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Insights into the ancestral flowers of Ranunculales

Authors

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Running title: Floral evolution in Ranunculales

Abstract

The question of the origin of petals has long been debated in the botanical literature. Ranunculales are characterized by a spectacular floral diversity, particularly at the perianth level. Recent progress in understanding the genetic bases of floral organ identity suggests a single origin for petals in Ranunculaceae contrasting with the traditional morphological hypothesis of repeated evolution. However, perianth evolution at order level remains incompletely understood. Recent advances in the elucidation of phylogenetic relationships within the order now provide a new opportunity to study character evolution with model-based methods. We used ancestral state reconstruction methods that take into account various sources of uncertainty to reconstruct the evolution of floral traits at the scale of Ranunculales using a consensus phylogenetic framework of 144 terminal species representing all families within the order. Ranunculales likely had ancestrally three trimerous whorls of perianth organs differentiated into two categories of petaloid organs differing in their shape. Each whorl was further lost or duplicated. Moreover, our results support the hypothesis of a single origin of highly specialised (elaborate) nectariferous petals within Ranunculaceae.

Keywords

Ancestral state reconstruction - elaborate petals - floral organ identity - Nektarblatt – Papaveraceae – Ranunculaceae

INTRODUCTION

Angiosperms are the most diverse clade of land plants in terms of species richness (90% of all described species). Morphological variation, especially of their flowers, reflects this taxonomic diversity. Flowers, defined here as a combination of stamens and carpels surrounded by sterile organs borne on a short axis, are the main synapomorphy of angiosperms, and are thought to have played a major role in their spectacular and rapid diversification radiation (Mulcahy, 1979). Flowers have evolved an almost endless array of forms, sizes, colours and scents, driven by the interaction with pollinating agents (mainly insects and wind, but also birds and mammals), while conserving their general organization. This organization most commonly consists in several concentric whorls of organs around the floral axis: two categories of sterile organs, the sepals and the petals (or sometimes a single category, the tepals), surrounding the fertile organs, the stamens (responsible for the male function) and the carpels (female function). When the sterile organs are differentiated into sepals and petals, the corolla usually plays a role in pollinator attraction. Based on mutant studies in two model eudicot species, *Arabidopsis thaliana* (L.) Heynh. and *Antirrhinum majus* L., a combinatorial model implicating four main functions (the ABCE model, Theißen & Saedler, 2001) has been proposed to account for the identity of each of the four floral organs. This model appears broadly conserved across angiosperms.

Ranunculales have been recognized in many traditional classification systems as a natural group (Cronquist, 1981; Kubitzki, 1995). However, the delineation of the order has varied due to the high floral diversity among families (reviewed in Wang *et al.* 2009). In particular, the core family of the order, the Ranunculaceae, has been qualified as a “famille par enchaînement” (meaning each genus is only linked to a few others by some similarities, the unity of the whole family being defined by the resulting network), and is famous for its lack of synapomorphies (Mangenot, 1973; Bonnier & Douin, 1934). This family was formerly

included in the order Ranales (Bentham & Hooker, 1867) together with seven other families, among which only two are now included in Ranunculales.

The order is currently taxonomically stable and strongly supported, and consists of seven families that are well described and well delineated. The current delineation was already in the first classification of the APG (Angiosperm Phylogeny Group, 1998). The current classification, based on a combined molecular and morphological analysis, is from Wang *et al.* (2009) and recognises the order as a fully supported monophyletic group. Ranunculales include seven families (ranked hereafter by decreasing species number): Ranunculaceae, Papaveraceae, Menispermaceae, Berberidaceae, Lardizabalaceae, Circaeasteraceae and Eupteleaceae (Angiosperm Phylogeny Group, 2016), containing ca. 4500 species (Stevens, 2001 onwards). Following Zhai *et al.* (2019), we use the term core Ranunculaceae to name the clade containing all Ranunculaceae *sensu* Wang *et al.* (2009) except the genera *Glaucidium* Siebold & Zucc. and *Hydrastis* J.Ellis, and the tribe Coptidoideae. Additionally, we introduce the term Historical Ranunculaceae to designate the clade containing all Ranunculaceae *sensu* Wang *et al.* (2009) except the genera *Glaucidium* and *Hydrastis*, which were assigned to their own families in several classifications during the 20th century (Tamura, 1972).

Ranunculales are exceptionally diverse in floral organization (Figure 1) (Endress, 1995; Endress & Matthews, 2006), making them an excellent model for floral evolution and evo-devo studies, as suggested by Damerval & Becker (2017). As currently defined, they display variation in many floral traits, such as the presence and number, form and differentiation of perianth parts (some being sepaloid, petaloid, or modified into nectar producing and storing organs), the symmetry (three types of symmetry are found in the group: actinomorphy, disymmetry and zygomorphy), the number of stamens, and the number and fusion of carpels. Variation concerns the sexual system, as well as phyllotaxy, which can be whorled or spiral,

or even chaotic (or irregular), the latter type being found in Eupteleaceae (Ren et al., 2007) and in Berberidaceae, characterizing the genus *Achlys* DC (Endress, 1989). Flowers are mostly bisexual, except in the genus *Thalictrum* L. (Ranunculaceae) where dioecy and androdioecy are found (Kaplan & Mulcahy, 1971).

Nectaries (defined here as nectar-secreting structures, not as organs) are present in several families, more noticeably Ranunculaceae, Papaveraceae and Berberidaceae. In Papaveraceae and some Berberidaceae (*Berberis* L. s.l.), nectaries are secreting glands located at the base of stamens and petals, respectively. In Ranunculaceae, nectaries are borne on highly modified organs traditionally called “Honigblätter” (Prantl, 1888) or more appropriately “Nektarblätter” (Janchen, 1949), considered as modified petals possibly of staminal origin that some authors excluded from the perianth organs (reviewed in Hiepko, 1965). In contrast, Hiepko (1965) considered that these organs are undoubtedly homolog to petals, based on features of their development (petal development is retarded with regard to the other floral organs) and their anatomy (petals are generally supplied with a single more or less ramified vascular bundle, while sepals are generally supplied with three bundles). This is supported by their constant position between outer perianth organs (either petaloid or sepaloid) and stamens (the position criterion for homology sensu Hennig, 1966)), and by the fact that they are delayed in their development compared to the other floral organs like most petals (Erbar, Kusma, & Leins, 1998; Tucker & Hodges, 2005; Zhao *et al.*, 2012a). It can be noted that the term “Honigblätter” or “Nektarblätter” was also used to qualify the nectary petals of Berberidaceae (Leinfellner, 1955). However, Hiepko (1965) advocated against the use of the term, for the above-mentioned reason that these organs are homolog to petals and should be called as such, in order to use a common terminology based on homology. Until recently, it was considered that petals in Ranunculaceae originated independently and were repeatedly recruited from stamens (Kosuge, 1994; Ronse De Craene, 2007), notably because

in the very early stages of their development, stamen and petal primordia are indistinguishable (e.g. Ren, Gu, & Chang, 2011; Zhao *et al.*, 2012a). Implicitly, it was assumed that the perianth organs of flowers with an undifferentiated perianth were sepals. This view was challenged recently by evo-devo studies investigating the expression of the ABCE model genes in several species of Ranunculales (reviewed in Damerval & Becker, 2017).

The phylogenetic position of Ranunculales as sister to all other eudicots is now consensual and strongly supported (Jansen *et al.*, 2007; Moore *et al.*, 2007, 2011; Drew *et al.*, 2014; Wickett *et al.*, 2014), making Ranunculales a key group to investigate floral evolution in one of the largest clades of angiosperms. Hypotheses concerning ancestral states for the eudicots were recently produced by Sauquet *et al.* (2017). According to this study, the ancestral eudicot flower was dimerous with three whorls of undifferentiated perianth organs, had an androecium composed of more than two whorls of stamens, and a gynoecium possibly composed of more than five whorled carpels, although much uncertainty remains concerning the phyllotaxy of this ancestral flower (Sauquet *et al.*, 2018).. Ancestral states for the order Ranunculales may have an impact on such reconstructions, which is why producing a well-supported hypothesis for the ancestral flowers of this key order is of major importance to discuss the evolution of floral traits in eudicots.

In this paper, our aim was to revisit floral evolution in the Ranunculales in a phylogenetic framework. Four plastid markers available from GenBank and used in published phylogenies (Wang *et al.*, 2009; Cossard *et al.*, 2016; Ortiz *et al.*, 2016; Sauquet *et al.*, 2017; Zhai *et al.*, 2019) were selected to produce a new comprehensive phylogeny of Ranunculales with Bayesian inference in order to take into account phylogenetic uncertainty.

Based on ancestral state reconstruction of 15 floral characters, we propose a new hypothesis for the ancestral flower of Ranunculales and discuss the evolution of each trait in the light of previous studies and genetic knowledge. This allowed us to produce an

evolutionary scenario for the flower of Ranunculales and examine hypotheses of adaptation, in particular for the modified nectariferous petals of Ranunculaceae.

MATERIALS AND METHODS

Taxonomic sampling

To obtain a phylogenetic framework representing both taxonomic and morphological diversity of the order Ranunculales, we selected 119 species for which sequences were available in GenBank for at least two of the four following plastid markers: *matK*, *rbcL*, *trnL-F* and *ndhF* (Supplementary Material 1). All families with more than two species (i.e., all except Eupteleaceae and Circaeasteraceae) were represented by a number of species corresponding to 2-3% of the total number of species of the family. Highly speciose genera were represented by two species. Twenty-seven additional species representing all major lineages of angiosperms (Amborellales, Nymphaeales, Austrobaileyales, magnoliids, monocots, basal eudicots, asterids and rosids) were selected to be used as outgroups to root the tree (Supplementary Material 1). The whole plastid genomes of *Acorus calamus* L., *Buxus sempervirens* Siebold & Zucc. and *Nymphaea alba* L. were used to obtain the sequences of the four targeted markers, identified with GenBank annotations.

