Establishment of zygomorphy on an ontogenetic spiral and evolution of perianth in the tribe Delphinieae (Ranunculaceae)

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- **Background and Aims** Ranunculaceae presents both ancestral and derived floral traits for eudicots, and as such is of potential interest to understand key steps involved in the evolution of zygomorphy in eudicots. Zygomorphy evolved once in Ranunculaceae, in the speciose and derived tribe Delphinieae. This tribe consists of two genera (Aconitum and Delphinium s.l.) comprising more than one-quarter of the species of the family. In this paper, the establishment of zygomorphy during development was investigated to cast light on the origin and evolution of this morphological novelty.

- **Methods** The floral developmental sequence of six species of Ranunculaceae, three actinomorphic (Nigella damascena, Aquilegia alpina and Clematis recta) and three zygomorphic (Aconitum napellus, Delphinium staphisagria and D. grandiflorum), was compared. A developmental model was elaborated to break down the successive acquisitions of floral organ identities on the ontogenic spiral (all the species studied except Aquilegia have a spiral phyllotaxis), giving clues to understanding this complex morphogenesis from an evo-devo point of view. In addition, the evolution of symmetry in Ranunculaceae was examined in conjunction with other traits of flowers and with ecological factors.

- **Key Results** In the species studied, zygomorphy is established after organogenesis is completed, and is late, compared with other zygomorphic eudicot species. Zygomorphy occurs in flowers characterized by a fixed merism and a partially reduced and transformed corolla.

- **Conclusions** It is suggested that shifts in expression of genes controlling the merism, as well as floral symmetry and organ identity, have played a critical role in the evolution of zygomorphy in Delphinieae, while the presence of pollinators able to exploit the peculiar morphology of the flower has been a key factor for the maintenance and diversification of this trait.

**Key words:** Delphinieae, development, evolution, evo-devo, nectar spurs, ontogenic spiral, Ranunculaceae, zygomorphy.

**INTRODUCTION**

To understand the origin, establishment and evolution of morphological novelties, it is crucial to study these new traits from a developmental point of view. A morphological novelty is considered a key innovation when the acquisition of the new trait is correlated with unexpectedly high rates of speciation in the clade housing the innovation (Sanderson and Donoghue, 1994). In the field of plant biology, floral symmetry is a well-known character for favouring evolutionary radiation and rapid diversification (Sargent, 2004). This trait is generally recorded for the perianth and androecium, the gynoecium often being characterized by loss of organs (Endress, 1999). Two principal types of symmetry are recognized among angiosperms: actinomorphy (radial symmetry or polysymmetry) and zygomorphy (bilateral symmetry or monosymmetry). In an actinomorphic flower, all organs of the same identity (sepal, petal or stamen) in a whorl exhibit equal (or almost equal) size and shape, and are inserted regularly on the receptacle. An actinomorphic flower is defined by at least two identical symmetry planes. By contrast, zygomorphy is defined by a supplementary identity superimposed on the basic organ identity, resulting in intra-whorl differentiation that generates a single symmetry plane, most often dorsoventrally oriented. Zygomorphy is a derived state that has arisen several times independently in flowering plants (Endress, 1999; Cubas, 2004). Exceptions to actinomorphy and zygomorphy are relatively rare and occur in only a few taxa, such as some Fumarioideae of Papaveraceae that have disymmetric flowers (characterized by two unequal planes of symmetry, e.g. Hypecoum and Dicentra sensu lato) or some Leguminosae, Caprifoliaceae and Cannaceae that possess asymmetric flowers (Dahlgren et al., 1985; Endress, 1999; Tucker, 2003).

Whereas in basal angiosperms transitions towards zygomorphy are scarce (e.g. Piperales), such transitions are much more frequent in the eudicots. Among basal eudicots, the order Ranunculales is sister to all other eudicots, and possesses features that are found both in basal angiosperms and in the more-derived eudicots. Three independent acquisitions of zygomorphy have been recorded within the Ranunculales, each in a different family (Papaveraceae, Menispermaceae and Ranunculaceae; Damerval and Nadot, 2007). Here, the focus is on Ranunculaceae (62 genera, 2525 species), a family that presents both ancestral and derived floral traits for eudicots,
and as such is of potential interest to understand key steps involved in the evolution of zygomorphy in eudicots. In Ranunculaceae, at least some floral organs are initiated on a spiral. Spiral phyllotaxis is an ancestral state and is traditionally associated with actinomorphy. Interestingly, in Ranunculaceae, zygomorphy takes place only in flowers with spiral phyllotaxis. The transition to zygomorphy occurred once in the family, in the ancestor of three sister genera, Aconitum, Delphinium and Consolida, which form the tribe Delphinieae. The genera Aconitum and Delphinium s.l. (Delphinium + Consolida) comprise approx. 300 and 365 species, respectively, which in total accounts for approx. 26% of all Ranunculaceae species [Tamura, 1993; also electronic databases: eFloras (www.efloras.org), Angiosperm Phylogeny Website (Stevens, 2001) and Delta (Watson and Dallwitz, 1992 onwards)]. In all species of Delphinieae, zygomorphy is apparent primarily in the calyx and corolla, where the dorso-lateral/ventral petals are reduced and the two adaxial ones develop into nectar spurs.

