SPURS IN A SPUR: PERIANTH EVOLUTION IN THE DELPHINIEAE (RANUNCULACEAE)

Florian Jabbour[1],* and Susanne S. Renner*

*Systematic Botany and Mycology, University of Munich (LMU), Menzinger-Str. 67, 80638 Munich, Germany

Delphinieae (Ranunculaceae) comprise ~650 species of temperate herbs in Eurasia, North America, and Africa. Their zygomorphic flowers have been the object of numerous studies in morphology, ecology, and developmental genetics, and new phylogenetic insights make it timely to synthesize knowledge about their evolution. Key features of Delphinieae flowers are unusual nectaries consisting of paired organs of the inner perianth whorl that are completely enclosed by a single dorsal organ of the outer whorl. We investigated the floral development of five annual, unicarpellate species of *Delphinium*, focusing on perianth organization. The results show that the nectar-storing organ in these species results from the postgenital fusion of two primordia of the internal perianth whorl. Eleven floral traits traced on a phylogeny of Delphinieae reveal only two homoplasies in the perianth, namely, the nightcap shape of the dorsal organ of the external perianth whorl and the reduction of the internal perianth whorl to two organs, traits that each evolved once in *Aconitum* and once in *Delphinium*. The length of the inner spur(s), the type of pollinator (bees, hummingbirds, hawkmoths), and species altitudinal ranges are unrelated, but most species are exclusively bumblebee adapted, and bee tongue lengths may match the internal nectar spur lengths. The paired inner spurs present in most Delphinieae require a back-and-forth movement of the tongue while the pollinator is inside the flower or hovering close to it. A new evolutionary scenario reconciles the diversity of perianth organization in Delphinieae with the tribe's conserved pollination mechanism.

Keywords: *Aconitum*, *Delphinium*, floral morphology, nested spurs, pollination, postgenital fusion.
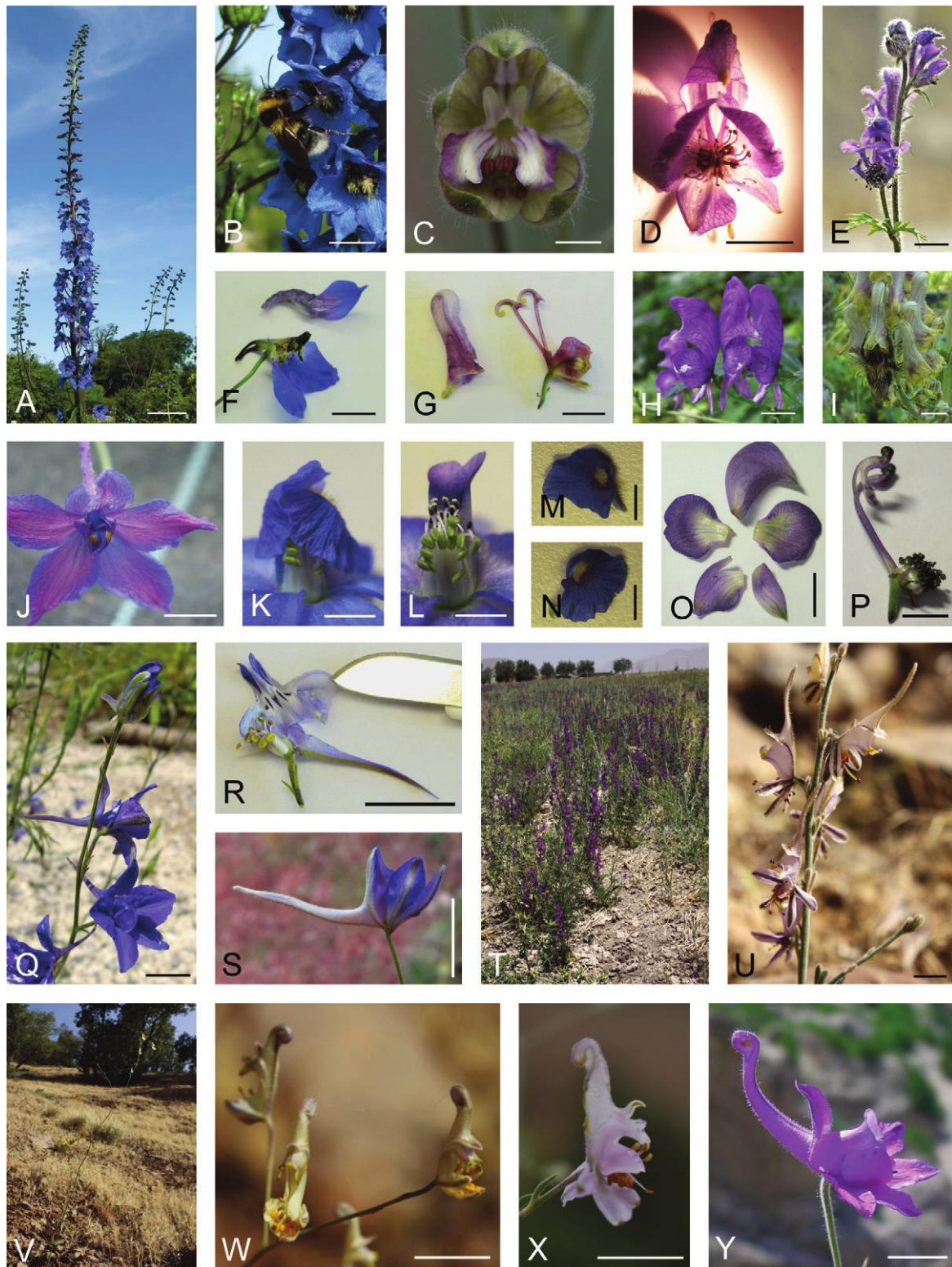
Online enhancement: video.