Alignment and phylogenetic reconstruction

For each marker, sequences were aligned with MAFFT version 7 (Katoh & Standley, 2013) using the default settings. Minor manual adjustments were done, and the four alignments were used each separately for phylogenetic reconstruction with Bayesian Inference. Because no highly supported incongruent relationships were identified among the four single gene tree topologies, a global alignment including all markers and totalizing 6325 bases was constructed and used for the phylogenetic analyses (Supplementary Material 2).

Unconstrained Bayesian phylogenetic reconstruction was performed with each marker separately and all markers combined (partitioned by marker), using MrBayes version 3.2.6 (Ronquist *et al.*, 2012) with the GTR+I+ Γ model selected with ModelTest version 2.3 (Nylander, 2004), with a burn-in of 25% of the total number of generations (2,000,000). Trees were rooted with *Amborella trichopoda*. Only nodes with posterior probability (PP) higher than 0.95 were considered as well supported, all other nodes were collapsed in the consensus tree. The resulting tree is presented in Supplementary Material 3. Since the topology from Zhai *et al.* (2019) was better resolved and supported than ours concerning phylogenetic relationships among tribes within core Ranunculaceae (this clade encompasses the subfamilies Thalictroideae and Ranunculoideae, the latter one being paraphyletic in both Zhai *et al.* (2019) and Cossard *et al.* (2016) studies), a constrained Bayesian analysis was performed with MrBayes version 3.2.6 (Ronquist *et al.*, 2012) on the CIPRES Gateway (Miller, Pfeiffer & Schwartz, 2010). The same parameters as for the unconstrained analyses were used, but with 5,000,000 generations. A total of 30 nodes were constrained.

Data scoring and coding

Fifteen (15) characters spanning the main floral variation observed in Ranunculales were defined. Because the perianth is highly variable in Ranunculales, most of the characters are focused on perianth structure. The structural perianth characters are as follows: phyllotaxy, symmetry, total number of perianth organs, merism (basic number of perianth organs), number of “whorls” (for simplicity reason, we chose to use a single term that applies here to whorls of morphologically identical organs or, in case of spiral or chaotic phyllotaxy, to categories of morphologically similar organs, formally series; in case of whorled phyllotaxy, there can be several whorls of morphologically identical organs), variability in the number of perianth parts (in Ranunculaceae, the number of perianth organs can vary among flowers

within several species), fusion of parts. We also defined several characters related to perianth aspect: differentiation (we call differentiation a situation where there are at least two categories of perianth organs differing by shape and/or colour, hereafter referred to as aspect), inner and outer organ aspect (petaloid, sepaloid or strongly modified), and presence of spurs (we chose to code the presence of spurs on any perianth organ, without specifying the category). The number of stamens, the number of carpels and their possible fusion were also scored, as well as the position of nectaries. Androecium and gynoecium phyllotaxy and merism were not scored because data are very scarce. All selected characters were coded as discrete. Data were obtained from detailed morphological and developmental studies, as well as from more superficial sources such as online floras, from online pictures if the identification could be trusted and from personal observations (Supplementary Material 3 and 4). We used an exemplar approach (Prendini, 2001), meaning that all terminals represent species, as opposed to coding supra-specific terminals. For each character, the number of states was defined according to the variation recorded in our sample of species. We limited the number of states to four to limit the number of models in ancestral state reconstruction (the higher the number of states, the higher the number of possible models), except for two characters (number of carpels and position of nectaries). Character states were first recorded in PROTEUS v1.26 (Sauquet, 2016) and then exported to Mesquite v3.6 (Maddison & Maddison, 2018) for further handling. The morphological matrix is presented in Supplementary Material 4, all associated references are presented in Supplementary Material 5.

Character states were optimized on the 50% consensus tree with nodes under 0.95 collapsed resulting from Bayesian inference with parsimony using Mesquite. The tree used for ancestral state reconstruction is the Bayesian consensus tree of Figure 2. For Bayesian Ancestral State Reconstruction (ASR), we used one thousand trees randomly sampled (using

Mesquite) from the posterior distribution of the Bayesian phylogenetic analysis (constrained four marker analysis).

Bayesian ASR was performed with BayesTraits v3 (Meade & Pagel, 2018) using the reversible jump Monte Carlo Markov Chain (rjMCMC) algorithm that takes into account both model and phylogenetic uncertainty (Pagel, Meade, & Barker, 2004; Pagel & Meade, 2006). The rjMCMC method permits to select the best-fit model (model selection), or to globally estimate parameter values and ancestral states in all sampled models (model averaging). This is a great advantage especially when uncertainty on the best-fit model is high (Pagel & Meade, 2006), which is the case here. For this reason, in the present study we chose to use the model averaging strategy. This method only reconstructs ancestral states for predetermined nodes. We selected the following nodes (called “key nodes” hereafter): Ranunculales and each of the seven families, to produce a hypothetical ancestral flower for the whole order and each family; [Berberidaceae + Ranunculaceae + Menispermaceae] and [Berberidaceae + Ranunculaceae] to investigate a possible common origin of nectar producing and toring organs; and core Ranunculaceae since this clade is the most species-rich and the most diversified in terms of flower morphology within the order. Each character was analysed separately with the MultiState option and mrca command for 20,000,000 generations sampled every 2,000 generations and uniform priors. The posterior probabilities and their 95% credibility intervals for each state at the nodes of interest were then computed with Tracer v1.7.1 (Rambaut *et al.*, 2018) after removing a 25% burn-in. For characters with up to three states, the results were interpreted using arbitrary thresholds as follows: for a given state at a given node, we consider that posterior probabilities of 0.5-0.6 indicate uncertain ancestral state, 0.6-0.7 indicate low confidence, 0.7-0.8 medium confidence and 0.8-1 high confidence. Characters with four states or more were interpreted differently, according to the confidence intervals overlaps (see Supplementary Material 6 for detailed explanations).

Results

The final alignment contained 15% of missing data. The consensus tree resulting from Bayesian inference is presented in Figure 2, which also indicates the 30 nodes constrained. There is a polytomy among Papaveraceae, Eupteleaceae, and the clade comprising all other families. Within the latter clade (core Ranunculales), Lardizabalaceae are sister to Circaeasteraceae, and both are sister to a clade formed by Menispermaceae and [Berberidaceae + Ranunculaceae]. Within Papaveraceae, Berberidaceae, and Menispermaceae, subfamilies are retrieved as monophyletic with good support. The unconstrained topology is presented in Supplementary Material 3.

The morphological matrix contains 8% of missing data. The results of ancestral state optimisation using parsimony for the characters “Number of perianth whorls”, “Differentiation”, “Inner perianth aspect” and “Position of nectaries” are presented in Figures 3 and 4, and in Supplementary Material 7 for the other characters. The detailed posterior probability (PP values) obtained with rjMCMC for ancestral states at key nodes (see Material and Methods section) are presented in Supplementary Material 8 together with the associated confidence intervals. A summary of the most likely and most parsimonious ancestral states is presented in Table 1.

In what follows, we present together the results obtained with both parsimony and Bayesian methods when they support the same hypothesis for the ancestral states at key nodes (in square brackets). More detailed results from parsimony only at other nodes are described as well. When the methods produced incongruent results, the result obtained with each method will be detailed.

Perianth phyllotaxy – Ancestral anthetic phyllotaxy of Ranunculales is whorled, with one transition to spiral in the ancestor of Circaeasteraceae, and at least one within the core Ranunculaceae.

Perianth symmetry – Ranunculales and all seven families were ancestrally actinomorphic except Papaveraceae which were ancestrally disymmetric. Another transition towards disymmetry occurred in the dimerous genus *Epimedium* L. (Berberidaceae), although visually the flowers are tetrasymmetric. Monosymmetry evolved once in Delphinieae (Ranunculaceae) and once or twice within Fumarioideae (Papaveraceae).

Number of perianth parts – With rjMCMC, Papaveraceae and core Ranunculaceae were reconstructed as having several perianth parts (5-10), Lardizabalaceae, Circaeasteraceae and Berberidaceae as having many (11 and more), Eupteleaceae as having none. The rjMCMC reconstructions were uncertain for the other key nodes. Parsimony reconstructions were ambiguous for every node except Papaveraceae, Eupteleaceae, Lardizabalaceae, Circaeasteraceae and Berberidaceae which had the same ancestral states as with the rjMCMC reconstructions. At least five transitions of the number of parts from several (5-10) to many (11 and more) occurred within Ranunculaceae, and at least two transitions from several to few (1-4).

Variability in the number of perianth parts – The character was reconstructed by rjMCMC as uncertain for every node except Papaveraceae and Lardizabalaceae (which have a constant number of parts). On the contrary, the character state was resolved as constant number of perianth parts with parsimony, with transitions towards a variable number of perianth parts within Papaveraceae, Menispermaceae, Berberidaceae and Ranunculaceae, followed by several reversals within Ranunculaceae.

Perianth merism – With parsimony, this character was reconstructed as ancestrally trimerous in Ranunculales with transitions to dimery in Papaveraceae, *Epimedium* and

Ranunculaceae, while the ancestral state of Ranunculaceae was uncertain with rjMCMC. Historical Ranunculaceae were ancestrally pentamerous and there were two transitions to tetramery in *Thalictrum* and *Clematis* and two transitions to trimery in *Eranthis* Salisb. and Anemoneae.

Number of perianth whorls (Figure 3a) – The ancestral state for the whole order was “three or more” whorls, from which one transition to the state “two whorls” occurred in the ancestor of the family Ranunculaceae, where it was followed by six to ten transitions towards a single whorl. Reductions in the number of whorls also occurred in Circaeasteraceae, twice within Menispermaceae and once within Lardizabalaceae.

