. (doi:10.1146/annurev.arplant.043008.092051)
- Kramer, E. M. & Irish, V. F. 2000 Evolution of the petal and stamen developmental programs: evidence from comparative studies of the lower eudicots and basal angiosperms. *Int. J. Plant Sci.* **161**, S29–S40. (doi:10.1086/317576)
- Kramer, E. M. & Jaramillo, M. A. 2005 The genetic basis for innovations in floral organ identity. *J. Exp. Zool. (Mol. Dev. Evol.)* **304B**, 526–535.
- Kramer, E. M. & Zimmer, E. A. 2006 Gene duplication and floral developmental genetics of basal eudicots. In *Advances in botanical research: developmental genetics of the flower*, vol. 44 (eds D. E. Soltis, P. S. Soltis & J. Leebens-Mack), pp. 354–385. London, UK: Academic Press.
- Kramer, E. M., Dorit, R. L. & Irish, V. F. 1998 Molecular evolution of genes controlling petal and stamen development: duplication and divergence within the *APETALA3* and *PISTILLATA* MADS-box gene lineages. *Genetics* **149**, 765–783.
- Kramer, E. M., Di Stilio, V. S. & Schluter, P. 2003 Complex patterns of gene duplication in the *APETALA3* and *PISTILLATA* lineages of the Ranunculaceae. *Int. J. Plant Sci.* **164**, 1–11. (doi:10.1086/344694)
- Kramer, E. M., Jaramillo, M. A. & Di Stilio, V. S. 2004 Patterns of gene duplication and functional evolution during the diversification of the *AGAMOUS* subfamily of MADS-box genes in angiosperms. *Genetics* **166**, 1011–1023. (doi:10.1534/genetics.166.2.1011)
- Kramer, E. M., Su, H.-J., Wu, J. M. & Hu, J. M. 2006 A simplified explanation for the frameshift mutation that created a novel C-terminal motif in the *APETALA3* gene lineage. *BMC Evol. Biol.* **6**, 30. (doi:10.1186/1471-2148-6-30)
- Kramer, E. M., Holappa, L., Gould, B., Jaramillo, M. A., Setnikov, D. & Santiago, P. 2007 Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot *Aquilegia* (Ranunculaceae). *Plant Cell* **19**, 750–766. (doi:10.1105/tpc.107.050385)

- Krizek, B. A., Prost, V. & Macias, A. 2000 *AINTEGUMENTA* promotes petal identity and acts as a negative regulator of *AGAMOUS*. *Plant Cell* **12**, 1357–1366. (doi:10.1105/tpc.12.8.1357)
- Lamb, R. S. & Irish, V. F. 2003 Functional divergence within the *APETALA3/PISTILLATA* floral homeotic gene lineages. *Proc. Natl Acad. Sci. USA* **100**, 6558–6563. (doi:10.1073/pnas.0631708100)
- Lee, J. Y., Baum, S. F., Oh, S. H., Jiang, C. Z., Chen, J. C. & Bowman, J. L. 2005 Recruitment of CRABS CLAW to promote nectary development within the eudicot clade. *Development* **132**, 5021–5032. (doi:10.1242/dev.02067)
- Litt, A. 2007 An evaluation of A-function: evidence from the *APETALA1* and *APETALA2* gene lineages. *Int. J. Plant Sci.* **168**, 73–91. (doi:10.1086/509662)
- Litt, A. & Irish, V. F. 2003 Duplication and diversification in the *APETALA1/FRUITFULL* floral homeotic gene lineage: implications for the evolution of floral development. *Genetics* **165**, 821–833.