In order to understand the developmental and evolutionary bases of zygomorphy in Delphinieae, floral organogenesis and organ development is analysed and compared in six species, three with actinomorphic flowers (Nigella damascena, Aquilegia alpina and Clematis recta) and three with zyomorphic flowers (Aconitum napellus, Delphinium staphisagria and D. grandiflorum). Full or partial descriptions have already been published for Aquilegia (Tucker and Hodges, 2005) and Aconitum (development of the petals: Kosuge, 1994; Kosuge and Tamura, 1988). This study concentrates on the timing of events leading to the establishment of zygomorphy and the formation of spurs. Moreover, the changes in organ identities during organogenesis are analysed in order to determine (a) the factors that can discriminate between two different and consecutive organs on the spiral and (b) the developmental origins of different structures in the corolla. The floral development of Delphinieae is compared with that of two zyomorphic species belonging to different eudicot lineages (the derived core eudicot Antirrhinum majus of Plantaginaceae and the basal eudicot Synaphea spinulosa of Proteaceae), in order to identify potential specificities of Delphinieae. In addition, the architectural and ecological contexts associated with the emergence of zygomorphy in Ranunculaceae are examined by looking at the evolution of floral and inflorescence architectural traits, the type of pollination and the sexual system.

Materials and methods

Species sampled and material collection

The taxa investigated are listed in Table 1. The six species were chosen for their morphological specificities (Fig. 1). All six species have petaloid sepals. The three actinomorphic species have a polysymmetric perianth but, in addition to this, Nigella damascena presents a series of bulged petals forming hairs and bearing nectaries, Aquilegia alpina has a whorled phyllotaxis and as many spurred petals as indicated by merism, and Clematis recta has no corolla. It should be noted that C. recta will be considered actinomorphic even though Endress (1987) described symmetry as bilateral in this genus because of the particular shape of the androecium. Except C. recta that exhibits staminodes between calyx and androecium, the five other species have petals more or less developed. Among the zygomorphic taxa, the genus Delphinium presents flowers with two dorsal spurred petals bearing nectaries inside the spurs. They also have two dorso-lateral hairy flat petals without nectaries and four reduced ventral petals. Spurred petals are short in Delphinium staphisagria, but long in D. grandiflorum, and both are wrapped in a single spurred sepal. Aconitum napellus presents a hood-shaped sepal enclosing two spurred nectariferous petals, and six reduced petals without nectaries. In addition to the dorsal sepal, the pentameric calyx of Delphinieae consists of two dorso-lateral and two ventral sepals.

Two eudicot species were chosen in order to represent the establishment of zygomorphy in taxa presenting various degrees of relatedness with the Ranunculaceae and their developmental sequence was reconstructed from literature. Synaphea spinulosa (Proteaceae; Douglas, 1997) was chosen because Proteales belong to the basal eudicot grade like Ranunculales, whereas Antirrhinum majus (Plantaginaceae; Vincent and Coen, 2004) belongs to a derived clade of the core eudicots.

Plants were grown in a greenhouse at UMR de Génétique Végétale, Gif-sur-Yvette, France. Floral buds were sampled in order to obtain an exhaustive representation of the developmental sequence. They were immediately fixed in FAA (85 mL 55% ethanol/5 mL glacial acetic acid/10 mL formaldehyde) and then stored in 70% ethanol. Transverse sections of A. napellus were observed with a binocular microscope Zeiss SV11.

Microscopy and calibration of development sequence over time

Buds were dissected with a Wild MZ8 stereomicroscope (Leica, Wetzlar, Germany), dehydrated in an ethanol-acetone series, and dried with a K850 critical point dryer (Emitech, Ashford, Kent, UK). Dried floral structures were mounted on aluminum stubs with colloidal graphite and coated with platinum using an Emitech K575X sputter coater (Emitech) and observed with a Supra 55VP scanning electron microscope (LEO Electron Microscopy, Cambridge, UK). Pickled reference material and platinum coated material are kept at UMR de Génétique Végétale.