Differentiation (Figure 3b) – The ancestor of Ranunculales had a differentiated perianth, from which an undifferentiated perianth evolved repeatedly within Menispermaceae, Circaeasteraceae, Berberidaceae and Ranunculaceae. The ancestral state was ambiguous only for Ranunculaceae and resolved as differentiated for all other families (except for the perianthless Eupteleaceae). Historical and core Ranunculaceae were both ancestrally differentiated.

Outer perianth aspect – There were discrepancies between parsimony and rjMCMC concerning this character, which was resolved as sepaloid with parsimony and petaloid with rjMCMC for Ranunculales, [Ranunculaceae + Berberidaceae + Menispermaceae], and Papaveraceae. [Ranunculaceae + Berberidaceae] and Berberidaceae were also petaloid with rjMCMC, and ambiguous with parsimony. Both methods were congruent for the ancestral state of Menispermaceae and Circaeasteraceae, which was resolved as sepaloid, while Lardizabalaceae and core Ranunculaceae were resolved as petaloid. Transitions from petaloid to sepaloid outer perianth organs occurred at least six times within Historical Ranunculaceae.

Inner perianth aspect (Figure 4a) – The ancestral state of Ranunculales was resolved as petaloid, as well as the ancestral state of Papaveraceae, Circaeasteraceae, Menispermaceae,

Berberidaceae, and Ranunculaceae (for this latter family, the ancestral state was uncertain with rjMCMC). Strongly modified inner perianth parts appeared in Fumarioideae, Lardizabalaceae, twice within Berberidaceae and in Historical Ranunculaceae, with at least four reversals within the latter.

Perianth parts fusion – All perianth parts were ancestrally free for almost all nodes of the tree, except two transitions towards a fused perianth within Fumarioideae (with petals partly or completely congenitally united in most species) and within Delphinieae (with petals congenitally united in *Consolida* (DC.) Gray).

Presence of spurs – Spurs evolved four times independently in the order, once in Papaveraceae (within Fumarioideae), once within Berberidaceae (*Epimedium* + *Vancouveria* C. Morren & Decne.), and twice within Ranunculaceae (*Aquilegia* and *Delphinieae*).

Stamen number – The ancestor of Ranunculales had few stamens (1-6), as well as the ancestor of Papaveraceae, Lardizabalaceae, Circaeasteraceae, Menispermaceae and Berberidaceae. An increase in the number of stamens from few (1-6) to many (13-49) occurred in Eupteleaceae, Papaveroideae and Ranunculaceae (from 1-6 to 13-49 with rjMCMC, from 1–6 to ≥ 50 with parsimony). Historical and core Ranunculaceae had ancestrally many stamens. Transitions from many (13-49) to more than fifty occurred several times within Papaveroideae and core Ranunculaceae.

Carpel number – The ancestral number of carpels of Ranunculales, Papaveraceae, Eupteleaceae, Circaeasteraceae, Lardizabalaceae and Ranunculaceae was unresolved with parsimony. With rjMCMC, the ancestral state was “>12” for Ranunculales, Eupteleaceae, Lardizabalaceae, Ranunculaceae, “2” for Papaveraceae, and “3-6” for Circaeasteraceae. Both methods reconstructed the ancestor of Menispermaceae as having three to six carpels, the ancestor of Berberidaceae as having a single carpel, and the ancestor of core Ranunculaceae

as having more than twelve carpels. Several transitions towards a lower number of carpels occurred within core Ranunculaceae.

Fusion of carpels – The character state was ambiguous in the ancestor of the order with parsimony but resolved as free carpels with rjMCMC. Papaveraceae was the only family with ancestrally congenitally united carpels in Ranunculales. Fusion of carpels evolved once in Ranunculaceae, in *Nigella*.

Position of nectaries (Figure 4b) – Nectaries evolved repeatedly in different clades and in various locations within the flower. They were ancestrally absent in Ranunculales (but the state was ambiguous with parsimony), Papaveraceae, Eupteleaceae and Menispermaceae. They appeared on the androecium once within Fumarioideae and twice within Berberidaceae. They appeared independently on the perianth of Lardizabalaceae and Circaeasteraceae (ambiguous state with parsimony), and on the perianth of Historical Ranunculaceae. For Ranunculaceae, the ancestral state was reconstructed as “absence of nectaries” with parsimony and “nectaries on the perianth” with rjMCMC.

DISCUSSION

Methodological remarks

Model-based methods for ancestral state reconstruction rely on phylogenetic trees containing information on genetic divergence (Pagel *et al.*, 2004) in addition to species relationships, i.e., with branch lengths. The two ways to obtain a tree with branch lengths is either to time-calibrate the tree using fossils, or to use only the inferred variation of genetic divergence contained in the molecular sequences used to reconstruct the phylogeny. We chose here to use a tree reflecting molecular branch lengths rather than absolute time, using molecular markers covering both the taxonomic and the morphological diversity of Ranunculales. Besides, there are few undisputed fossils of Ranunculales, except two in

Berberidaceae but very young (Rodríguez-de la Rosa, Cevallos-Ferriz & Silva-Pineda, 1998; Sun *et al.*, 2011), making calibration unreliable (Sauquet *et al.*, 2012) . However, the choice of phylograms relies on the assumption that morphological and molecular evolutionary rates are correlated with each other, which can be questioned when the chosen molecular markers are from the maternally inherited chloroplast genome only (as it is the case here). Plastid markers are known to have limited power in resolving phylogenetic relationships depending on taxa and taxonomic scale. It is therefore not surprising that some nodes of the unconstrained resulting tree are poorly supported. This is also the case for the relationships among tribes within core Ranunculaceae. As mentioned in the Material and methods section, we used the topology of the recently published phylogeny of Ranunculaceae based on plastid genomes (Zhai *et al.*, 2019) in which relationships among tribes are resolved to constrain several nodes in our phylogenetic analyses.

Ancestral states reconstructed with rjMCMC and parsimony are most often similar. However, in several cases the results obtained with the two methods are different, with two situations. In some cases, the ancestral state is resolved with one method and unresolved with the other one. There are also a few cases where both methods give conflicting hypotheses for the ancestral state. Different/conflicting results between rjMCMC and parsimony can be accounted for by the sensitivity of each method to various biases. While parsimony is highly sensitive to the tree topology, the distribution of missing data and the choice of outgroups, Bayesian inference can be biased by character state frequency represented in the taxon sample versus in nature, and by artefacts in branch length estimates which would be exacerbated by the choice of phylograms vs. chronograms (Lewis, 2001). We considered relevant to use both parsimony and rjMCMC because the results can be discussed in light of these possible biases.

Trait evolution

As previously mentioned, both methods used for ancestral state reconstruction (rjMCMC and parsimony) gave congruent results for the vast majority of characters and nodes, with some exceptions where the ancestral state was resolved with one method and unresolved with the other. This was the case for example for the character perianth differentiation, for which rjMCMC could not resolve the ancestral state at some nodes, or for the character number of carpels, for which parsimony gave an ambiguous state. In what follows we will discuss the results obtained for each character, comparing the methods, in light of the current knowledge on floral development and genes involved in floral architecture in Ranunculales.

Structural perianth characters

Phyllotaxy – Ideally, distinguishing between cyclic and spiral phyllotaxy for a flower requires a study of the whole floral development, but such information is far from being available for the ca. 300,000 species of flowering plants. Secondary criteria can help infer floral phyllotaxy from mature flowers, such as the concentric distribution of floral organs around the floral axis (Endress & Matthews, 2006). We considered perianth phyllotaxy at anthesis, and reconstructed this character as ancestrally cyclic for all nodes except Circaeasteraceae, which are described as having spiral flowers (Tian *et al.*, 2007). In Papaveraceae, Lardizabalaceae, Berberidaceae and Menispermaceae, the underlying floral development is cyclic in the species for which it has been described (Endress & Doyle, 2009), except in the oligomerous flowers of the genus *Hypserpa* from Menispermaceae, which have a spiral phyllotaxy (Endress, 1995). Interestingly, the perianth of Ranunculaceae and Core Ranunculaceae was reconstructed as ancestrally cyclic, as previously suggested (Endress & Doyle, 2009), a character state that is present for example in *Aquilegia* (Tucker & Hodges, 2005) or *Clematis* L. (Ren *et al.*, 2010), although many species have spiral phyllotaxy (see Payer (1857), Schöffel (1932) and Hiepko (1965) for overviews of morphology and ontogeny

in Ranunculaceae, and for more detailed studies see for example Jabbour *et al.* (2009) for several Delphinieae, Zhao *et al.* (2011) for *Nigella* L. and *Helleborus* L., Zhao *et al.* (2012a) for Ranunculeae). Within the tribes Ranunculeae and Anemoneae, both spiral and whorled (or irregularly whorled) phyllotaxy coexist (Zhao *et al.*, 2012a), although the number of transitions is unknown. Spiral organ inception may have prevented organ synorganisation in Ranunculaceae (contrary to Fumarioideae for example) but may in turn have promoted the huge variability in floral organisation in the family, in terms of number of organs (see below). It has to be noted that our conclusions on perianth phyllotaxy may not apply to the fertile organs of the flower, since phyllotaxy can vary among floral whorls (as shown by Zhao *et al.*, (2012a) in some species of the tribe Ranunculeae), as discussed by Ronse De Craene, Soltis, & Soltis (2003) and more recently by Sauquet *et al.* (2017, 2018) and Sokoloff *et al.* (2017).