- Liu, Y., Nakayama, N., Schiff, M., Litt, A., Irish, V. F. & Dinesh-Kumar, S. P. 2004 Virus induced gene silencing of a *DEFICIENS* ortholog in *Nicotiana benthamiana*. *Plant Mol. Biol.* **54**, 701–711. (doi:10.1023/B:PLAN.0000040899.53378.83)
- Mara, C. & Irish, V. 2008 Two GATA transcription factors are downstream effectors of floral homeotic gene action in *Arabidopsis*. *Plant Phys.* **147**, 707–718. (doi:10.1104/pp.107.115634)
- Martin, C. & Gerats, T. 1993 Control of pigment biosynthesis genes during petal development. *Plant Cell* **5**, 1253–1264. (doi:10.1105/tpc.5.10.1253)
- Martin, C., Bhatt, K., Baumann, E., Jin, H., Zachgo, S., Roberts, K., Schwarz-Sommer, Z., Glover, B. & Perez-Rodriguez, M. 2002 The mechanics of cell fate determination in petals. *Phil. Trans. R. Soc. Lond. B* **357**, 809–813. (doi:10.1098/rstb.2002.1089)
- Miikeda, O., Kita, K., Handa, T. & Yukawa, T. 2006 Phylogenetic relationships of *Clematis* (Ranunculaceae) based on chloroplast and nuclear DNA sequences. *Bot. J. Linn. Soc.* **152**, 153–168.
- Miller, R. B. 1981 Hawkmoths and the geographic patterns of floral variation in *Aquilegia caerulea*. *Evolution* **35**, 763–774. (doi:10.2307/2408246)
- Noda, K., Glover, B. J., Linstead, P. & Martin, C. 1994 Flower color intensity depends on specialized cell-shape controlled by a Myb-related transcription factor. *Nature* **369**, 661–664. (doi:10.1038/369661a0)
- Okamuro, J. K., Caster, B., Villarroel, R., van Montagu, M. & Jofuku, K. D. 1997 The AP2 domain of *APETALA2* defines a large new family of DNA binding proteins in *Arabidopsis*. *Proc. Natl Acad. Sci.* **94**, 7076–7081. (doi:10.1073/pnas.94.13.7076)
- Orr, H. A. & Coyne, J. A. 1992 The genetics of adaptation—a reassessment. *Am. Nat.* **140**, 725–742.
- Park, J.-H., Ishikawa, Y., Yoshida, R., Kanno, A. & Kameya, T. 2003 Expression of *AODEF*, a B-functional MADS-box gene, in stamens and inner tepals of the dioecious species *Asparagus officinalis* L. *Plant Mol. Biol.* **51**, 867–875. (doi:10.1023/A:1023097202885)
- Park, J.-H., Ishikawa, Y., Ochiai, H., Kanno, A. & Kameya, T. 2004 Two *GLOBOSA*-like genes are expressed in second and third whorls of homochlamydeous flowers in *Asparagus officinalis* L. *Plant Cell Phys.* **45**, 325–332. (doi:10.1093/pcp/pch040)
- Piwarzyk, E., Yang, Y. & Jack, T. 2007 Conserved C-terminal motifs of the *Arabidopsis* proteins *APETALA3* and *PISTILLATA* are dispensable for floral organ identity function. *Plant Phys.* **145**, 1495–1505. (doi:10.1104/pp.107.105346)
- Prantl, K. 1887 Beitrage zur Morphologie und Systematik der Ranunculaceen. *Bot. Jahrb. Syst.* **9**, 225–273.
- Prazmo, W. 1961 Cytogenetic studies on the genus *Aquilegia* II. Crosses between *Aquilegia ecalcarata* Maxim. and *Aquilegia chrysantha* Gray. *Acta Soc. Bot. Pol.* **30**, 57–77.
- Prazmo, W. 1965 Cytogenetic studies on the genus *Aquilegia*. III. Inheritance of the traits distinguishing different complexes in the genus *Aquilegia*. *Acta Soc. Bot. Pol.* **34**, 403–437.
- Preston, J. C. & Hileman, L. C. 2009 Developmental genetics of floral symmetry evolution. *Trends Plant Sci.* **14**, 147–154. (doi:10.1016/j.tplants.2008.12.005)
- Rasmussen, D. E., Kramer, E. M. & Zimmer, E. A. 2009 One size fits all? Molecular evidence for a commonly inherited petal identity program in the Ranunculales. *Am. J. Bot.* **96**, 1–14.