The successive developmental stages were represented along an axis of relative time representing the development of the flower, from the initiation of the first sepal primordium

<table>
<thead>
<tr>
<th>Species</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aconitum napellus L.</td>
<td>Horticultural</td>
</tr>
<tr>
<td>Aquilegia alpina L.</td>
<td>Horticultural</td>
</tr>
<tr>
<td>Clematis recta L.</td>
<td>Horticultural</td>
</tr>
<tr>
<td>Delphinium grandiflorum L.</td>
<td>Horticultural</td>
</tr>
<tr>
<td>Delphinium staphisagria L.</td>
<td>Accession 02-58, Natural History Museum, Paris</td>
</tr>
<tr>
<td>Nigella damascena L.</td>
<td>Accession 04-98, Natural History Museum, Paris</td>
</tr>
</tbody>
</table>
on the floral apex to the bud just before blooming. Time was calibrated according to the size of the floral receptacle. This organ grows continuously and homogeneously, thus giving a reliable image of the pace of development (see Douglas, 1997). The relative time was calculated as the proportion between the diameter of the receptacle of the bud and that of the adult open flower. The diameter of the floral receptacle for buds and adult flower was measured for several individuals and plants (three to seven from different plants).

Reconstruction of character evolution

Morphological data (phyllotaxis, merism, number of nectar spurs, sexual system and type of inflorescence) were compiled from Tamura (1993) and electronic databases: eFloras (www.efloras.org), Angiosperm Phylogeny Website (Stevens, 2001 onwards) and Delta (Watson and Dallwitz, 1992 onwards). Pollination data were compiled from the literature (Table 2). A matrix of 31 taxa including all characters was built using Mesquite 2.01 (Maddison, 2000). Character states were plotted on the most recent phylogenetic tree of Ranunculaceae, comprising 31 genera among the 62 identified (Hoot, 2008). Ancestral states were not resolved due to the lack of resolution at the deepest nodes of the tree.

**RESULTS**

Comparative developmental analysis was done in six species of Ranunculaceae, in order to describe precisely the organogenesis and further differentiation of floral organs preceding the establishment of zygomorphy. Three species of Delphinieae (one species of Aconitum and two of Delphinium differing by the length of the spur), one species of Nigella, that is considered the sister genus of Delphinieae or at least being a part of their sister clade, one species of Aquilegia (Aq. alpina) that shares architectural traits with Nigella but is phylogenetically distant from Delphinieae, and one species of Clematis where a corolla is lacking (C. recta) were selected.

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**Table 2. Species of Delphinieae and Nigella and available data about their pollination compiled from the literature**

<table>
<thead>
<tr>
<th>Species</th>
<th>Spur length (mm)</th>
<th>Diptera</th>
<th>Hymenoptera</th>
<th>Apodiformes</th>
<th>Lepidoptera</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. nuttallianum</em></td>
<td>8–23</td>
<td>Solitary bees</td>
<td>Queen bumblebees</td>
<td>Hummingbirds</td>
<td></td>
<td>Bosch and Waser, 1999; Williams et al., 2001; Flora of North America (eFloras: <a href="http://www.efloras.org">www.efloras.org</a>)</td>
</tr>
<tr>
<td><em>D. barbei</em></td>
<td>10–18</td>
<td></td>
<td>Bumblebees</td>
<td>Hummingbirds</td>
<td></td>
<td>Williams et al., 2001; Flora of North America (eFloras)</td>
</tr>
<tr>
<td><em>D. nudicaule</em></td>
<td>26–33.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Guerant, 1982</td>
</tr>
<tr>
<td><em>D. leroyi</em></td>
<td>30–40</td>
<td></td>
<td>Queen and worker bumblebees</td>
<td></td>
<td></td>
<td>Johnson, 2001; Flora Zambesiaca (eFloras)</td>
</tr>
<tr>
<td><em>Ac. columbianum</em></td>
<td>approx. 11</td>
<td></td>
<td>Queen and worker bumblebees</td>
<td></td>
<td></td>
<td>Bosch and Waser, 1999; Flora of North America (eFloras)</td>
</tr>
<tr>
<td><em>Ac. lycocotonum</em></td>
<td>16–20</td>
<td></td>
<td>Bombus gerstaeckeri</td>
<td></td>
<td></td>
<td>Utelli et al., 2000</td>
</tr>
<tr>
<td><em>Ac. gymnandrum</em></td>
<td>approx. 18</td>
<td></td>
<td>Bumblebees</td>
<td></td>
<td></td>
<td>Zhang et al., 2006; Flora of China (eFloras)</td>
</tr>
<tr>
<td><em>Ac. septentrionale</em></td>
<td>approx. 24</td>
<td></td>
<td>Specialist Bombus consobrinus</td>
<td></td>
<td></td>
<td>Thostesen and Olesen, 1996; Flora of China (eFloras)</td>
</tr>
<tr>
<td><em>Nigella arvensis</em></td>
<td>No spur</td>
<td>Apis mellifera</td>
<td>Pyrobombus lapidarius, Polistes dominulus, Eumenes pedonculatus</td>
<td></td>
<td></td>
<td>Weber, 1995</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Photographs of the species studied. Actinomorphic species: *Clematis recta* (A1, A2), *Nigella damascena* (B1, B2) and *Aquilegia alpina* (C1, C2); zygomorphic species: *Delphinium grandiflorum* (D1, D2), *D. staphisagria* (E1, E2) and *Aconitum napellus* (F1, F2). Arrows indicate: the bulged petals in *N. damascena* (B); the spurred petals in *Aq. alpina* (C); the location of the spurred sepal and of the pair of spurred petals in *D. staphisagria* (E1, E2, respectively). Scale bars: (A, B) = 0.5 cm; (C–F) = 1 cm.
Timing of events during floral development