Merism – The trimerous ancestral state reconstructed for Ranunculales, and for most key nodes in our study (except Papaveraceae and possibly Ranunculaceae), as in Ronse De Craene *et al.* (2003) and Endress & Doyle (2009), was also hypothesized to be ancestral for mesangiosperms (Endress & Doyle, 2009; Sauquet *et al.*, 2017), suggesting that the state could be plesiomorphic for Ranunculales and possibly homologous to the trimery of monocots and magnoliids. The dimerous ancestral state reconstructed for Papaveraceae is unambiguous, all members of the family sharing this state. It is less clear for Ranunculaceae, since the ancestral state is unresolved with rjMCMC. It cannot be excluded that the dimerous state reconstructed with parsimony for the family Ranunculaceae is biased by the dimerous flowers of *Glaucidium* and *Hydrastis*, successively first and second diverging genera within the family. In the case of *Hydrastis*, dimerous and trimerous flowers have been recorded (Tobe & Keating, 1985; Endress & Doyle, 2009). If the first Ranunculaceae were indeed dimerous, there would have been a shift to pentamery at the base of Historical Ranunculaceae, the whole situation representing a convergence with the transition from dimery to pentamery

at the base of Pentapetaleae (Sauquet *et al.*, 2017). Pentamery had already been proposed as the ancestral state for Historical Ranunculaceae, although with a very limited number of representatives for the clade (Ronse De Craene *et al.*, 2003). It has to be stressed that in Ranunculaceae, merism is a very complex character, with frequent variation among whorls of organs within the perianth (like in Delphinieae for example), in addition to the variation observed among genera, making this character sometimes difficult to code for the perianth as a whole.

Number of parts – While the ancestral number of perianth parts is resolved with both methods for most families (except Menispermaceae and Ranunculaceae), it remains unresolved for the Ranunculales. However, since a trimerous perianth composed of at least three whorls of perianth organs was identified as the ancestral state for the order, and considering that the ancestor of all eudicots likely possessed three whorls of perianth organs although in a dimerous context (Sauquet *et al.*, 2017), it seems reasonable to suggest that the ancestral flower of Ranunculales possessed nine perianth organs organized in three trimerous whorls (see below for the discussion on differentiation among these three whorls). From this ancestral state, transitions towards dimery occurred in Papaveraceae and within Berberidaceae in *Epimedium*, and the perianth was completely lost in Eupteleaceae (Ren *et al.*, 2007), and within Berberidaceae in *Achlys* (Endress, 1989). Increase in the number of perianth parts occurred in the ancestor of [Lardizabalaceae + Circaeasteraceae] and in Berberidaceae. Since the ancestral state for Menispermaceae is unresolved, it is not possible to know whether these increases happened independently or in the ancestor of “core” Ranunculales (all families except Papaveraceae and Eupteleaceae). The ancestral state is unresolved for Ranunculaceae as currently defined, but Historical Ranunculaceae (all Ranunculaceae except *Hydrastis* and *Glaucidium*) were reconstructed as having a perianth likely composed of ten organs in two whorls of five organs each, followed by repeated reduction or increase in organ number

during the diversification of the family. The character “variability of perianth organ number” is debatable, but its merit is to highlight the contrast between clades with ancestral cyclic phyllotaxy, reconstructed as having a constant number of perianth organs, and clades with ancestral spiral phyllotaxy, reconstructed as having a variable number of perianth organs. This supports previous works arguing that spiral development allows flexibility in organ number (Endress & Doyle, 2007). The superimposition of monosymmetry on spiral phyllotaxy, which characterizes the tribe Delphinieae, can however restrict organ number variability. In this tribe, the number of perianth organs is fixed and is one of the features that differentiates genera (Jabbour and Renner, 2012).

Number of whorls – As a reminder, we chose in this study to apply the term “whorl” to both whorled and spiral flowers, in order to have a common term to designate all morphologically similar organs, although we are aware that the term series should normally be used in the case of spiral flowers. The present study adds further precision to previous works in which the ancestral number of perianth whorls for Ranunculales was uncertain (Ronse De Craene *et al.*, 2003) or partially resolved as “more than two” (Endress & Doyle, 2009), by suggesting that the ancestral state would be “three or more” whorls. Because flowers with strictly more than three whorls occur only in Berberidaceae (Zhao *et al.*, 2016) and Lardizabalaceae (Zhang & Ren, 2011), we suggest that the ancestral state for Ranunculales was three whorls, with further increase in these two families. Transitions from three to two whorls occurred in Menispermaceae and at the base of Ranunculaceae, with further repeated losses of one whorl within Ranunculaceae. This pattern suggests that the transition from three to two whorls is due to a single loss of perianth organs in the ancestor of Ranunculaceae; therefore, two whorls, when present, are likely homologous throughout all Ranunculaceae. Concerning species with a single whorl of perianth organs, even if traditionally the uniseriate perianth of Ranunculaceae was considered as a calyx (Tobe, 1980),

from a strict morphological point of view the homology is difficult to assess because the identity is blurred by aspects in relation to function. From an evolutionary point of view, the use of the term “petal” to describe the internal perianth organs in Ranunculales is controversial, not because of their possible staminodial origin but because they may not be homologous to the petals of the core eudicots (Endress & Matthews, 2006). Issues related to the origin (from bracts or stamens), homology, and identity of perianth organs across angiosperms have been discussed (Ronse De Craene *et al.*, 2003).

Evo-devo studies of the floral identity genes are precious to guide homology hypotheses between whorls and thus produce hypotheses on which whorls were lost and which whorls were duplicated when their number increases. According to the classical ABCE model, perianth organs are determined by the expression of A and B functions and exclusion of the C function. The outer whorl (usually the calyx) is determined by the A function that appears to include different actors and processes across angiosperms (Monniaux & Vandebussche, 2018). In the Ranunculaceae species *Nigella damascena* L. a role of the *AGL6* gene in sepal identity has been found (Wang *et al.*, 2016). In the Ranunculales as a whole, the A function has been little studied yet (Damerval & Becker, 2017) and further investigations would help to understand the extensive variation of the perianth across the order. Petal identity most generally involves the action of the B-class genes. In *Arabidopsis thaliana* and other model species, B function is carried out by two paralogous lineages of transcription factor genes, the *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) lineages, issued from a duplication of an ancestral gene before angiosperm diversification. In Ranunculales, *AP3*-like genes underwent two successive duplications (Rasmussen *et al.*, 2009). Expression analyses of genes of the three lineages strongly suggest that genes of the *AP3-III* lineage (identified in all Ranunculales except Eupteleaceae) are specifically expressed in petals in Papaveraceae, Ranunculaceae, Berberidaceae and Lardizabalaceae species (Rasmussen, Kramer, & Zimmer, 2009; Hu *et al.*,

2012; Lange *et al.*, 2013). *AP3-3* genes are expressed in all perianth organs of *Menispermum dauricum* DC. (Menispermaceae), while no data are available for Circaeasteraceae (Rasmussen *et al.*, 2009). *AP3-3* genes are not expressed and/or are disrupted in apetalous species of Papaveraceae and Ranunculaceae (Arango-Ocampo *et al.* 2016; Zhang *et al.* 2013). In addition, functional studies in *Papaver somniferum* L. (Drea *et al.* 2007), *Aquilegia caerulea* E.James (Sharma *et al.*, 2011) and *Nigella damascena* (Gonçalves *et al.* 2013; support the hypothesis of a role of *AP3-3* genes in petal identity. The expression patterns of *AP3-3* therefore provides arguments towards the homology of the inner whorls of Ranunculaceae and the inner whorls of Berberidaceae (Rasmussen *et al.*, 2009). Based on these results, we suppose that the additional perianth whorl of the ancestor of Berberidaceae was gained by the duplication of the inner whorl in their ancestor, and that Ranunculaceae lost the outermost whorl. In the Lardizabalaceae species *Sinofranchetia chinensis*, which has a 4-whorled perianth, *AP3-3* is expressed in both inner whorls, which would then be homologous to the inner perianth organs of Ranunculaceae and Berberidaceae, suggesting a duplication of the inner whorl. The outer whorls, in which the gene is not expressed, would be homologous to the outer perianth organs of Ranunculaceae and Berberidaceae (Hu *et al.*, 2012). Interestingly, in *Akebia trifoliata* (Thunb.) Koidz. (Lardizabalaceae) where the perianth is reduced to three petaloid organs interpreted as sepals by Hiepko (1965), the ortholog of the *AP3-3* gene is not expressed (Shan *et al.*, 2006; Hu *et al.*, 2012). The authors argue that based on the development the perianth organs are indeed likely homologous to sepals and that *AP3* genes would be responsible for the petaloidy of the sepals. In spite of multiple origins of petals in eudicots, floral evo-devo studies show that the same classes of genes have been repeatedly recruited for the development and specialisation of these organs (see above), leading some authors to introduce the concept of deep homology among all eudicot petals (Irish, 2009).

Functional perianth characters

Contrary to core eudicots, which have a differentiated perianth with usually green and inconspicuous sepals at anthesis (sepaloidy) while petals are usually showy and hold the function of attracting pollinators (petaloidy), the flowers of Ranunculales are much more variable as to the location of the function of attraction. As previously mentioned, the outer and inner perianth whorls can be all coloured and showy as in *Aquilegia chrysantha* A.Gray, all small, greenish and inconspicuous as in *Menispermum dauricum*, green – sepaloid (outer) and coloured – petaloid (inner) as in *Papaver rhoeas* L., or coloured and showy (outer) and strongly modified (inner) *i.e.*, elaborate and less conspicuous as in *Nigella damascena*. In the latter case, the function of visual attraction has shifted towards the outer perianth organs, whereas the petals have acquired the new function of producing and storing nectar. In spite of the presence of a nectariferous spur, we considered the petals of *Aquilegia* as petaloid because their upper part is flat and showy. Interestingly, it has been recently found that the B-class genes would be involved in sepal petaloidy in *Aquilegia* (Sharma & Kramer, 2017).