- Rauscher, M. D. 2008 Evolutionary transitions in floral color. *Int. J. Plant Sci.* **169**, 7–21. (doi:10.1086/523358)
- Ro, K. E. & McPheron, B. A. 1997 Molecular phylogeny of the *Aquilegia* group (Ranunculaceae) based on internal transcribed spacers and 5.8S nuclear ribosomal DNA. *Biochem. Syst. Ecol.* **25**, 445–461. (doi:10.1016/S0305-1978(97)00029-X)
- Rosin, F. M. & Kramer, E. M. 2009 Old dogs, new tricks: regulatory evolution in conserved genetic modules leads to novel morphologies in plants. *Dev. Biol.* **332**, 25–35. (doi:10.1016/j.ydbio.2009.05.542)
- Sablowski, R. W. M. & Meyerowitz, E. M. 1998 A homolog of *NO APICAL MERISTEM* is an immediate target of the floral homeotic genes *APETALA3/PISTILLATA*. *Cell* **92**, 93–103. (doi:10.1016/S0092-8674(00)80902-2)
- Schwinn, K. et al. 2006 A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant Cell* **18**, 831–851. (doi:10.1105/tpc.105.039255)
- Smith, G. H. 1928 Vascular anatomy of ranalian flowers. *Bot. Gaz.* **82**, 85, 1–29. 152–177.
- Stellari, G. M., Jaramillo, M. A. & Kramer, E. M. 2004 Evolution of the *APETALA3* and *PISTILLATA* lineages of MADS-box containing genes in basal angiosperms. *Mol. Biol. Evol.* **21**, 506–519. (doi:10.1093/molbev/msh044)
- Streisfeld, M. A. & Rauscher, M. D. 2009 Altered trans-regulatory control of gene expression in multiple anthocyanin genes contributes to adaptive flower color evolution in *Mimulus aurantiacus*. *Mol. Biol. Evol.* **26**, 433–444. (doi:10.1093/molbev/msn268)
- Takhtajan, A. 1991 *Evolutionary trends in flowering plants*. New York, NY: Columbia University Press.
- Tamura, M. 1965 Morphology, ecology, and phylogeny of the Ranunculaceae IV. *Sci. Rep. Osaka Univ.* **14**, 53–71.
- Tamura, M. 1993 Ranunculaceae. In *The families and genera of vascular plants. 2. Flowering plants. Dicotyledons. Magnoliid, Hamamelid and Caryophyllid families*, vol. 2 (eds K. Kubitzki, J. G. Rohwer & V. Bittrich), pp. 563–583. Berlin, Germany: Springer.
- Taylor, R. J. 1984 Floral anthocyanins of *Aquilegia* and their relationship to distribution and pollination biology of the species. *Bull. Torrey Bot. Club* **111**, 462–468. (doi:10.2307/2995896)
- Tucker, S. C. & Hodges, S. A. 2005 Floral ontogeny of *Aquilegia*, *Semiaquilegia*, and *Isopyrum* (Ranunculaceae). *Int. J. Plant Sci.* **166**, 557–574. (doi:10.1086/429848)
- Vandenbussche, M., Theissen, G., Van de Peer, Y. & Gerats, T. 2003 Structural diversification and neo-functionalization during floral MADS-box gene evolution by C-terminal frameshift mutations. *Nucl. Acids Res.* **31**, 4401–4409. (doi:10.1093/nar/gkg642)
- Walker-Larsen, J. & Harder, L. D. 2000 The evolution of staminodes in angiosperms: patterns of stamen reduction,

- loss, and functional re-invention. *Am. J. Bot.* **87**, 1367–1384. (doi:10.2307/2656866)
- Wang, Z. *et al.* 2008 Genetic control of floral zygomorphy in pea (*Pisum sativum* L.). *Proc. Natl Acad. Sci. USA* **105**, 10 414–10 419. (doi:10.1073/pnas.0803291105)
- Wasserthal, L. T. 1997 The pollinators of the Malagasy star orchids *Angraecum sesquipedale*, *A. sororium* and *A. compactum* and the evolution of extremely long spurs by pollinator shift. *Bot. Acta* **110**, 343–359.
