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**ONE SIZE FITS ALL? MOLECULAR EVIDENCE FOR A COMMONLY INHERITED PETAL IDENTITY PROGRAM IN RANUNCULALES.**

**DAVID A. RASMUSSEN**, **ELENA M. KRAMER**, and **ELIZABETH A. ZIMMER**

Dept. of Organismic and Evolutionary Biology, Harvard University, Cambridge MA 02138

2 Current Address: Dept. of Biology, Duke University, Durham, NC 27708

4 Current Address: Department of Botany and Laboratories of Analytical Biology, Museum Support Center, Smithsonian Institution, Suitland, MD 20746

3 Author for Correspondence: 16 Divinity Ave, Cambridge MA 02138; ekramer@oeb.harvard.edu; phone: 617-496-3460, fax: 617-496-5854

*These authors contributed equally to this work.
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ABSTRACT

Petaloid organs are a major component of the floral diversity observed across nearly all major clades of angiosperms. The variable morphology and development of these organs has led to the hypothesis that they are not homologous but, rather, have evolved multiple times. A particularly notable example of petal diversity, and potential homoplasy, is found within the order Ranunculales, exemplified by families such as Ranunculaceae, Berberidaceae and Papaveraceae. To investigate the molecular basis of petal identity in Ranunculales, we used a combination of molecular phylogenetics and gene expression analysis to characterize APETALA3 (AP3) and PISTILLATA (PI) homologs from a total of 13 representative genera of the order. One of the most striking results of this study is that orthologs of a single AP3 lineage consistently exhibit petal-specific expression across both Ranunculaceae and Berberidaceae. We conclude from this finding that these supposedly homoplastic petals in fact share a developmental genetic program that appears to have been present in the common ancestor of the two families. We discuss the implications of this type of molecular data for long-held typological definitions of petals and, more broadly, the evolution of petaloid organs across the angiosperms.

Key Words: APETALA3, PISTILLATA, MADS box genes, petal evolution, Ranunculales
INTRODUCTION

The early evolution of angiosperms remains shrouded in mystery in part because we lack a clear understanding of how flowers and their associated organs evolved. Reproductive organs such as stamens and carpels are key morphological innovations in angiosperm biology and it seems likely that they represent modifications of pre-existing structures, although the exact nature of this modification is controversial (Theissen et al., 2002; Baum and Hileman, 2006; Frohlich and Chase, 2007). Most angiosperm flowers have a sterile perianth composed of petaloid and/or protective organs, but unlike stamens and carpels, which are widely believe to have evolved only once, it remains unclear whether petaloid organs evolved once in a common ancestor or independently in different lineages (Baum and Whitlock, 1999; Kramer and Jaramillo, 2005). Since the evolution of petaloid organs facilitated the morphological diversification and ecological specialization of flowers, a resolution to issues regarding their evolutionary origins may provide general insights into early angiosperm diversification.

Character state reconstructions based on modern phylogenetic relationships have shown that petaloid organs likely evolved early during the crown angiosperm radiation (Zanis et al., 2003; Hileman and Irish, this issue pp. ###). Consistent with this, many angiosperms of the ANITA grade, as well as many magnoliids, possess petaloid perianths (Endress, 1994; Endress, 2003). However, while most early angiosperms have a perianth composed entirely of petaloid or weakly differentiated organs known as tepals, other taxa possess a bipartite perianth composed of morphologically distinct sepals and petals. In flowers where the perianth is bipartite, the second whorl of petaloid organs are often thought to resemble sterilized stamens in aspects of their development and morphology (Takhtajan,
Fundamental morphological and developmental differences between these putative staminoid petals, or “andropetals”, and petaloid tepals thought to be derived from bracts, or “bracteopetals”, have been interpreted as evidence against a single derivation of petaloid organs (Eames, 1961; reviewed in Takhtajan, 1991). Furthermore, the evolutionary distribution of andropetals and bracteopetals across different lineages of flowering plants suggest that petaloid organs evolved many times independently (Bierhorst, 1971; Takhtajan, 1991).

One of the most diverse clades in terms of perianth morphology is the eudicot order Ranunculales, particularly the family Ranunculaceae. Many genera of Ranunculaceae have bipartite perianths with second-whorl petals that strongly resemble modified stamens (Tamura, 1965; Kosuge, 1994). Cited similarities between the petals and stamens include their phyllotactic pattern, vasculature, developmental kinetics (e.g., their timing of initiation), appearance of the early primordia, and final morphology. At the same time, the morphology of the petals and the entire perianth varies greatly within the family (Fig 1; Tamura, 1965; Kosuge and Tamura, 1989; Kosuge, 1994). Many taxa actually possess two types of petaloid organs: large, showy sepals in the first whorl and highly variable, often nectiferous petals in the second. This perianth type is exemplified by Aquilegia L., Xanthorrhiza Marshall, and Trollius L. (Fig 1B-D and F-H). However, in genera such as Ranunculus L., petals do not resemble stamens at maturity and the sepals are leaf-like (Fig. 1M-N). At the same time, it is very common to find taxa that have petaloid sepals but lack second whorl petals all together, as in Anemone L. or Caltha L. (Fig. 1I-J). This variation is even observed within genera, such as in Clematis L. where some species possess petals (Fig. 1K-L) while other species are apetalous. It should be noted that in contrast to the term tepal defined above, the outer
perianth organs of the Ranunculaceae are always referred to as sepals, even if they are not part of a truly bipartite perianth (Tamura 1965). The considerable variation observed in petals within and among genera of Ranunculaceae has been considered to be consistent with a homoplastic pattern of petal evolution, with staminoid petals gained independently on multiple occasions (Prantl, 1887; Worsdell, 1903; Hoot, 1991; Kosuge, 1994; Hoot and Crane, 1995).