Differentiation – Ranunculales are reconstructed as being ancestrally differentiated, which is congruent with previous work (Endress & Doyle, 2009). However, our reconstruction does not allow us to know which whorl was different from the two others. If eudicots were indeed ancestrally undifferentiated as suggested in Sauquet *et al.* (2017), differentiation therefore evolved independently in Ranunculales and in other eudicots, with repeated reversals to an undifferentiated perianth among Ranunculales, although in none of the key nodes. In many cases, the loss of differentiation is simply due to a reduction of the perianth to only one whorl, such as in *Circaeaster* Maxim. (Circaeasteraceae), *Akebia* (Lardizabalaceae), and *Anemonella* Spach., *Thalictrum*, *Caltha* L., *Trautvetteria* Fisch. & C.A. Mey., *Anemone* L. *Clematis* (Ranunculaceae). Loss of differentiation also evolved in taxa along with a transition from

three to two whorls of perianth organs, such as *Abuta* (Menispermaceae), and *Glaucidium*, *Hydrastis*, and *Laccopetalum* Ulbr. (Ranunculaceae). This is the sign that the lost whorl had a different aspect than the remaining two and had also probably a different function. In this regard, differentiation (functional trait) and the number of whorls (structural trait) are interdependent traits in Ranunculales. In the case of Berberidaceae, the perianth repeatedly became undifferentiated but it is not possible to correlate this functional transition to the structural change in the number of whorls since the number of whorls can vary in a way our coding does not take into account (they can have more than three whorls of perianth organs), and because there is no hypothesis on their ancestral state in the literature (Zhao *et al.*, 2016).

Perianth aspect – According to our results, the inner perianth was ancestrally petaloid in Ranunculales and all families except Lardizabalaceae, with three evolutionary transitions towards a modified inner perianth within individual families in the order. One took place at the base of Lardizabalaceae, which usually have strongly reduced nectariferous petals (Zhang & Ren, 2011). The second transition towards modified inner perianth organs occurred within Papaveraceae at the base of subfamily Fumarioideae (although this is supported only with parsimony), where the petals are spurred and coherent at the top, hiding the reproductive organs at anthesis (Lidén, 1986). The single transition towards modified inner perianth organs that occurred at the base of the Core Ranunculaceae suggests that the ranunculacean petals, traditionally called Nektarblätter (Hiepko, 1965) or “nectary organs” (Kosuge, 1994; Erbar *et al.*, 1998) because they act as nectar producing and storing organs rather than as visual cues for pollinators, would all be homologous in spite of their remarkably diverse shapes (tubular in *Eranthis* and *Helleborus*, stalked and spurred in *Delphinium* L. and *Aconitum* L., or spatulate in *Actaea* (Zhai *et al.*, 2019), supporting the position of Hiepko (1965) who considered these Nektarblätter as homolog to petals. The petaloid inner perianth organs of *Adonis* L., *Aquilegia* and of the ancestor of the clade [Callianthemaceae + Helleboreae +

Anemoneae + Ranunculeae] would result from repeated reversals towards petaloidy. Whereas both ancestral state reconstruction methods gave congruent results for the inner perianth aspect, they are conflicting for the outer perianth. The PP of the Bayesian analysis supports petaloid sepals as the ancestral state for all Ranunculales, contrary to the situation found in most eudicots, including the outgroups selected for the present analysis. If petaloid sepals were indeed the ancestral state for Ranunculales, the sepaloid sepals of Menispermaceae, Circaeasteraceae and Papaveroideae would then be derived. From an evolutionary point of view and based on the number of perianth whorls, our results suggest that the outer perianth organs of Ranunculales would be homologous to the sepals of all other eudicots, in spite of their different aspect.

Elaborate petal and nectary evolution – The strong modification of the petals, leading to the so-called Nektarblätter originated at the base of Historical Ranunculaceae, representing a synapomorphy for this latter clade. This is in contradiction with the previous hypothesis that these modified organs would have evolved multiple times in Ranunculaceae, recruited from modified stamens (Ronse De Craene & Smets, 1995; Erbar *et al.*, 1998), because their youngest stages are indistinguishable from those of the stamens (as in *Asteropyrum* J.R. Drumm. & Hutch. (Zhao *et al.*, 2012b), *Helleborus* and *Nigella* (Zhao *et al.*, 2011), or also *Adonis* (Ren *et al.*, 2009)), but it is in agreement with evo-devo studies as discussed above. The distribution of the “strongly modified” petals within Ranunculaceae mirrors the one of nectaries that were ancestrally present on the perianth of the ancestor of Historical Ranunculaceae (reconstructed only with parsimony) (Figure 4). The functional role of these elaborate nectar-producing petals in pollination, and their importance in the evolution of Ranunculaceae was already stressed, but under the hypothesis of their multiple origin (Endress, 1995; Erbar *et al.*, 1998; Endress & Matthews, 2006). Within the family, there are a few records of secretory regions located elsewhere in the flower, represented in our dataset by

Caltha palustris L. and *Anemone nemorosa* L. (borne on the gynoecium in both cases) and *Anemone pulsatilla* L. (borne on the androecium), all having a perianth composed of a single whorl of petaloid organs. Their closest relatives have two whorls of perianth organs with nectaries located on the perianth, when present. The production of nectar could have been transferred to the gynoecium or to the androecium, to compensate for the loss of the whorls bearing the nectaries in their ancestors. Nevertheless, it should be noted that gynoecial nectar can sometimes not be formally nectar but lipidic stigmatic secretions, as it is probably the case for *Caltha palustris* (Endress, 1995), supporting a different origin of secretory region in this species. The petals were also modified independently in Berberidaceae into nectar-storing organs, like in *Epimedium* or *Vancouveria* C. Morren & Decne. for example (Terabayashi, 1979; Erbar, 2014). The evolution of nectaries within Berberidaceae appears somewhat complex in this study. Variation of the presence or absence of nectaries within the family was already discussed in developmental studies (Zhao *et al.*, 2016), although without considering their position. Several species in our dataset were scored following sources such as floras or pictures from biodiversity databases, when no developmental or morphological study could be found, and it is possible that the position of nectaries was erroneously scored, resulting in an overestimation of the number of origins of nectaries. Nevertheless, the results obtained here highlight the presence of variation in this family. The uncertainty and discrepancies between parsimony and rjMCMC for the key nodes Berberidaceae and [Berberidaceae + Ranunculaceae] leave open the question of a common origin of nectaries borne on petals in these two families. Recently in Ranunculaceae, homologs of the *STYLISH* gene family were found to control nectary development in *Aquilegia* (Min, Bunn, & Kramer, 2019). Investigations targeted on these genes could help answer this question. Nectaries also appeared on the perianth of Lardizabalaceae and of Circaeasteraceae (Figure 4b). These two families being sister groups, it may be hypothesized that the nectaries evolved on the perianth

of their common ancestor. The “nectar leaves” present in some Lardizabalaceae were sometimes described as staminodes (Zhang & Ren, 2011), but in this family too evo-devo studies suggest a petal identity for these organs, since a homolog of *AP3-3* is found to be expressed (Hu *et al.*, 2012), confirming Hiepko’s view that these very small scale-like organs are homolog to petals, notably due to their vascularization (Hiepko, 1965). In Papaveraceae subfamily Fumarioideae, although the petals are not nectariferous, the outer whorl evolved a highly elaborate three dimensional shape, acting as nectar-storing organs (Endress & Matthews, 2006). The nectaries are located at the base of the stamens and the nectar is collected and stored in the pouch developed by the outermost whorl of petals. These nectaries appeared independently from the nectaries of other Ranunculales and would represent a synapomorphy of the clade containing all Fumarioideae except *Hypecoum* L. and *Pteridophyllum* Siebold & Zucc..

Symmetry – Unsurprisingly, the pattern obtained here for the character symmetry is consistent with previous studies (Damerval & Nadot, 2007; Reyes, Sauquet, & Nadot, 2016). The ancestral state for Ranunculales, and all key nodes except Papaveraceae is fully resolved as actinomorphy. Transitions towards disymmetry occurred once in the ancestor of Papaveraceae, and once in *Epimedium* (Berberidaceae). Bilateral symmetry evolved at least once from disymmetry within subfamily Fumarioideae (Papaveraceae) as already shown (Sauquet *et al.*, 2015), and twice from actinomorphy, in the genus *Cissampelos* L. (Menispermaceae), and in the ancestor of tribe Delphinieae (Ranunculaceae), where it could have triggered species diversification, considering the large number of species composing this tribe (ca. 650 species) with regard to the rest of the family (2525 species), as already investigated before (Jabbour *et al.*, 2009).

Reproductive characters

Number of stamens – Although this character is extraordinarily variable across angiosperms (from one stamen in *Piper* to over a thousand in *Adansonia* L., for example) even within families (in *Arecaceae* for example, (Nadot *et al.*, 2011)), it is often overlooked in studies on floral evolution. In some descriptions, the information given on stamen number can be “numerous” when the number is higher than 10-15. We show here that stamen number increased repeatedly in Ranunculales: in *Papaveroideae*, *Eupteleaceae*, within *Menispermaceae* and *Berberidaceae* and in the ancestor of *Ranunculaceae*, in agreement with previous studies (Damerval & Nadot 2007; Endress & Doyle 2009). The ancestral state (1-6) fits with the hypothesis of ancestral trimerous flowers for Ranunculales, *Menispermaceae* and *Berberidaceae*. In *Historical Ranunculaceae* and *Core Ranunculaceae*, the number of stamens is >12, which means the transition to pentamery does not completely account for this increase, as ten stamens would be expected since they seem to be organized in two whorls in Ranunculales (Endress & Doyle, 2009). A transition towards a spiral development of the androecium could explain this number, as reconstructed before (Endress & Doyle, 2009).

Number of carpels: The ancestral number of carpels is ambiguous and surprisingly, even the ancestral state for *Papaveraceae* is not resolved, although it seems to be due to the low level of variation in this group (Sauquet *et al.*, 2015). *Berberidaceae* had a single carpel, which is consistent with all the descriptions of the family and it was already considered as a synapomorphy for the family (Zhao *et al.*, 2016 and references within), *Menispermaceae* probably had ancestrally (3-6) carpels, and *Core Ranunculaceae* had 12 or more carpels, adding precision to previous works. For example, in Wang *et al.* (2009), the state for the highest possible number of carpels was “more than three”. We found that carpels were ancestrally fused in Ranunculales with rjMCMC. Fusion of carpels is common in monocots and eudicots, and may be ancestral in monocots (Sokoloff *et al.*, 2013). According to Endress

& Doyle (2009), post-genital fusion between carpels could be ancestral for mesangiosperms, followed by multiple losses.