The developmental sequence of the six species studied is presented schematically in Fig. 2, highlighting the various times when zygomorphy is established and spurs are formed in the Delphinieae species. Developmental stages are shown for the three zygomorphic species (Figs 3, 4 and 5, and Fig. S1 in Supplementary data, available online).

The initiation of all organs is most generally completed during the first quarter of development. In all species studied floral organ primordia initiate in a helical fashion (Figs 3 and 6, and Fig. S2 in Supplementary data), with the exception of petals, stamens and carpels in *Aquilegia alpina* which initiate in a whorled fashion (Fig. S2 in Supplementary data), and sepals in *Clematis recta* that appear as two opposite decussate primordia (data not shown).

In Delphinieae and *Nigella*, the perianth is composed of five petaloid sepals and eight more or less reduced petals. Sepal, petal and stamen initiation consists of four, seven and *n* − 1 plastochrons, respectively, *n* being the number of stamens. The sepals and petals initiate on respectively two and three revolutions of the spiral, meaning that phyllotaxis changes from 2/5 to 3/8 from calyx to corolla. The time devoted to the initiation of the three types of organs is equivalent (Fig. 2). This means that four plastochrons (separating the initiation of five sepals) must last as long as seven plastochrons (separating the initiation of eight petals). Since calyx and corolla appeared whorled in adult flowers, the plastochron between the last sepal and the first petal must be longer. It can therefore be concluded that petals initiate more rapidly than sepals and that stamens initiate even more rapidly than petals (if *n* > 8).

*Aquilegia alpina* bears five petals that initiate at the same speed as sepals. However, stamens initiate more rapidly, as in the other species studied. This developmental sequence is identical to that described by Tucker and Hodges (2005) for *Aquilegia olympica*. As noticed by Kramer and Hall (2005), staminodes are generally found between stamens and carpels.

After initiation, petals in *Delphinieae, Nigella damascena* and *Aquilegia alpina* appear to be delayed in development compared with the other organs. In *Aquilegia alpina* and *Nigella damascena*, they acquire their adult form at around the first quarter of the developmental sequence, after organogenesis is completed. In zygomorphic species, the protuberances preceding the spurs appear on the limb of the petals after all organs have been initiated. The dorsal sepal becomes hooded (*Aconitum napellus*) and spurred (*Delphinium grandiflorum*) a quarter time of development after the emergence of spurs in the two dorsal petals. Kosuge and Tamura (1988) noticed that zygomorphy is established when the bud is approx. 2 mm, which is consistent with the present results. Spurs in the dorsal petals and in the dorsal sepal of *Delphinium staphisagria* appear later than in *Delphinium grandiflorum. Zygoma plays its cards close to its chest

Zygomorphy in the three Delphinieae species is established late in development, compared with the situation in the two outgroup species *Synaphea spinulosa* and *Antirrhinum majus*, where zygomorphy is established during initiation of the calyx (Douglas, 1997; Vincent and Coen, 2004). In the tribe Delphinieae, the floral apex remains a circular and