Despite differences in morphology, molecular studies of floral developmental genetics have provided evidence for a conserved genetic program promoting petaloidy across most of the angiosperms. In core eudicots such as Arabidopsis and Antirrhinum, the B class MADS-box genes APETALA3 (AP3) and PISTILLATA (PI) are critical to the specification of petal and stamen identity within the developing flower (Coen and Meyerowitz, 1991). Comparative genetic studies have shown that AP3/PI homologs are commonly expressed in petaloid organs of other taxa, including monocots, magnoliids and angiosperms of the ANITA grade (reviewed Kim et al., 2005). Outside of the core eudicots this expression is more variable, spatially and temporally, than within the core eudicots (Kim et al, 2005; Kramer and Irish, 1999, 2000; Soltis et al, 2006). However, functional studies in the eudicots Papaver L. and Aquilegia and the grasses Zea L. and Oryza L. indicate that AP3/PI homologs are required for the identity of petals or petal-derived organs (Ambrose et al., 2000; Drea et al., 2007; Kramer et al, 2007; Nagasawa et al., 2003). These findings suggest that the function of AP3/PI genes may be conserved across angiosperms to a large degree, although not invariably so (reviewed in Kramer and Jaramillo, 2005; Kramer and Zimmer, 2006). It therefore seems plausible that even if petaloid organs have been lost and regained in different lineages, a commonly inherited genetic program has played a role in their
evolution. This reflects a kind of process homology that can exist even in the absence of historical homology of the organs (Gilbert and Bolker, 2001). An alternative interpretation of these results is that homologs of the AP3/PI lineage have been independently recruited many times to function in the development of truly independently derived petaloid organs. Under this model, any similarity in the genetic programs controlling petaloidy would be due to convergence rather than common inheritance. Our current difficulty is to clearly distinguish between these models.

A commonly inherited petal identity program could also account for the putatively homoplastic pattern of petal evolution observed within Ranunculaceae. Complex patterns of gene duplication within Ranunculales AP3 lineage may actually help us to determine whether the association of B gene homolog expression with petaloid organs is homologous or convergent. Previous studies have identified three paralogous AP3 lineages, referred to as AP3-I, -II and -III, which were produced by two duplication events that clearly predate the last common ancestor of Ranunculaceae and may have occurred very early in Ranunculales (Kramer, Di Stilio, and Schlüter, 2003). Overall, considerable variation in the expression patterns of these paralogs has been observed, with the AP3-III lineage representing an intriguing pattern (Kramer, Di Stilio, and Schlüter, 2003; Kramer et al., 2007). In Aquilegia, AqAP3-3 expression is petal specific, and across the family the orthologs are generally not expressed in species or mutant cultivars that lack petals (Kramer, Di Stilio, and Schlüter, 2003; Kramer et al., 2007). In Papaver, the AP3-III ortholog is required for petal identity although it is not petal specific in its expression (Drea et al., 2007). These findings raise the possibility that a typical B class function was ancestral in the order and was later subdivided into the petal-
specific expression of the *AP3-III*, which would represent subfunctionalization (sensu Force et al., 1999) of a commonly inherited petal identity program. Unfortunately, current data are not sufficient to clearly support this model.

As a first step towards understanding whether a conserved petal identity program exists within Ranunculaceae (and more broadly, Ranunculales), we identified *AP3* and *PI* homologs from 11 previously unsampled taxa and used RT-PCR to determine the expression patterns of all known homologs in 13 different genera. These target taxa were chosen to represent a broad range of petal and perianth morphology, as well as to span multiple families in the order. While previous studies have focused on the expression patterns of *AP3/PI* genes from distantly related taxa, the current study allows us to use expression patterns to analyze variation in the petal identity program on a narrower scale. Of particular interest is the fact that the *AP3-III* orthologs were found to be largely petal-specific across Ranunculaceae and Berberidaceae. We interpret this finding as evidence for process homology, a commonly inherited petal identity program utilizing *AP3-III*, which contradicts the traditional hypothesis of independently derived petals. The implications of these results for larger questions of petal evolution across the angiosperms are discussed.

**MATERIALS AND METHODS**

**Plant materials** – A broad developmental range of floral tissue was obtained from the following taxa: *Aconitum sinomontanum* Nakai (Ranunculaceae, AA 394-95), *Caltha palustris* L. (Ranunculaceae, EMK 130), *Clematis alpina* L. (Ranunculaceae, AA 337-2006), *Delphinium exaltatum* Aiton (Ranunculaceae, EMK 131), *Xanthorrhiza*
simplicissimina Marshall (Ranunculaceae, AA 17610), Hydrastis canadensis L. (Ranunculaceae, EMK 132), Epimedium grandiflora L. (Berberidaceae, EMK 133), Jeffersonia diphylla L. (Berberidaceae, AA 490-95), Holboellia coriacea Diels (Lardizabalaceae, Scott Arboretum 96-087), Menispermum dauricum DC (Menispermaceae, AA 548-94), and Eupeilea polyandra Siebold & Zucc. (Eupeileaceae, AA 1610-77). Dissected floral organs were photodocumented using a Kontron Elektronik ProgRes 3012 digital camera mounted on a Leica WILD M10 dissecting microscope (Harvard Imaging Center).