Scenario of loss and specialization

Elaborating on these ancestral state reconstructions, we produced a scenario for perianth evolution in Ranunculales based on a combination of specialisations, gains and losses of perianth whorls (Figure 5). From an ancestral Ranunculales flower with at least three differentiated petaloid perianth whorls, the outermost organs would have evolved twice towards sepaldoidy, in the ancestral flower of Papaveraceae and in Menispermaceae, and the whole perianth would have been lost in the ancestor of Eupteleaceae. The ancestral perianth would have been retained in the ancestor of [Lardizabalaceae + Circaeasteraceae], with subsequent loss of two perianth whorls in Circaeasteraceae, which would have evolved towards spiral phyllotaxy of the remaining whorl (the ancestral aspect, petaloid or sepaldoid, is unclear), while in Lardizabalaceae, one whorl of organs was gained and the two inner whorl became specialised and modified as “nectary leaves”, both outer whorls remaining petaloid. The ancestor of [Menispermaceae + Berberidaceae + Ranunculaceae] and of [Berberidaceae + Ranunculaceae] would have retained the ancestral perianth of Ranunculales, with further specialisation towards sepaldoidy of the outer whorl in Menispermaceae, like in Papaveraceae. The ancestral flower of Berberidaceae would have gained an extra whorl of petaloid organs, resulting in their typical perianth described as having two whorls of petaloid sepals and two whorls of petals (or more in some Berberidaceae), while one of the outer whorls would have been lost in the ancestor of Ranunculaceae, both remaining whorls being different but petaloid. Within Ranunculaceae, the ancestor of Historical Ranunculaceae would have evolved the strongly modified typical ranunculacean petals, which would have been retained (or lost) in Core Ranunculaceae.

The ancestral differentiation of the perianth at the level of Ranunculales could result from different origins of the organs (bracts or staminodes), as already extensively discussed based on morphology (Hiepko, 1965; Kosuge, 1994; Endress, 1995; Ronse De Craene & Brockington, 2013), and as suggested by developmental genetics (Rasmussen *et al.*, 2009; Zhang *et al.*, 2013). The possible loss of one whorl from an ancestral perianth with three whorls in Ranunculaceae had been briefly suggested by (Ronse De Craene *et al.* (2003) but little discussed. The scenario proposed here questions the notion of petal, since the same word is used to designate organs that are analogous with similar functions, or organs that are homologous with different functions as already discussed by (Ronse De Craene & Brockington, 2013). The same rationale applies to the term “sepal” used to designate the outermost homologous organs with a protective function in Papaveraceae and Menispermaceae, and to designate the petaloid outer organs of Ranunculaceae, which have a different origin in this latter case since they are probably homologous to the innermost outer organs, the outermost whorls having been lost.

This scenario is of course a hypothesis but, because it relies on ancestral state reconstructions that take into account phylogenetic uncertainty, we believe it is a reasonable hypothesis for perianth evolution in Ranunculales. This scenario can prove useful to guide future evo-devo studies aiming at testing the identity of the different perianth organs and their homology across families. For example, homologs of *AP3-3* are expected to be expressed in both inner perianth whorls of Berberidaceae but not in the two outer whorls, and they are expected to be expressed also in the inner perianth organs of Menispermaceae but not in both outer whorls although the innermost outer whorl is petaloid.

Characters linked to pollination

Studying floral evolution is crucial to understand the natural history of angiosperms, since the flower has played a major role in shaping the extraordinary diversity of this group of land plants. This has taken place largely through the interaction with pollinating agents. Most of the traits examined in this study likely have an adaptive value with regard to pollination, and some (like bilateral symmetry, pentamery, or perianth differentiation) have even been proposed as key innovations (Endress, 2011), i.e., their origin in a clade would have triggered species diversification. To construct evolutionary scenarios based on adaptive hypotheses, it is primordial to identify ancestral states, and hence give a direction to evolution, to ensure that the traits identified as adaptations are indeed evolutionary novelties. In what follows, we will discuss traits directly related to the interaction with pollinators, and the potential adaptive value of these traits. It is generally admitted that the perianth has two main functions in the reproduction of flowering plants: i) protect floral organs during development - this function is assumed by the sepals in eudicots, or by the outer tepals in monocots, or by the whole perigone in flowers with a single whorl of perianth organs, ii) attract pollinators by visual cues - this function is usually assumed by the petals in eudicots, and by the whole perianth in monocots with undifferentiated perianth. In the case of species presenting elaborate petals, a third function is assumed, reward floral visitors by the production of nectar (Endress & Matthews, 2006).

In Ranunculales, as in most angiosperms, the characters concerning perianth features and nectary location are the most closely linked to pollination, hence to reproductive success. In Eupteleaceae, the loss of perianth combined with an increase in stamen number (potentially leading to an increase in pollen production) suggest adaptation to wind pollination, which has indeed been observed (Ren *et al.*, 2007). The sepaloïdy of the outer perianth organs of Papaveraceae, Circaeasteraceae and Menispermaceae, derived from an ancestral petaloïdy hypothesized here, could indicate a change of strategy favouring protection, leaving the

function of attraction restricted to the inner organs. The low specific diversity of Circaeasteraceae could result from their spiral phyllotaxy at anthesis, which seems to be a major selective disadvantage (Reyes *et al.*, 2018), or could be accounted for by their mode of reproduction. In the monotypic genus *Kingdonia* Balf. F. & W.W. Sm., reproduction is predominantly clonal (Wang *et al.*, 2012) although fly pollination has been observed (He, Zhang, & Ren, 2006). The states for the ancestral flower of Ranunculales would correspond to a fly pollination syndrome, according to Endress (1995), which would have been retained in Lardizabalaceae and Menispermaceae.

The possible roles of modified petals have been discussed by Endress & Matthews (2006). In Ranunculales, the elaborate petal is usually thought to serve as a reservoir for nectar enhancing the storage capacity of the flower and the amount of reward available for the pollinators, whether nectaries are located on the petal itself or elsewhere in the flower. A recent study conducted on the pollination of *Ranunculus* L. species (Min *et al.*, 2019) showed that the flowers, which typically have flat petals bearing a nectary scale, are pollinated by a few insect categories, suggesting some specialisation in pollination, although buttercup flowers, with their open corolla, numerous stamens and presence of nectar, are usually viewed as generalists (Tamura, 1993). More generally, the diversity of shapes of elaborate petals in Ranunculaceae, at the intergeneric level but also among species of the same genus, like in *Nigella* or *Aquilegia*, could reflect specialisation in pollination.

The diversity of perianth traits in Ranunculales and particularly in Ranunculaceae, which have evolved nectar spurs and zygomorphy, raises the question of whether some of these traits may have played a role in species diversification. This has been shown in the genus *Aquilegia*, where spur length had an effect on species diversification (Hodges, 1997). Spurs combined with zygomorphy and a relatively low number of stamens are also present in tribe Delphinieae (Jabbour & Renner, 2012) and Fumarioideae. Interestingly, this combination of traits has been

described as one of the most performant combination across angiosperms as a whole (Litt *et al.*, 2016), which could explain the large species diversity of Delphinieae that gather almost a quarter of the total number of Ranunculaceae species (Jabbour *et al.*, 2009) and the Fumarioideae that encompass more than twice more species than Papaveroideae (Sauquet *et al.*, 2015).

These hypotheses call for field studies since pollinators are confidently known for only a very small number of species and approximations through hypothetical pollination syndromes can be hazardous, especially in the case of insect pollination (Ollerton *et al.*, 2009). Furthermore, all of these hypotheses could be investigated in the context of diversification studies if a reliable timeframe were available for the whole order. Indeed, the correlation between innovation and an increase in diversification rates is a better argument for adaptation than simple discussion of the traits and taxonomic diversity such as here (O'Meara *et al.*, 2016).

Ancestral flower

To our knowledge, even though floral trait evolution in Ranunculales has been addressed in several previous studies (e.g. Endress & Doyle, 2009), there has never been a formal hypothesis for the ancestral flower of the order as a whole. For the first time, we propose that this ancestral flower was actinomorphic, possessed a differentiated perianth composed of a minimum of three trimerous whorls, an androecium composed of six stamens at least, and a gynoecium composed of >12 carpels. All organs were free. This challenges the view that phyllotaxy in the ancestral perianth of Ranunculales, was spiral, as already found with phylogeny-based analyses (Endress & Doyle, 2009). In our hypothesis for the ancestral flower of Ranunculales, the trimerous perianth is shared by the ancestral flower reconstructed for monocots, magnoliids, and angiosperms as a whole (Sauquet *et al.*, 2017). This is in contrast

with the dimerous state reconstructed for eudicots, although with uncertainty. There could have been a transition from trimery to dimery at the base of all eudicots, followed by a reversal at the base of the early-diverging Ranunculales. Alternatively, reconstructions could have been influenced by the dimery of Papaveraceae and Proteaceae.

Conclusion

Although there are still many uncertainties in the results presented in this study due to the phylogenetic framework (incomplete sampling, phylogenetic uncertainty due to the limited number of markers, lack of information for some floral traits), we propose a new, clear working hypothesis on the evolution of the flower in the highly diversified Ranunculales order. In particular, we show that nectar-storing inner organs of the perianth (“petals”) have a single origin in the ancestor of Historical Ranunculaceae. This family is traditionally distinctive as having flowers with a combination of spirally arranged perianth parts in one or two series, numerous stamens, and an apocarpous gynoecium. Here we find that the strongly modified morphology associated with nectar production and storage of the inner parts of the perianth is a synapomorphy of the family. This work brings further support, in a complementary manner, to recent evo-devo hypotheses on the origin of petals in Ranunculales and proposes a more comprehensive view of the evolution of the flower as a whole including reproductive organs, providing a robust basis to further explore the molecular bases of floral variation in an evo-devo perspective. The study presented here also provides a solid basis to address future questions of adaptation and to elaborate evolutionary scenarios by testing correlated evolution between traits and diversification rates, using phylogenetic comparative methods.