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**Fig. 2.** Timing of events during floral development in the six species of Ranunculaceae studied and in two eudicot species [*Synaphea spinulosa* (Proteaceae) and *Antirrhinum majus* (Plantaginaceae)]. The relative time axis indicates the percentage of development already accomplished (calculated as floral base width of the bud/floral base width of the adult flower). Striped rectangle in *S. spinulosa* refers to the initiation of tepals.
Fig. 3. SEM micrographs of the developmental sequence of *Aconitum napellus*. (A) Young inflorescence; (B) floral primordium showing the first three sepals (after removal of the bract and of the two prophylls); (C, D) floral primordia showing the spiral initiation of sepals (numbers indicate the order of initiation of sepals); (E) floral primordium showing the second and fifth petals (seventh and tenth perianth members, P7 and P10) initiating in front of the dorsal sepal, and a very young stamen primordium (St); (F–H) floral primordia showing spiral initiation of stamens; (I) basal view of a floral bud showing reduced petals (Rp); (J) side view of a floral bud showing the two spurred petals of different size (P7 and P10); (K) apical view of a floral bud showing three carpels in central position surrounded by stamens; (L) side view of a floral bud showing a spurred petal and stamens. Scale bars: (A–K) = 100 μm; (L) = 1 mm.
regular mound until the end of androecium initiation. Subsequently, dorsoventral zygomorphy is first generated by reduced growth of petals in the abaxial region compared with the dorsal petals, with the reduction of six petals (one facing each dorso-lateral sepal and a pair facing each ventral sepal) in the genus *Aconitum* and four (one pair facing each ventral sepal) petals in the genus *Delphinium* (Figs 2 and 5, and Fig. S1 in Supplementary data). The two dorsalmost petals further develop into spurs. Interestingly, zygomorphy becomes conspicuous from the outside of the flower still later, when the stalks of the spurred petals begin to grow (Fig. 4), and the dorsal sepal differentiates into a helmet in *Aconitum* (Figs 3 and 4) and into a single spur in *Delphinium* (Fig. 5, and Fig. S1 in Supplementary data). The two dorsalmost petals further develop into spurs.

Historical associations among floral and ecological characters

From the comparison of the evolution of symmetry and other floral traits, it is shown that, in Ranunculaceae, zygomorphy evolved in a relatively fixed ground-plan (at least with a stable number of sepals or petals; Fig. 7) associated with pentamery of the calyx and racemose inflorescences. The three zygomorphic genera have polyanthous, bisexual flowers with a spiral phyllotaxis and a perianth differentiated between calyx and corolla.

According to the literature (see references in Table 2), short-spurred flowers of Delphinieae are pollinated by bees and bumblebees, those with medium-sized spurs by hummingbirds and those with long spurs by hawkmoths (Table 2). In most cases, effective pollinators are not specialized visitors. In the genus *Nigella*, visitors/pollinators were only identified for the species *N. arvensis* and consist of *Apis mellifera* and representatives of three genera of Hymenoptera: *Pyrobombus*, *Polistes* and *Eumenes* (Weber, 1995). Delphinieae are thus visited by a broader spectrum of animals (hawkmoths and hummingbirds in addition to Diptera and Hymenoptera).

**DISCUSSION**

Partial descriptions of floral organogenesis and organ development in Ranunculaceae have already been published (Payer, 1857; Mair, 1977; Kosuge and Tamura, 1988; Kosuge, 1994; Tucker, 1997; Tucker and Hodges, 2005; Erbar et al., 1999; Ren et al., 2004; Song et al., 2007). The organogenesis of the flower was not dealt with in a chronologically detailed way in these studies, and the present work is the first one to tackle this issue from the floral symmetry point of view. In addition, the present study provides data on new species and
full developmental sequences which help to understand the establishment of zygomorphy in a derived tribe, and the distribution of different organ identities on an ontogenic spiral.

**Acquisition of floral organ identities on an ontogenic spiral in Nigella and Delphinieae**

All the species studied have a centripetal organogenesis (Scho¨ffel, 1932), but they differ in the way organs are initiated, on a full ontogenic spiral or not. Even though *Aquilegia* species have a spirally arranged calyx, the corolla is whorled and the stamens are initiated on orthostichies, a characteristic of whorled phyllotaxis (Endress and Doyle, 2007). Petals are the first organs initiated at the base of orthostichies. The four sepals of *Clematis* species are initiated in pairs and not on a spiral, as is the case for the androecium. Outer stamens arise in whorls, but initiation becomes spiral higher on the apex (Ronse De Craene and Smets, 1996). In *Nigella* and
Delphinieae, all organs are initiated on a same ontogenic spiral. In most species studied, the various organs share characteristics (like colour, cell type, shape, initiation timing, appendages, etc.) during development resulting in blurred identities, which is all the more problematic when all organs are initiated on the same ontogenic spiral. In this context, comparative analysis of the developmental process in the various species may help define criteria for identifying boundaries between spirally arranged organs.

In the early stages of development of the species bearing petals, petal primordia are different from sepal primordia in shape and size. Moreover, the cell type may also help distinguish between both (Kramer et al., 2007). In contrast, petal primordia are indistinguishable from stamen primordia, exhibiting similar shape and size (Figs 3 and 5, and Fig. S1 in Supplementary data; Payer, 1857; Tepfer, 1953; Hiepko, 1965; Sattler, 1973; Kosuge and Tamura, 1988, 1989; Kosuge, 1994). This similarity was also shown at the anatomical level (Payer, 1857; Smith, 1928; Hiepko, 1965; Tamura, 1965, 1984; Erbar et al., 1999). The link of petals with staminodes (andropetals) in Ranunculaceae was hypothesized by Ronse De Craene and Smets (1994, 1995) and Erbar et al. (1999), based on morphological and developmental observations. Petals in Ranunculaceae were considered as the outermost whorl of the androecium, and interpreted in a phylogenetic sense as heterotopic staminodes (Ronse De Craene and Smets, 2001; see Ronse De Craene, 2003). An alternative hypothesis has been presented by a recent developmental genetic study of Rasmussen et al. (2009). The authors have shown that petals in the whole order Ranunculales must not be seen as homoplastic derived staminodes but rather as the result of a genetic developmental programme, common to Berberidaceae and Ranunculaceae. Gradients of gene expression may lead to the morphological grades (Kunst et al., 1989) between stamens and petals that were interpreted in the past as reproducing the evolutionary history between stamens and petals.