**RNA extraction, RT-PCR and Homolog Characterization** – As described in Kramer, Di Stilio, and Schlüter (2003), total RNA and poly(A) RNA were extracted from frozen floral buds and first strand cDNA was prepared. The cDNA was used as template in PCR reactions and the products cloned and analyzed as previously described (Kramer, Di Stilio, and Schlüter, 2003; Stellari, Jaramillo, and Kramer, 2004). Homology of sequenced clones was initially determined using BLAST (Altschul et al., 1997) and putative loci were delimited based on sequence identity and phylogenetic analyses (see below). A total of 21 AP3 and 12 PI homologs were identified in this study (4 of those loci appear to have two alleles) and their sequences are deposited in GenBank under accession numbers EU481781-EU481820.

**Phylogenetic Analysis** – In addition to the 21 new AP3 homologs identified in this study, we assembled a nucleotide dataset of 64 additional homologs based on GenBank accessions: 42 Ranunculales homologs, nine eudicot homologs, nine core
eudicot homologs (both euAP3 and TM6 lineages) and four magnoliid homologs to serve as the outgroup (see Suppl. Table 1 for all accession information and Suppl. File 1 for the final alignment). The alignment was initially compiled using CLUSTALW and subsequently refined manually using MacClade 4.06 (Suppl. File 1; Maddison and Maddison, 2000). Maximum likelihood (ML) phylogenetic analyses were performed using PAUP* (Swofford, 2002). We used Modeltest (Posada and Crandall, 1998) to determine the simplest and most appropriate evolutionary model for our dataset using the Akaike Information Criterion (AIC). The model selected was a general time-reversible model (GTR) with a proportion of invariable sites (I) and a gamma approximation for the rate of variation among sites (Γ). The ML analysis used a single heuristic search with 100 random addition replicates, TBR branch swapping, and MULPARS options (Swofford, 2002). Branch support was estimated by performing 100 replicates of nonparametric bootstrapping (Felsenstein, 1985) using the same parameters as the original analysis. A similar analysis was conducted on a nucleotide alignment of all available Ranunculales PI homologs using the Euptelea homolog EupPI as the designated outgroup (Suppl. File 2). This homolog was chosen due to the fact that Euptelea appears to be sister to all other Ranunculales (Soltis et al., 2003).

**Expression Studies** – Total RNA was extracted from dissected floral organs using PureLink Plant RNA Reagent (Invitrogen, Carlsbad CA). RNA samples with excessive genomic DNA contamination were treated with Turbo™ DNase (Ambion, Austin TX). A small number of samples required further purification using Illustra RNAspin Mini Isolation columns (GE Healthcare Bio-Sciences Corp., Piscataway NJ) to enable robust
PCR amplification. Locus specific RT-PCR was performed as described in Kramer, Di Stilio, and Schlüter (2003). In order to control for template concentration, we made a common master mix with each cDNA sample and aliquoted the mix across multiple primer pairs. For every target locus, specific primers were designed to flank multiple introns (based on conserved MADS gene intron positions Stellari, Jaramillo, and Kramer, 2004; Suppl. Table 2). In the process of designing primers for the PI homologs of *Cimicifuga racemosa* (Kramer, Di Stilio, and Schlüter, 2003), we discovered that what had previously been described as three separate paralogs are more accurately considered two loci with two alleles each. The locus deposited in GenBank as *CirPI-2* appears to be a hybrid of two of these alleles. This error has been corrected and the original “*CirPI-3*” locus has been designated as *CirPI-2* (see Suppl. Table 1 for correct accession numbers).

In two cases, that of *Xanthorhiza simplicissima* and *Berberis gilgiana*, the PI paralogs were judged to be too similar in sequence to accurately distinguish using RT-PCR so the primers were designed to amplify both copies simultaneously. The following PCR program was used: 12 min. at 95°C followed by 25-28 cycles of 1 min. at 95°C, 30 sec. at 55°C and 1 min. at 72°C followed by 10 min. at 72°C. 25-28 cycles was found to be within the linear range of amplification for a diverse selection of primer pairs and templates so this was used throughout the experiment. All RT-PCR products were confirmed initially based on size and subsequently by direct sequencing from the positive control reactions, which used the original floral cDNA from which the loci were isolated. 20 µl of each reaction was run on a 1% agarose gel and digitally photographed using a ChemiDoc™ XRS System (Bio-Rad Laboratories, Hercules CA). All sets of reactions were repeated 2-5 times to test for consistency in amplification.
Character state reconstruction – For the reconstruction of perianth organs across Ranunculales, a composite tree was obtained by assembling published trees of Ranunculales (Hoot, 1995; Hoot, Magallon, and Crane, 1999; Soltis et al., 2003; Kim et al., 2004; Ortiz, Kellogg, and Van der Werff, 2007). We assessed two characters. The first was the phenotype of the first whorl organs, which are generally termed sepals in Ranunculales regardless of the presence of petals (see text for further discussion). There were three character states in this case: absent (0), green (1) or petaloid (2). The second character was absence (0) or presence (1) of petals in the second whorl, which we defined simply as whether there were morphologically distinct, sterile organs positioned between the sepals and stamens. Character states were compiled from a number of different sources: (Cheng-Yih and Kubitzki, 1993; Endress, 1993; Kessler, 1993; Loconte, 1993; Tamura, 1993; Stevens, 2001 onwards; Ren et al., 2004). Ancestral character states were reconstructed in MacClade 4.06 using parsimony with equal cost of state changes to reconstruct all states at each node (Maddison and Maddison, 2000).