- Weigel, D. 1995 The *APETALA2* domain is related to a novel type of DNA binding domain. *Plant Cell* **7**, 388–389. (doi:10.1105/tpc.7.4.388)
- Whibley, A. C., Langlade, N. B., Andalo, C., Hanna, A. I., Bangham, A., Thebaud, C. & Coen, E. 2006 Evolutionary paths underlying flower color variation in *Antirrhinum*. *Science* **313**, 963–966. (doi:10.1126/science.1129161)
- Whitney, H. M., Chittka, L., Bruce, T. J. A. & Glover, B. J. 2009 Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. *Curr. Biol.* **19**, 948–953. (doi:10.1016/j.cub.2009.04.051)
- Whittall, J. B. & Hodges, S. A. 2007 Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**, 706–710. (doi:10.1038/nature05857)
- Whittall, J. B., Medina-Marino, A., Zimmer, E. A. & Hodges, S. A. 2006a Generating single-copy nuclear gene data for a recent adaptive radiation. *Mol. Phylogenet. Evol.* **39**, 124–134. (doi:10.1016/j.ympev.2005.10.010)
- Whittall, J. B., Voelckel, C., Kliebenstein, D. J. & Hodges, S. A. 2006b Convergence, constraint and the role of gene expression during adaptive radiation: floral anthocyanins in *Aquilegia*. *Mol. Ecol.* **15**, 4645–4657. (doi:10.1111/j.1365-294X.2006.03114.x)
- Worsdell, W. C. 1903 The origin of the perianth of flowers, with special reference to the Ranunculaceae. *New Phyt.* **2**, 42–48.
- Xu, L., Chen, H., Hu, X. H., Zhang, R. M., Zhang, Z. & Luo, Z. W. 2006 Average gene length is highly conserved in prokaryotes and eukaryotes and diverges only between the two kingdoms. *Mol. Biol. Evol.* **23**, 1107–1108. (doi:10.1093/molbev/msk019)
- Yang, J. Y., Counterman, B. A., Eckert, C. G. & Hodges, S. A. 2005 Cross-species amplification of microsatellite loci in *Aquilegia* and *Semiaquilegia* (Ranunculaceae). *Mol. Ecol. Notes* **5**, 317–320. (doi:10.1111/j.1471-8286.2005.00913.x)
- Zachgo, S., de Andrade Silva, E., Motte, P., Trobner, W., Saedler, H. & Schwarz-Sommer, Z. 1995 Functional analysis of the *Antirrhinum* floral homeotic *DEFICIENS* gene in vivo and in vitro by using a temperature-sensitive mutant. *Development* **121**, 2861–2875.
- Zahn, L. M., Kong, H., Leebens-Mack, J. H., Kim, S., Soltis, P. S., Landherr, L. L., Soltis, D. E., Depamphilis, C. W. & Ma, H. 2005a The evolution of the *SEPALLATA* subfamily of MADS-Box genes: a preangiosperm origin with multiple duplications throughout angiosperm history. *Genetics* **169**, 2209–2223. (doi:10.1534/genetics.104.037770)
- Zahn, L. M., Leebens-Mack, J., dePamphilis, C. W., Ma, H. & Theissen, G. 2005b To B or not to B a flower: the role of *DEFICIENS* and *GLOBOSA* orthologs in the evolution of the angiosperms. *J. Hered.* **96**, 225–240. (doi:10.1093/jhered/esi033)
- Zanis, M., Soltis, P. S., Qiu, Y.-L., Zimmer, E. A. & Soltis, D. E. 2003 Phylogenetic analyses and perianth evolution in basal angiosperms. *Ann. Mo. Bot. Gard.* **90**, 129–150. (doi:10.2307/3298579)