In Nigella, Aquilegia and Delphinieae, the growth pace and differentiation of petal and stamen primordia differ following organogenesis. Stamen primordia soon differentiate into filament and anther, while the growth of petal primordia is delayed. The retarded development of petals is a common feature of other plant families (Payer, 1857; Sattler, 1973) and has been explained as the arrested condition of original stamens (e.g. de Candolle, 1817; Troll, 1928; Arber, 1937). Petals develop once all organs have been initiated. The late development of three-dimensional structures such as the spurred/bulged petals confers a specific and supplementary identity to petals of Nigella, Aquilegia and Delphinieae among Ranunculaceae, which appears to be linked to the presence of nectaries on petals.

Thus, the present study in Ranunculaceae species helps us define morphological and developmental criteria to distinguish the identity of initiating organs juxtaposed on an ontogenic spiral: (a) the relative position of organs on the spiral (depending on this position the organ which initiates after a sepal is either a sepal or a petal); (b) the longer plastochron between two organs of two different identities compared with two similar organs; (c) the pace of organogenesis (petals and stamens initiates more rapidly than sepals); and (d) the delay in petal development compared with other floral organs.

A developmental model is proposed that synthesizes these changes in floral identities along the ontogenetic spiral, and hypothesizes the acquisition of supplementary specific identities for petals that account for the emergence of zygomorphy in tribe Delphinieae (Fig. 8). The sister taxon to Delphinieae...
Fig. 7. Evolution of perianth merism represented on the phylogenetic tree of Ranunculaceae in Hoot (2008). Dark blue, dimerous; light blue, tetramerous; yellow, trimerous; green, pentamerous; black, variable. The tribe Delphinieae is highlighted in pink.
was considered to be *Nigella* (Hoot, 1991, 1995), even though the clade formed by *Nigella, Actaea* and *Cimicifuga* has also been suggested by some authors (Johansson and Jansen, 1993). After organogenesis and when ‘sepal’, ‘petal’, ‘stamen’ and ‘carpel’ identities are established (Fig. 8A), *Nigella* flowers retain an actinomorphic corolla, while flowers of Delphinieae acquire a dorsoventral asymmetry as a result of the unidirectional growth of petals (see Figs 5F and 8B): the abaxial petals are reduced and the adaxial ones develop into petaloid structures. This bilateral symmetry is not perfect, however, due to the spiral initiation of petal primordia. This phenotype could be determined by a morphogen present in a dorsoventral concentration gradient (see Fig. 5f) acting either as a growth repressor, which is more abundant in the abaxial region, or conversely acting as an activator, which is more abundant in the dorsal region. The asymmetry pattern thus differs from the pattern described in *An. majus*, where development is retarded in the dorsal petals and stamen due to asymmetric early expression of the *CYCLOIDEA (CYC)* growth repressor gene (Luo et al., 1996). The effect of CYC in *Antirrhinum* appears to be organ and stage specific: retarded/suppressed growth of the dorsal stamen, retarded dorsal petal growth during early development but enlargement of dorsal petals compared with dorso-lateral/ventral ones during late development. The expression of CYC-like genes has been recorded in ventral petals of a few core eudicots (Howarth and Donoghue, 2006; Broholm et al., 2008) suggesting that these genes may also play a role in ventral identity in some species.

As development proceeds, all petals of *Nigella* become bulged and nectariferous, and form hairs on two flat appendages (Fig. 9B). These two features are observed in different petals in *Delphinium*. The petals in dorso-lateral position become hairy but harbour a flat shape. The two dorsalmost petals of *Delphinium*, and also of *Aconitum* take a structure similar to that of bulged petals of *Nigella*, the petal claws probably being homologous, as well as the bulge and the growing spur of dorsal petals (Fig. 9). Hairs are absent from all petals in *Aconitum* (Fig. 8C). If the *Nigella* morph is indeed ancestral, the corolla of Delphinieae would result from the reduction of four (*Delphinium*) or six (*Aconitum*) petals in conjunction with the differentiation of partly new petal identities. The distinct identities ‘spurred and nectariferous’ (in *Aconitum* and *Delphinium*) and ‘flat, hairy and non-nectariferous’ (in *Delphinium*) could be considered as a split of a composite identity (‘bulged, hairy and nectariferous’) specific to the petals of *Nigella*.