For the reconstruction of expression evolution in the AP3 lineage of Ranunculales, the ML tree obtained above was pruned to remove loci for which we did not have expression data. We scored expression of each locus in the sepals, petals and stamens using a simple presence (+ or ++ in Table 1) vs. absence (- or +/- in Table 1) binary code (see also Table 1 legend). For the petals, there was an additional character state for absence of the petals themselves. The expression data were drawn from the current study and several previous analyses (Kramer and Irish, 1999, 2000; Kramer, Di Stilio, and Schüchter, 2003; Shan et al., 2006; Kramer et al., 2007). The ancestral
character states for each organ type were reconstructed using the same technique as described above.

RESULTS

The evolution of the perianth across Ranunculales – Perianth morphology in Ranunculales varies greatly in terms of the showiness of the sepals and the presence or absence of second whorl petals (Fig. 1). In addition, the petals exhibit very diverse morphologies – some closely resembling the neighboring stamens but others are large and showy (Fig. 1). Before considering the evolution of these structures, it is important that we clearly define the terms we will use to discuss them. We use “petaloidy” to indicate general showiness, including bright coloration (other than green). Petaloidy is commonly associated with pollinator attraction but can occur in many different types of organs in different positions within the flower. When we use “sepal” and “petal”, however, we are referring to specific positions in the flower: first whorl for the sepals and second whorl for the petals. As noted above, in the Ranunculaceae the term “sepal” is commonly used to denote outer perianth organs regardless of whether the perianth is bipartite (Tamura 1965; Tamura 1993). For example, the perianth organs of Caltha (Fig. 1I) are called sepals even though petals are absent (Song, Tian, and Ren, 2007). For consistency sake, we have used this terminology for all Ranunculales we examined.

We used a simplified, composite phylogeny of Ranunculales to track changes in the two primary perianth characters: petaloidy of the sepals and presence/absence of the petals. Petaloid sepals are ancestral in most families, evolving after the split with Papaveraceae (Fig. 2). For petals, we simply considered whether there were
morphologically distinct sterile organs positioned between the sepals and stamens. Reconstruction of the presence or absence of petals suggests that they were present at least as far back as the node after the split with the Eupteleaceae, but have been lost many times independently and perhaps regained in isolated cases (Fig. 2). The state of the ancestor of the sister families Ranunculaceae and Berberidaceae is unresolved due to the presence in both families of early branching lineages that lack petals, but we cannot rule out the possibility that *Nandina*, *Glaucidium* and *Hydrastis* represent independent losses. Similarly, in the clade including the tribes Ranunculeae and Anemoneae (small box in Fig. 2), it is unclear whether petals were lost and then regained in *Ranunculus* and the *Clematis* subgenus Atragene (represented by *C. alpina*) or simply lost several times (see Discussion). As discussed above, the petals across the order have been considered homoplasious based on many different morphological and developmental criteria. It is worth noting, however, that many features are shared across the different petal types, including their position, early developmental kinetics, vascular patterning and the presence of nectaries (Kosuge and Tamura, 1989; Endress, 1995; Ronse De Craene and Smets, 1995; Erbar, Kusma, and Leins, 1998). While these characters had previously been used to associate the petals with stamens and support a model of multiple recent derivations, we could also consider them evidence of a commonly inherited syndrome.

**Identification and phylogenetic analysis of B gene homologs** – To better understand whether petals were independently derived or homologous at some level, we sought to characterize their organ identity programs using gene expression patterns. The first step was to identify homologs of the genes normally associated with petal and stamen identity, the MADS box containing genes *APETALA3 (AP3)* and *PISTILLATA*
(PI). We expanded previous sampling of Ranunculales with the addition of 21 new AP3 and 12 new PI homologs from 11 taxa representing five families of the order. The PI homologs exhibit all of the typical characteristics of the lineage and, as has been previously demonstrated (Kramer, Di Stilio, and Schlüter, 2003), have experienced many recent duplication events (Suppl. Fig. 1). Similarly, additional sampling of the AP3 lineage supports earlier studies that found evidence for three deeply conserved, paralogous AP3 lineages within Ranunculales (Kramer, Di Stilio, and Schlüter, 2003). Maximum likelihood analysis of the current dataset recovered a phylogeny with strong support for Ranunculaceae or Ranunculaceae + Berberidaceae clades within these lineages (AP3-I, AP3-II, and AP3-III; Fig. 3). Although the early branching nodes within each lineage do not have strong statistical support, they do reflect the expected taxonomic relationships (Fig. 2). The Euptelea representative, EupAP3, is positioned as sister to the three lineages in the current topology. Again, this position lacks significant statistical support, but it would suggest that two duplication events occurred after the last common ancestor of Eupteleaceae and all other Ranunculales. All three AP3 lineages were not recovered in all taxa. In particular, the AP3-II lineage has not been recovered from any representative of the Papaveraceae and the AP3-III lineage has not been found in the Lardizabalaceae. While the sequencing in this study and previous studies was extensive, it is important to remember that it is based on RT-PCR and therefore detects only expressed genes. These loci may have been genuinely lost from some genomes but it is also possible that that they are just not expressed at appreciable levels.