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Tables

Table 1 - Interpretation of the results obtained with rjMCMC and parsimony for each character and each key node. NA = Non-applicable; level of confidence for characters up to three states: *=low, **=medium, ***=high (based on mean PP); level of confidence for characters with four states or more: °=low, °°=medium, °°°=high (based on confidence interval overlaps).

Key Nodes	Perianth phyllotaxy		Perianth symmetry		Number of perianth parts	
	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony
Ranunculales	Whorled ***	Whorled	Actinomorphic *	Actinomorphic	Uncertain	Ambiguous
Ran+Ber+Men	Whorled ***	Whorled	Actinomorphic ***	Actinomorphic	Uncertain	Ambiguous
Ran+Ber	Whorled ***	Whorled	Actinomorphic ***	Actinomorphic	Uncertain	Ambiguous
Papaveraceae	Whorled ***	Whorled	Disymmetric ***	Disymmetric	5-10 °°	5-10
Eupteleaceae	NA	Whorled	NA	Actinomorphic	None °°°	None
Circaeasteraceae	Spiral ***	Spiral	Actinomorphic ***	Actinomorphic	≥11 °	≥11
Lardizabalaceae	Whorled ***	NA	Actinomorphic ***	Actinomorphic	≥11 °	≥11
Menispermaceae	Whorled ***	Whorled	Actinomorphic ***	Actinomorphic	Uncertain	Ambiguous
Berberidaceae	Whorled ***	Whorled	Actinomorphic ***	Actinomorphic	≥11 °°	5-10
Ranunculaceae	Whorled **	Whorled	Actinomorphic ***	Actinomorphic	Uncertain	Ambiguous
Core Ranunculaceae	Whorled *	Whorled	Actinomorphic ***	Actinomorphic	5-10 °	5-10

Key Nodes	Perianth merism		Number of perianth whorls		Differentiation		Variability perianth parts	
	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony
Ranunculales	Trimerous °	Trimerous	≥3 °°°	≥3	Differentiated **	Differentiated	Uncertain	Constant
Ran+Ber+Men	Trimerous °°	Trimerous	≥3 °°	≥3	Uncertain	Differentiated	Uncertain	Constant
Ran+Ber	Trimerous °	Trimerous	≥3 °	≥3	Uncertain	Differentiated	Uncertain	Constant
Papaveraceae	Dimerous °°°	Dimerous	≥3 °°°	≥3	Differentiated ***	Differentiated	Constant *	Constant
Eupteleaceae	NA	NA	NA	NA	NA	NA	NA	NA
Circaeasteraceae	NA	NA	1 °°	1	Differentiated *	Differentiated	Uncertain	Variable
Lardizabalaceae	Trimerous °°°	Trimerous	≥3 °	≥3	Differentiated **	Differentiated	Constant *	Constant
Menispermaceae	Trimerous °°°	Trimerous	≥3 °	≥3	Differentiated ***	Differentiated	Uncertain	Constant
Berberidaceae	Trimerous °°	Trimerous	≥3 °°°	≥3	Uncertain	Differentiated	Uncertain	Constant
Ranunculaceae	Uncertain	Dimerous	2 °°	2	Uncertain	Ambiguous	Uncertain	Constant
Core Ranunculaceae	Pentamerous °	Pentamerous	2 °°	2	Differentiated **	Differentiated	Uncertain	Variable

Key Nodes	Outer perianth aspect		Inner perianth aspect		Perianth parts fusion		Spurs	
	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony
Ranunculales	Petaloid ***	Sepaloid	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Ran+Ber+Men	Petaloid ***	Sepaloid	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Ran+Ber	Petaloid ***	Sepaloid	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Papaveraceae	Petaloid ***	Sepaloid	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Eupteleaceae	NA	NA	NA	NA	NA	NA	NA	NA
Circaeasteraceae	Sepaloid ***	Sepaloid	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Lardizabalaceae	Petaloid ***		Strongly modified ***	Strongly modified	Free ***		Absent ***	
Menispermaceae		Petaloid				Free		Absent
	Sepaloid ***	Sepaloid	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Berberidaceae	Petaloid ***	Ambiguous	Petaloid ***	Petaloid	Free ***	Free	Absent ***	Absent
Ranunculaceae	Petaloid ***	Ambiguous	Uncertain	Petaloid	Free ***	Free	Absent ***	Absent
Core Ranunculaceae	Petaloid ***		Strongly modified ***	Strongly modified	Free ***		Absent ***	
		Petaloid				Free		Absent
Key Nodes	Stamen number		Carpel number		Fusion of carpels		Position of nectaries	
	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony	rjMCMC	Parsimony
Ranunculales	0-6 °	0-6	≥12 °	Ambiguous	Free ***	Ambiguous	Uncertain	Absent
Ran+Ber+Men	0-6 °°	0-6	≥12 °	Ambiguous	Free ***	Free	Uncertain	Absent
Ran+Ber	0-6 °	0-6	≥12 °	Ambiguous	Free ***	Free	Perianth °	Absent
Papaveraceae	0-6 °	0-6	2 °	Ambiguous	Fused ***	Fused	Absent °	Absent
Eupteleaceae	13-49 °°°	13-49	≥12 °	Ambiguous	Free ***	Free	Absent °°°	Absent
Circaeasteraceae	0-6 °°°	0-6	3-6 °	Ambiguous	Free ***	Free	Perianth °°°	Ambiguous
Lardizabalaceae	0-6 °°°	0-6	≥12 °	Ambiguous	Free ***	Free	Perianth °°°	Ambiguous
Menispermaceae	0-6 °	0-6	3-6 °°°	3-6	Free ***	Free	Absent °°°	Absent
Berberidaceae	0-6 °°°	0-6	1 °°°	1	NA	NA	Uncertain	Absent
Ranunculaceae	13-49 °	≥50	≥12 °°	Ambiguous	Free ***	Free	Perianth °	Absent
Core Ranunculaceae	13-49 °	13-49	≥12 °°	≥12	Free ***	Free	Perianth °°°	Perianth

Figures captions

Figure 1 - Examples of floral diversity in Ranunculales. a. Lardizabalaceae. *Akebia quinata*. Male flowers in the back, female flower in the front. b. Menispermaceae. *Menispermum canadense* L.. Male flowers. c. Papaveraceae. *Papaver orientale* L.. d. Papaveraceae. *Fumaria capreolata* L.. e. Berberidaceae. *Epimedium alpinum* L.. f. Ranunculaceae. *Helleborus niger* L.. g. Ranunculaceae. *Clematis alpina* (L.) Mill.. h. Ranunculaceae. *Aconitum carmichaelii* Debeaux. - Photographs: Laetitia Carrive.

Figure 2 – Bayesian constrained consensus tree. Numbers above branches are posterior probabilities (PP). All nodes with PP values lower than 0.95 (our threshold value for good support) were collapsed. Subfamilies and tribes within Ranunculaceae are indicated on the right-hand side of the tree. The 30 constrained nodes are shown in yellow. Key Nodes are highlighted in dark blue.

Figure 3 – Ancestral state reconstructions using parsimony on the constrained Bayesian tree. The key nodes are indicated (BER=Berberidaceae, CIR=Circaeasteraceae, EUP=Eupteleaceae, LAR=Lardizabalaceae, MEN=Menispermaceae, PAP=Papaveraceae, RAN=Ranunculaceae, HRAN=Historical Ranunculaceae, CRAN=Core Ranunculaceae). a. Character “Number of whorls”. b. Character “Differentiation”.

Figure 4 – Ancestral state reconstructions using parsimony on the constrained Bayesian tree. The key nodes are indicated (BER=Berberidaceae, CIR= Circaeasteraceae, EUP=Eupteleaceae, LAR=Lardizabalaceae, MEN=Menispermaceae, PAP=Papaveraceae, RAN=Ranunculaceae, HRAN=Historical Ranunculaceae, CRAN=Core Ranunculaceae). a. Character “Inner perianth aspect”. b. Character “Location of nectar production”.

Figure 5 - Evolutionary scenario of the evolution of perianth structure and differentiation. Ancestral states concerning fertile organs are not shown.