The development of the petal spurs in *Delphinium* and *Aconitum* precedes the acquisition of a spur in the dorsal sepal (Fig. 8D). Indeed, the growth of this sepal may depend on the same morphogenetic factor that induces the formation of nectar spurs, but expressed later. At the end of organ development, the calyx of all species studied becomes petaloid in shape and pigmentation (Fig. 8D).

In model core eudicot species, petal identity is controlled by the interplay between different MADS-box genes, more specifically the so-called B-class genes (Coen and Meyerowitz, 1991). They consist of two main lineages, *APETALA3/DEFICIENS*.
(AP3/DEF) and PISTILLATA/GLOBOSA (PI/GLO) that have originated from a duplication predating the emergence of angiosperms (Kramer et al., 1998). Both lineages have been extensively studied, especially in the Ranunculaceae by the group of E. Kramer (Kramer et al., 1998, 2003, 2007; Kramer and Irish, 1999, 2000; Rasmussen et al., 2009). Two duplications took place in the AP3 lineage before the diversification of the Ranunculales, giving rise to three paralogous lineages, while the PI lineage underwent several more recent duplications (Kramer et al., 2003; Rasmussen et al., 2009). Expression of AP3-I and AP3-II in sepals, together with PI, evolved early in Ranunculales but was lost many times, and lines of evidence for a role of B-gene expression in sepal petaloidy are faint. Expression of AP3-III lineage genes was found closely correlated with the presence of petals in Ranunculales, and a petal-specific expression of AP3-III genes appears in both Berberidaceae and Ranunculaceae. Recent expression studies suggest that AP3-III orthologues are more expressed in dorsal petals than in dorsi-lateral ones in Delphinium exaltatum (Rasmussen et al., 2009). Whether this asymmetric expression could correspond to our postulated morphogen gradient or could just be a response to it remains open. Moreover the genetic and molecular background for three-dimensional appendages of petals such as spurs, have still not been deciphered, although the redeployment of expression of KNOX genes controlling petal tube formation has been suggested in Antirrhineae (Golz et al., 2002).

Specificities of floral symmetry in Ranunculaceae

Floral symmetry in Ranunculaceae (whether actinomorphic or zygomorphic) is actually imperfect due to spiral initiation (Endress, 1999) and a homogeneous growth resulting in unequal size among organs sharing a same identity [in Aconitum (Fig. 3) and Delphinium (Fig. 5, and Fig. S1 in Supplementary data) the growth of one spurred petal is delayed compared with the other]. This situation is specific to Ranunculaceae.

Zygomorphy follows the same architectural plan in the three genera (of which only two were studied here) of Delphinieae. In the corolla, it is linked to asymmetric growth and the development of two dorsal spurs. Interestingly, flowers with paired spurs are extremely rare among angiosperms. As far as is known, the genus Diascia (Scrophulariaceae) is the only other example of a flower with paired spurs. In the calyx, zygomorphy is linked to a single spur whose development may be promoted by the genetic programme already responsible for the dorso-ventral differentiation in the corolla. Different spur lengths have been recorded in the

Fig. 9. SEM micrographs of the changes in the morphology of the petals in Nigella damascena (A–C) and Aconitum napellus (D–F) through development. Abbreviations: Sk, Stalk; Lp, lip; Bg, bulges. Scale bars: (A, B, D–F) = 100 μm; (C) = 200 μm.
genus Delphinium. The difference between D. grandiflorum and D. staphisagria can be interpreted as a heterochrony since the spurs emerge later in D. staphisagria than in D. grandiflorum but grow at the same rate in both species according to the observations made (Fig. 2). Another case of heterochrony was recorded in D. nudicaule, where the corolla resembles the buds of many other Delphinium species (Guerrant, 1982).

In Delphinieae, the floral apex is actinomorphic until the end of organogenesis, and zygomorphy appears after all organs have been initiated (also observed by Tucker, 1997), which is quite late compared with eudicot zygomorphic species (see the developmental sequence of the other basal eudicot species S. spinulosa and of the core eudicot An. majus in Fig. 2). For example, in the model species An. majus (Plantaginaeae), zygomorphy is established at the 15th plastochron among the 58 identified by Vincent and Coen (2004), i.e. between 0% and 9% of the developmental sequence, with the differentiation of dorsal, lateral and ventral sepals. The study of Feng et al. (2006) on Lotus japonicus (Fabaceae) shows that zygomorphy is established at the very beginning of floral development when the floral organs initiate in a unidirectional order (Dong et al., 2005). It has been suggested that late establishment of zygomorphy during development characterizes species occurring in groups with predominantly actinomorphic flowers (Endress, 1999), which is the case in Ranunculaceae. Similarly, in Iberis amara, a species that belongs to a family with otherwise actinomorphic (or disymmetric) flower species (Brassicaceae, Rosidae), petals begin to grow in a heterogeneous way only after the onset of stamen initiation (Busch and Zachgo, 2007).