**Expression analysis of B gene homologs** – The presence of these three ancient AP3 paralogs across Ranunculales has the potential to inform our understanding of petal
evolution within the group. Earlier studies found that representatives of the AP3-III lineage were rarely expressed in taxa lacking petals but were always detected when petals are present (Kramer, Di Stilio, and Schlüter, 2003). The underlying cause of this pattern has become clearer with detailed expression studies of Aquilegia, where the AqvAP3-3 locus is expressed specifically in the petals from inception, with only weak stamen expression detected at very late developmental stages (Kramer et al., 2007). The association between AP3-III expression and petals is generally supported in the current analysis. Anemone nemerosa remains the only taxon studied to date that lacks petals but still expresses an AP3-III ortholog at significant levels. We now have one taxon, Holboellia coriacea, that is described as having petals but in which we cannot recover an AP3-III ortholog (notably, the petals of Holboellia are described as minute; Cheng-Yih and Kubitzki, 1993). If the petals across the Ranunculales are truly independently derived, however, one might not expect to see conservation of the AP3-III/petal correlation. In order to better understand this aspect of petal identity, we conducted a comparative expression study of thirteen taxa from Ranunculaceae, Berberidaceae, and Menispermaceae, which complements previous analyses of Ranunculaceae, Lardizabalaceae and Papaveraceae (Kramer and Irish, 1999, 2000; Kramer, Di Stilio, and Schlüter, 2003; Shan et al., 2006; Kramer et al., 2007). We used locus specific RT-PCR on RNA from dissected floral organs to determine gene expression of all known AP3 and PI homologs in each taxon (Fig. 4 and Suppl. Fig. 2). This approach has the benefit of being quick and relatively easy but sacrifices a level of detail, particularly in terms of temporal dynamics that have been observed in more exhaustive studies using in situ hybridization (Kramer et al., 2007). To control for the extreme sensitivity of a PCR-
based approach, we used relatively low cycle numbers (25-28 cycles) to stay within the linear range of amplification.

Our findings are summarized in Table 1 and Figure 5, together with results from other studies. The results for the *AP3* lineages are of particular interest since they have the potential to inform deeper evolutionary questions. In Figure 5, the evolution of these expression patterns has been reconstructed within the context of the gene lineage phylogeny in Figure 3. We did not map expression in carpels because, although placental and ovule expression of B gene homologs is common (Kramer and Irish, 2000; Kim et al., 2005), no functional role has yet been ascribed to this expression pattern (Jack, Brockman, and Meyerowitz, 1992; Vandenbussche et al., 2004; de Martino et al., 2006; Rijpkema et al., 2006; Kramer et al., 2007). Along these lines, it is unclear whether *AP3/PI* contributes to the petaloidy of the sepals in Ranunculales (Kramer, Di Stilio, and Schlüter, 2003; Kramer et al., 2007), but since this cannot currently be ruled out, sepals were included in the analysis.

There are several noticeable trends for each organ and lineage. Overall, we see that the reconstructed ancestral expression domain is as expected for B class genes – petals and stamens (Fig. 5). Expression in sepals evolved early within both the *AP3-I* and –II lineages but is rarely observed for the *AP3-III* lineage, *Menispermum* being the only exception (Fig. 4). Within the *AP3-I* and –II lineages, sepal expression has been lost many times, representing the most dynamic aspect of *AP3* paralog expression (Fig. 5). These transitions occur even within recent evolutionary timescales. For instance, the *AP3-I* and –II orthologs of *Clematis* differ in their sepal expression between the two sampled species (Fig. 5 and Table 1). There is no obvious correlation between petaloidy
of the sepals and B homolog expression (Fig. 2 and 5), but there are only two taxa in the dataset with non-showy sepals so we do not have the statistical power to test this rigorously. Most taxa express detectable levels of at least one AP3 and one PI paralog in their sepals, regardless of whether these organs are petaloid or not, but in a few cases the AP3 expression is weak (especially Xanthorhiza, Fig. 4 and Cimicifuga, Suppl. Fig. 2).

Expression in petals is common across all homologs, but has been lost in several members of the AP3-II lineage (Delphinium DleAP3-2, Cimicifuga CirAP3-2, Xanthorhiza XsAP3-2; Fig. 4, 5 and Suppl. Fig. 2). One complication of this analysis is that the expression pattern reconstruction recovers petal expression as ancestral for several nodes where the actual presence of petals was equivocal in Fig. 2. Suffice to say, it appears that if petals were present, these genes were expressed, but if petals were not, the genes must have been reactivated when the organ reappeared. As with petals, expression in stamens is almost universal among AP3 homologs, with the major exception of the AP3-III lineage (Fig. 5). In this lineage stamen expression was lost in the common ancestor of Ranunculaceae + Berberidaceae and is not regained until the common ancestor of the tribes Ranunculeae and Anemoneae (asterisk Fig. 5). This strong stamen expression appears to be the reason why AP3-III is detected in the otherwise apetalous Anemone (Suppl. Fig. 2). Thus, the petal-specific expression pattern of the AP3-III lineage was present in the last common ancestor of both Ranunculaceae and Berberidaceae and could represent a synapomorphy that unifies the two families, assuming petals were in fact present in their ancestor.