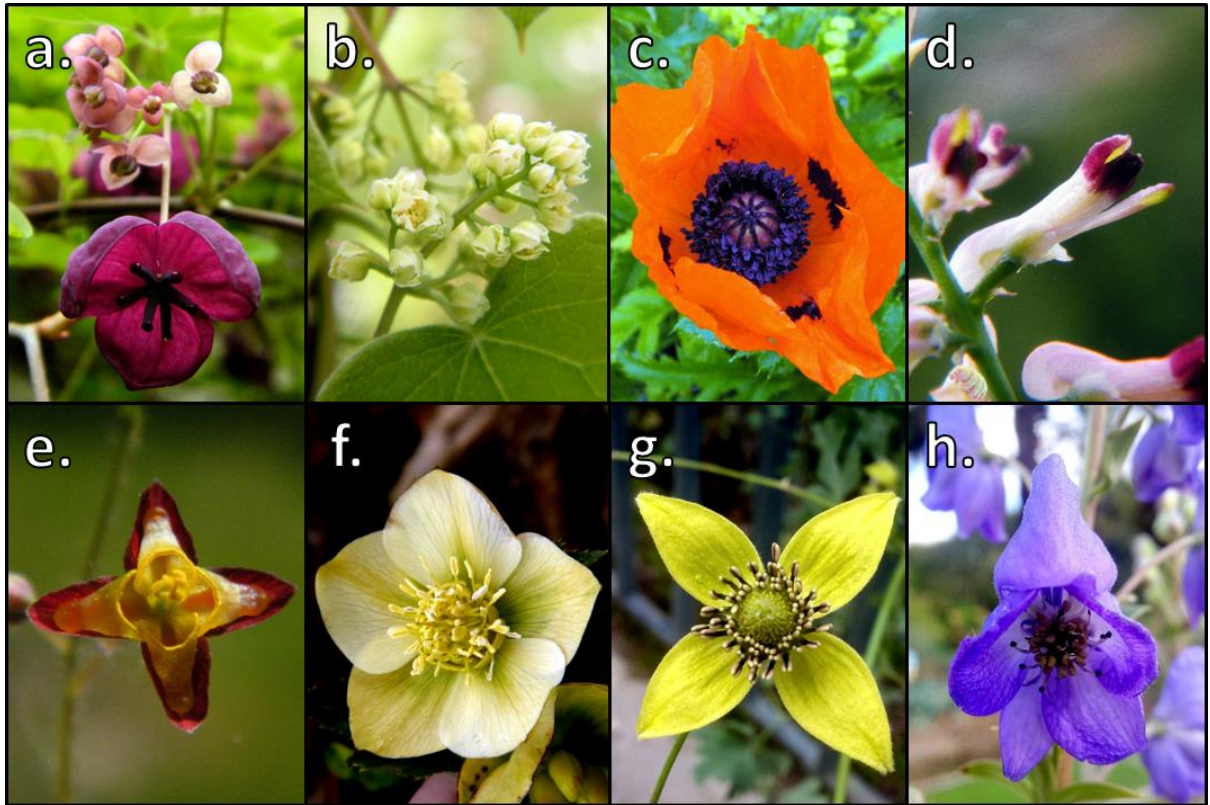
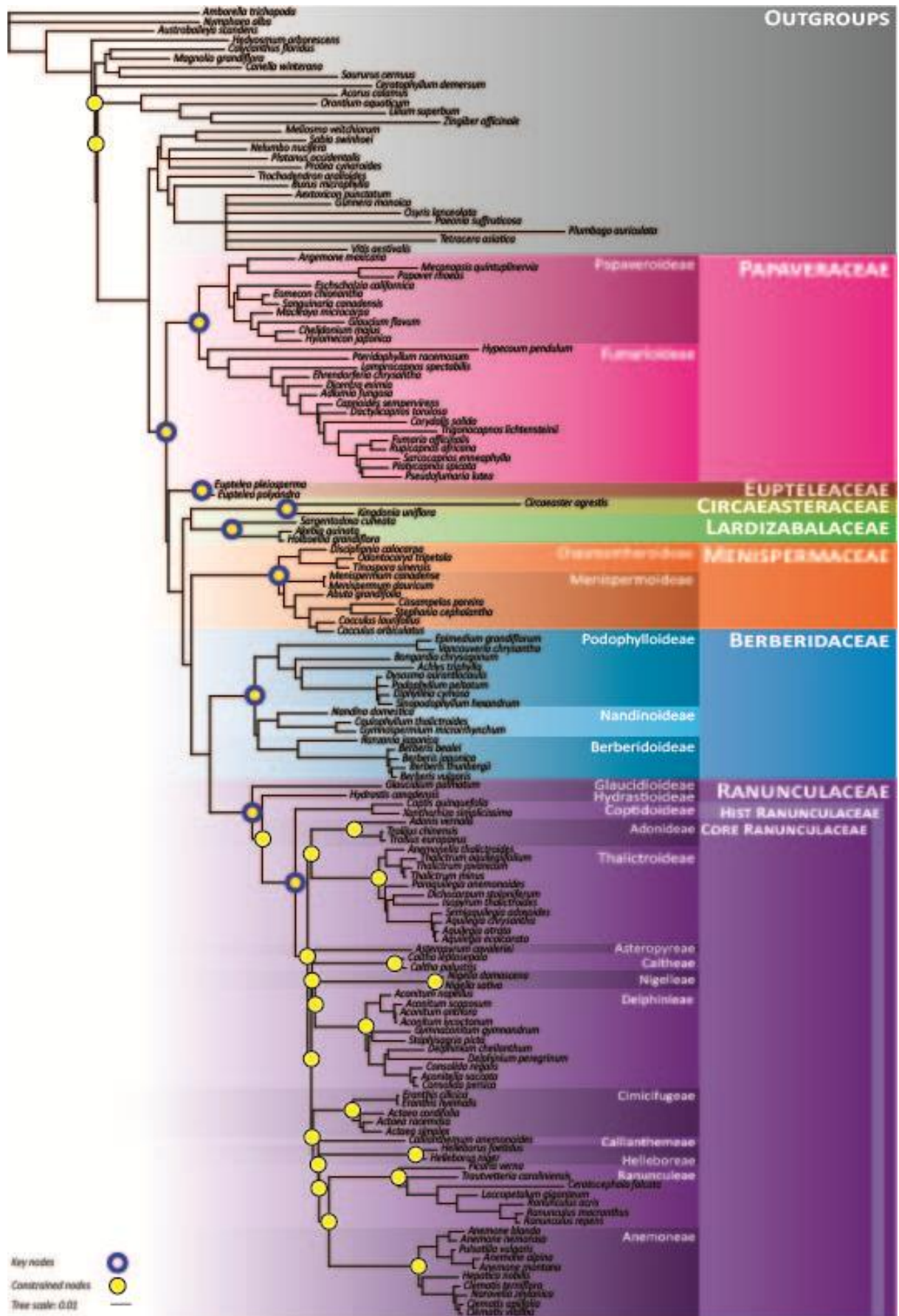


Figure 1



Figure

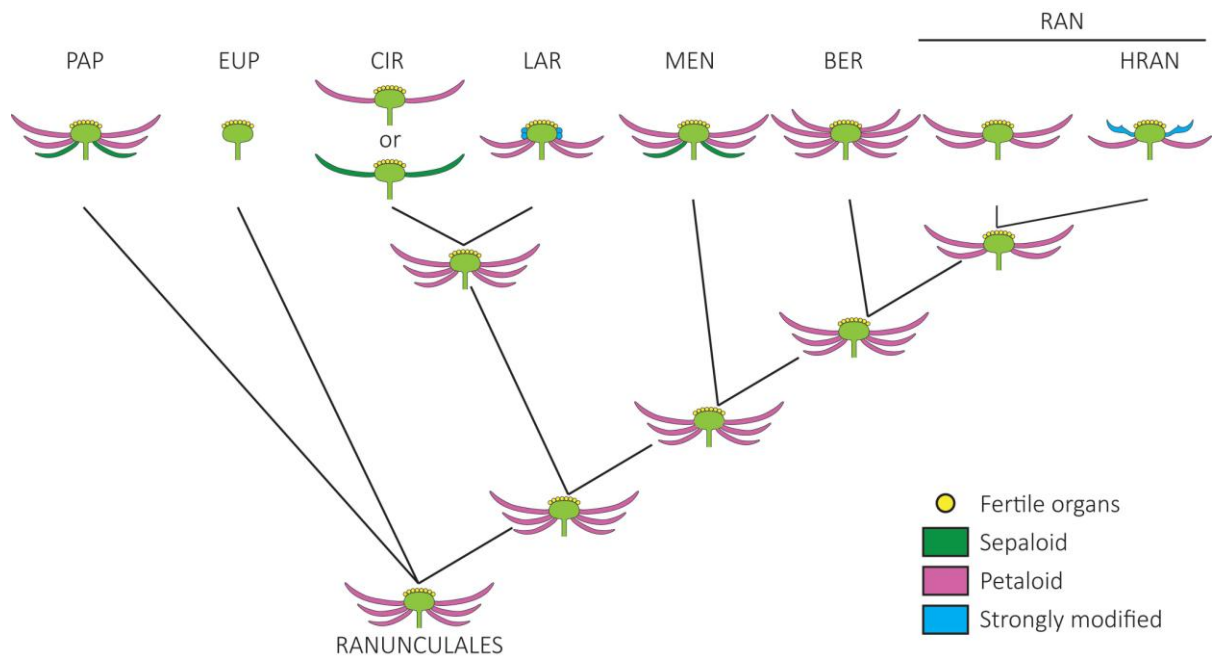


Figure 5

Supplementary Material

S1. Taxonomic sampling. List of species included in the phylogenetic analyses, with GenBank accession of the sequences used. Information on family and order is given for each species.

S2. Alignment used for the phylogenetic reconstructions

S3. Bayesian unconstrained consensus tree. Numbers above branches are posterior probabilities (PP). All nodes with PP values lower than 0.95 (our threshold value for good support) were collapsed. Subfamilies and tribes within Ranunculaceae are indicated on the right-hand side of the tree. (BER=Berberidaceae, CIR= Circaeasteraceae, EUP=Eupteleaceae, LAR=Lardizabalaceae, MEN=Menispermaceae, PAP=Papaveraceae, RAN=Ranunculaceae, HRAN=Historical Ranunculaceae, CRAN=Core Ranunculaceae).

S4. Morphological matrix

S5. Sources of morphological data

S6. Explanations on the rjMCMC results interpretations for characters with four states or more. State shown here in the number of stamens. a. Uncertain result. b. Low confidence result. c. Medium confidence result. d. High confidence result.

S7. Parsimony results. Ancestral state reconstruction using parsimony of the characters on the Bayesian consensus tree. Legend is given in the blue box on each tree.

S8. Ancestral states reconstructions for the key nodes. a. Raw rjMCMC results. The values are the mean PP and the 95% confidence interval of the PP between square brackets. b. Interpreted rjMCMC results (NA = Non-applicable; level of confidence: *=low, **=medium, ***=high c. Parsimony results. Most parsimonious state.