**Accounting for the evolutionary success of Delphinieae: evolutionary forces driving floral symmetry in Ranunculaceae**

The ancestral states for the flowers in Ranunculaceae include spiral phyllotaxis (Hirmer, 1931; Schöffel, 1932) and an open floral ground-plan (with a variable number of organs of each type), two conditions traditionally only associated with actinomorphy (Endress, 1987). Fixed merism evolved at least nine times in the family (Fig. 7) but only once, in conjunction with partial reduction of the corolla, in the ancestor of Delphinieae. In this case, the spurred petals bearing nectaries can develop and grow in the bud with less spatial constraints, generating flower zygomorphy. Even if the sister taxa of Delphinieae may present flowers with a number of sepal different from five and a number of petals different from eight, it is assumed that a fixed merism preceded the evolution towards zygomorphy and is a prerequisite to it [consistent with the hypothesis of Endress (2001); see Jabbour et al. (2008)].

Zygomorphy evolved in Delphinieae in conjunction with nectar spurs. Both of these traits are considered key innovations in angiosperms (Hodges, 1997; Hodges and Arnold, 1995; Sargent, 2004). Disentangling the respective evolutionary role of zygomorphy and nectar spurs would require studies assessing which is the initial attractor for pollinators: is it the volatile chemical components of nectar (chemical attraction) or the zygomorphy of the flower (visual attraction)? Data are lacking in this field of study and it is consequently impossible to know which character played the primary role in species diversification. Nectar spurs and zygomorphy will therefore be considered as a single morphological novelty. The question is whether this morphological novelty represents a key innovation in this tribe. No case of reversal to actinomorphy is recorded among the 665 species that make up the Delphinieae, suggesting that ‘zygomyph + nectar spurs’ can be considered a key innovation according to the qualitative definition proposed by Endress (2001). Nevertheless, accelerated speciation can simply be due to stochastic processes, and the hypotheses of key innovation must then be statistically tested for changes in speciation rates (Raup et al., 1973; Slowinski and Gayer, 1989, 1993). Using several statistical tests, Hodges and Arnold (1995) showed that the answer to the question is dependent on the sister group of Delphinieae, emphasizing the need for a better resolved phylogenetic tree of Ranunculaceae to definitely clarify this point.

It is tempting to suggest that the evolution of zygomorphy is dependent on the animals able to pollinate these new-shaped flowers. Some regular flowers of Ranunculaceae may have evolved repeatedly to shapes with deviations from actinomorphy, but ecological conditions may have been unfavourable for the maintenance of zygomorphy in most cases except in Delphinieae. Accordingly, spur length appears to be a critical factor in animal–plant coevolution. According to the ‘pollinator shift’ hypothesis of Ennos (2008), spur length remains stable or increases through coevolution with pollinators. It would be very interesting to test this assertion using a detailed phylogeny of Delphinieae, to see if species with the longest spurs are indeed the most derived in the tree. The available phylogenies of the genus Aconitum (Utelli et al., 2000; Kita and Ito, 2000; Luo et al., 2005) are not resolved enough to detect any lengthening of spurs in the course of evolution.

The pattern of association between spur length and pollinator type in Delphinium and Aconitum (Table 2) is very similar to the one described in Aquilegia (Whittall and Hodges, 2007): Delphinium species with the shortest nectar spurs are pollinated by bees or bumblebees, those with intermediate nectar spurs by hummingbirds, and those with the longest nectar spurs by hawkmoths. In Delphinium, flat petals act also as pollinator filters and/or attractors, in addition to the spurred petals. They are used as a visual cue for pollinators due to the hairs that turn yellow at maturity. Apart from bumblebees that use them as a landing platform (Laverty, 1980), these petals probably prevent all access to the nectar except to the heaviest and strongest visitors (Guerrant, 1982).

While successive shifts in function or expression of genes controlling merism, as well as floral symmetry and organ identity may have played a critical role in the evolution of zygomorphy in Delphinieae, the presence of pollinators able to exploit the peculiar morphology of the flower has probably been a key factor for the maintenance and diversification of this trait.

**SUPPLEMENTARY DATA**

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following two figures. Fig. S1. SEM micrographs of the developmental sequence of
Delphinium staphisagria. Fig. S2. SEM micrographs of the spiral and whorled phyllostaxis in flowers of Ranunculaceae

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