**DISCUSSION**
The petals of Ranunculales have been considered to be independently derived from stamens many times throughout the order. Previously cited evidence for homoplasious petals include the similarity of stamen and petal primordia at inception, as well as their frequent similarity at maturity; the presence of a single vascular trace; position on the same phyllotactic axis; interconversion by homeosis; and the existence of chimeric intermediates in some taxa (Prantl, 1887; Worsdell, 1903; Tamura, 1965; Kosuge, 1994). At the same time, petals across Ranunculaceae (and Ranunculales) share characteristics that distinguish them from stamens, such as the presence of nectaries and the common development of spurs or pockets (Endress, 1995; Stevens, 2001 onwards). Contrary to the traditional viewpoint, when we map the presence of simply defined petals on the phylogeny of the Ranunculales it appears that they may well have been ancestral in the order with many losses.

Our modern understanding of floral developmental genetics requires us to significantly revise the way we think about the characters used to assess petal homology. First, it has become clear that phyllotaxy, merosity and early primordium development are controlled independently of the floral organ identity program (reviewed in Kramer, 2005) and, therefore, these features can evolve separately from one another. Second, we now know that morphological grades between organ types (e.g., petals into stamens) can be produced by gradients of gene expression (Kunst et al., 1989) and, therefore, may not be related to the evolutionary history of the organs. Last, the ability of homeotic genetic programs to rapidly transform organ identity makes evolutionary transitions between identities, such as stamens and petals, a simpler matter than our traditional models of
gradual modification could envision. Furthermore, this last factor means that positional correspondence and genetic identity can be unlinked in some taxa (reviewed in Jaramillo and Kramer, 2007). In this context terms like andropetals and bracteopetals become less meaningful, as they are based on characteristics that are not necessarily evolving in concert. It must be recognized that these concepts often rely on an “ontogeny (or morphology) recapitulates phylogeny” frame of thinking, which has been seriously questioned in the animal developmental evolution field (largely because of its tendency to promote oversimplification, for extensive discussion see Raff and Kaufman, 1983; Gould, 1985; Raff, 1996).

Given the difficulties of interpreting morphological and developmental characters, we have sought to add molecular characters to the evaluation process, specifically the expression patterns of AP3 paralogs. As discussed above, the positive detection of B gene homolog expression may be of limited utility for homology assessment since the data do not clearly distinguish between a commonly inherited petal identity program and the convergent recruitment of the B gene homologs to this function. In the case of the Ranunculales, however, we have a fortuitous situation that may assist us in this process – there are three ancient AP3 paralogs, one of which (AP3-III) has been shown to be specifically expressed in the petals of Aquilegia (Kramer et al., 2007). Our study finds that such AP3-III expression in petals is the rule rather than the exception across representatives of both Ranunculaceae and Berberidaceae. The pattern breaks down outside of these families. In the few sampled representatives of the Menispermaceae and Papaveraceae, expression is broad, and we have yet to identify an AP3-III ortholog in the Lardizabalaceae (which could be due to either primer mismatch, low expression or actual
loss). The most likely explanation for this pattern of gene expression is that the petals of Ranunculaceae and Berberidaceae share a kind of process homology in the form of a commonly inherited petal identity program that involves orthologs of the AP3-III lineage. While process homology can exist in the absence of true historical homology (Bolker and Raff, 1996; Gilbert and Bolker, 2001), our character state analysis (Fig. 2) also raises the possibility that these organs are genuinely homologous.

Before we accept this conclusion at face value, we should consider the alternatives. If petals evolved completely de novo from stamens on multiple occasions, it would require petal specific expression of the same paralog to have evolved in at least eight separate instances across the two families. Given that AP3-I and –II are typically expressed in stamens, we do not expect that AP3-III orthologs would always evolve to be a petal-specific paralog unless the loci were somehow predisposed to such recruitment. For instance, in animal systems homologs of the Pax6 gene lineage have repeatedly been found to control eye development, even though these structures were long believed to have evolved independently (Treisman, 2004; Gehring, 2005; Kozmik, 2005). One suggested explanation for the observed pattern is that eyes typically evolve at the anterior ends of organisms and perhaps Pax6’s ancestral function is in anterior development, which predisposed it for recruitment for eye development (Harris, 1997). This model preserves the homoplasy of the eyes while explaining their molecular genetic similarity through convergent recruitment of genetic orthologs (although this is not the currently accepted model; Kozmik, 2005). In plants, the genetic control of zygomorphy in the core eudicots appears to have evolved in just this way, repeatedly recruiting homologs of the Antirrhinum gene CYCLOIDEA (Luo et al., 1996; Feng et al., 2006; Busch and Zachgo,
The analogous argument in our case would be that perhaps, early on, *AP3-III* became restricted to the outer whorl of stamens, thereby making it likely to be recruited each time these organs independently evolved into petals. However, this interpretation would lead us to expect that *AP3-III* should be expressed in outer stamens across all taxa, while our data show that the orthologs are *not* typically expressed in flowers that lack petals.

Thus, the most parsimonious explanation for the evolution of this character is the presence of a homologous petal identity program that specifically utilizes *AP3-III* orthologs, likely dating back to the last common ancestor of Ranunculaceae and Berberidaceae. It would seem that when petals are lost, possibly by homeotic transformation into stamens, this petal identity program is turned off and *AP3-III* expression is coordinately lost. It is worth noting that the *AP3-III* ortholog of *Papaver* has been shown to function in petal and stamen identity (Drea et al., 2007), raising the possibility that the later evolution of petal-specific expression in the gene lineage was a subfunctionalization event. We must emphasize that our expression data speaks most directly to the question of process homology (a commonly inherited genetic program). Since such programs can potentially be turned off and on, it remains possible that the petals of Ranunculaceae + Berberidaceae are not all historically homologous. One could reasonably argue that it should be easier to turn off a genetic program several times than to turn it off and then reactivate it, which would support a model where the common ancestor of the two families did have petals. The genus *Clematis*, however, represents one clear example where petals must have been re-evolved. While most species lack petals, they are present in members of the subgenus Atragene L. Moreover, a recent
phylogenetic analysis placed the petalous genus *Naravelia* DC within an otherwise apetalous clade (Miikeda et al., 2006). It will be important to investigate these examples to test the hypothesis that an ancestral genetic program was simply reactivated. Because all of our studies to date are based on identification of expressed loci, questions also remain as to what happens to *AP3-III* orthologs in the genomes of apetalous taxa that do not express detectable levels of the genes in flowers.

There are many other aspects of petal evolution in Ranunculaceae that are unresolved. Many petals across the family are strikingly similar to stamens, even developing as morphological grades in genera such as *Clematis* and *Myosurus* L. (Tamura, 1965). However, this kind of morphology could easily be produced by something analogous to the ‘fading borders’ model (Buzgo, Soltis, and Soltis, 2004; Soltis et al., 2007). Along these lines, it has been suggested that some level of *AGAMOUS* expression, which normally promotes stamen identity, could contribute to the development of very staminoid petals (Erbar, Kusma, and Leins, 1998). While this is not observed in *Aquilegia* (Kramer et al., 2007), the hypothesis should be tested in a genus with strongly staminoid petals.

We can also ask why petal morphology is so variable in Ranunculaceae, or even across the Ranunculales. From a molecular genetic perspective, it seems that even if the contributions of *AP3-III* orthologs are conserved, other aspects of the developmental program must be highly variable. This variation could be in other components of the organ identity program, in parallel genetic pathways (such as organ polarity) or in downstream targets of *AP3-III*. Just in terms of organ identity, we see that the expression of *AP3-II* varies in petals of different taxa and we have no idea (yet) if this relates to
morphological diversity. Also, in *Aquilegia* we know that there is significant temporal variation, especially for *AP3-I* and –II orthologs (Kramer et al. 2007), which is lost in the simplistic form of expression analysis we have used here. It will be critical to extend full *in situ* characterization to a wider sampling across the order to capture more of this type of information.

The question regarding diversity in petal morphology can also be addressed from a more general evolutionary viewpoint: what selective conditions might underlie the diversification of petal morphology in the Ranunculales? One possible explanation is the almost universal presence of petaloid sepals across the order. In other angiosperm lineages with petaloid sepals, it has been observed that the second whorl petals are often reduced or lost (Ronse De Craene, 2007). A similar trend may hold here where petaloid sepals take over the function of pollinator attraction, allowing the second whorl petals to be reduced to nectaries or lost altogether. For the family Ranunculaceae, Kramer, D’Stilio and Schlüter (2003) hypothesized that the presence of multiple *AP3* paralogs facilitated the evolution of two types of petaloid organs in separate whorls. The current study has found that sepal expression of *AP3-I* and –II evolved on the same internode of the tree as petaloid sepals, after the divergence of Papaveraceae (Fig. 5), and *PI* homologs are always expressed in these organs as well (Table 1). The combined expression of *AP3* and, *PI* homologs in the petaloid sepals would be consistent with the hypothesis that B gene promote petaloid features. However, in *Aquilegia* the sepal expression detected at late stages with RT-PCR could not be confirmed during critical early developmental phases, and *PI* silencing had only subtle effects on sepal development (Kramer et al. 2007). Thus, we do not know whether the petaloidy of sepals
in Ranunculales is actually dependent on $AP3/PI$ function. In fact, other studies have observed a lack of $AP3/PI$ expression in first whorl-derived petaloid organs of diverse taxa (Park et al., 2003, 2004; Jaramillo and Kramer, 2004; Geuten et al., 2006), so we cannot rule out the existence of novel mechanisms for producing petaloidy both in this order and across the angiosperms.

In summary, this study has uncovered clear evidence for a conserved developmental program involving the specific expression of $AP3$-$III$ orthologs that underlies the diverse petals found in Ranunculaceae and Berberidaceae. These organs at least share process homology and may, in fact, be historically homologous. We realize that this runs contrary to well over a century of botanical theory (Prantl, 1887; Worsdell, 1903) and, therefore, will require further data collection and analysis before it can be taken as certain. Molecular characters are in no way a cure-all to our difficulties with assessing homology in plants, but when used in concert with modern phylogenies and developmental/morphological data, they can provide new insights. The utility of $AP3$ paralog expression in this case is quite serendipitous and, unfortunately, is not likely to be applicable to other groups with similar questions. When considering the angiosperms as a whole, we still have the difficulty of distinguishing between a commonly inherited $AP3/PI$ petal identity program and convergent recruitment of these genes. The field does seem to be reaching a point where the preponderance of the data supports a model of deeply conserved petal identity, although there are novel mechanisms promoting petaloidy in some cases and much more functional data is needed (see Hileman and Irish, this issue pp. ### and Soltis et al, this issue pp. ###). Members of Ranunculaceae are widely accepted as examples of independently derived petals, so if this is not the case, we
must allow ourselves to consider alternative hypotheses for the evolution of petaloidy at deeper phylogenetic levels as well.
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**Clematis integrifolia**

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(Kramer, Di Stilio, and Schlüter, 2003)

**Delphinium exaltatum**

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**Epimedium grandiflora**

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**Menispermaceae**

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**Papaveraceae**

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- = no detectable expression; +/- = barely detectable expression; + = weak to moderate expression; ++ = moderate to strong expression; n/a = not applicable (organs not present); (Shan et al., 2006) (Kramer and Irish, 1999) (Kramer and Irish, 2000)
nd = not done. Ref = Reference for expression data. See Materials and Methods for accession numbers and original citations of sequences.
**Figure Legends**

Fig. 2. Composite phylogeny of Ranunculales showing optimized character states for petaloidy of sepals (left) and presence of petals in second whorl (right). On left, blue indicates petaloid sepals; green, non-petaloid sepals; grey, absence of sepals; and orange, unresolved ancestral states. On right, pink indicates presence of second whorl petals; grey, their absence; and orange, unresolved ancestral states. The small box delimits the Ranunculeae + Anemoneae (see text). Taxa included in current study shown in bold. *T.* = *Thalictrum*. Each family delimited by box. Berber = Berberidaceae, Menisp = Menispermaceae, Lardiz = Lardizabalaceae, Cir = Circaeasteraceae, Papav = Papaveraceae, Eup = Eupteleaceae.

Fig. 3. Maximum likelihood phylogeny of the *AP3* lineage of Ranunculales. ML bootstrap values (above 50%) are placed at nodes. Stars indicate inferred gene duplication events. Branch colors indicate plant families. Red = Ranunculaceae, Orange = Berberidaceae, Purple = Menispermaceae, Blue = Lardizabalaceae, Green = Papaveraceae, Brown = Eupteleaceae. Outgroup sequences are described in Materials and Methods and Suppl. Table 1.


Fig. 5. Evolution of expression patterns in *AP3* gene lineage across Ranunculales. ML phylogeny shown in Fig. 3 pruned to exclude loci without expression data. Expression pattern character states indicated for sepals (S column or first box), petals (P column or second box) and stamens (A column or third box). Terminal character states based on observed expression patterns and shown in a column on right (Table 1, Fig. 4, Suppl. Fig. 2). Filled boxes represent clearly detectable expression (+ or ++ in Table 1); open boxes, undetectable or barely detectable expression (- or +/- in Table 1). Reconstructed ancestral character states shown for major internal nodes. Half filled box indicates unresolved ancestral character state for gene expression. Colors of the boxes correspond to morphological characters reconstructed in Fig. 2. First box/column S coded green = non-petaloid sepals, blue = petaloid sepals or orange = unresolved sepal appearance. Second box/column P coded pink = petals present, grey = petals absent, or orang = presence of petals equivocal. All boxes corresponding to stamens, yellow. An asterisk marks the node along which stamen expression is regained in the *AP3-III* lineage. Along right side of the figure, corresponding families of loci are indicated: Ran = Ranunculaceae, Ber = Berberidaceae, Men = Menispermaceae, Lar = Lardizabalaceae, and Pap = Papaveraceae.
Figure 2
Figure 3
Figure 4
Figure 5
Supplemental Figure Legends

Suppl. Fig. 1. Maximum likelihood phylogeny of PI lineage of Ranunculales. ML bootstrap percentages (above 50) placed at nodes. Stars indicate inferred gene duplication events. Branch colors indicate plant families. Red = Ranunculaceae, Orange = Berberidaceae, Purple = Menispermaceae, Blue = Lardizabalaceae, Green = Papaveraceae, Brown = Eupteleaceae. EupPI was designated as the outgroup.

Suppl. Figure 2

A. Aconitum
- AcsAP3-1
- AcsAP3-2
- AcsAP3-3
- AcsPl
- ACTIN

B. Anemone
- AnnAP3-1
- AnnAP3-2
- AnnAP3-3
- AnnPl-1
- AnnPl-2
- ACTIN

C. Caltha
- CapAP3-1
- CapAP3-2
- CapPl
- ACTIN

D. Cimicifuga
- CirAP3-1
- CirAP3-2
- CirAP3-3
- CirPl-1
- CirPl-2
- ACTIN

E. Clematis alpina
- ClaAP3-1
- ClaAP3-2
- ClaAP3-3
- ClaPl-1
- ClaPl-2
- ACTIN

F. Ranunculus
- RfAP3-1
- RfAP3-2
- RfAP3-3
- RfPl-1
- RfPl-1b
- RfPl-2
- RfPl-3
- ACTIN

G. Trautvetteria
- TrcAP3-1
- TrcPl-1
- TrcPl-2
- ACTIN

H. Trollius
- TlAP3-1
- TlAP3-2
- TlAP3-3
- TlPl-1
- TlPl-2
- TlPl-3
- TlPl-4
- ACTIN

I. Berberis
- BgAP3-1
- BgAP3-2
- BgPl-1/2
- ACTIN