Introduction

Recent years have seen much progress in our understanding of the developmental and molecular bases of floral organ identity (Causier et al. 2010; Litt and Kramer 2010; Rijpkema et al. 2010; Dornelas et al. 2011) and zygomorphy (Citerne et al. 2010 for a review; Bartlett and Specht 2011; Chapman et al. 2012; Preston and Hileman 2012). Especially the basal eudicot family Ranunculaceae has been the focus of studies on these topics (Payer 1857; Kosuge and Tamura 1988, 1989; Erbar et al. 1998; Jabbour et al. 2009; Rasmussen et al. 2009; Sharma et al. 2011; Puzey et al. 2012). While zygomorphy evolved only once in this family, in the stem lineage of the tribe Delphinieae (600–700 species), it is associated with elaborate perianths and nectar spurs (fig. 1). The complexity and unclear derivation of Ranunculaceae perianths and nectar-producing and nectar-storing organs has led to diverse terms for the organs in whorl 1 (W1) and whorl 2 (W2) in this family (table 1). Regardless of terminology, the nectar spurs of Delphinieae are unusual among angiosperms in being paired parallel structures (fused or not). Paired floral spurs are otherwise found only in the Scrophulariaceae *Diascia*, where they offer oil that is taken up by the two legs of the pollinating bee (Vogel 1984; Vogel and Michener 1985).

[1] Author for correspondence; e-mail: florian.jabbour@gmail.com.

The organogenesis, function, and evolution of the perianth and paired nectar spurs of Delphinieae are the main focus of this study. We first present original data on the development of the perianth in a subclade of *Delphinium* (traditionally treated as *Aconitella/Consolida*), and we then reinterpret the evolution of perianth organization using a new molecular phylogeny for the Delphinieae combined with data on floral function, especially the role of the nectar-storing organs. To avoid the sepal/petal terminology problem, we use the unambiguous terms "W1 organs" and "W2 organs" to refer to the first (outer) and second (inner) perianth organs, respectively.

The basic Delphinieae perianth consists of two whorls of petaloid organs. The outer whorl is composed of five organs borne on an ontogenic spiral (Jabbour et al. 2009): two ventral, two lateral, and a spurred or hooded dorsal one (fig. 1C, 1D, 1J, 1O). The internal whorl comprises one, two, or four organs (fig. 1C, 1F, 1G, 1J–1N, 1P, 1R), all in the dorsal half of the flower. Other W2 organs stop developing shortly after organogenesis or develop into slender and petaloid staminodes (fig. 1P; Kosuge and Tamura 1989; Erbar et al. 1998; Jabbour et al. 2009). One or two organs in the dorsalmost position of the inner whorl become nectariferous and are enclosed by the dorsal W1 organ (figs. 1D, 1F, 1G, 2). The nectaries consist of a stalk bearing a limb, the latter consisting of the nectar-containing spur and a labium (terminology of Kosuge and Tamura 1988; see our fig. 2). To reach the nectar at the tip of the spur(s) in *Delphinium*, bees land on the W2 lateral organs (Bosch et al. 1997; Liao et al.

Fig. 1 Inflorescences and flowers of Delphinieae. *A*, Inflorescence of *Delphinium elatum* L. *B*, Flower of *D. elatum* with a visitor. *C*, Face view of a young flower of *Staphisagria macrosperma* Spach showing the five W1 organs and the two spurred dorsal and two flat lateral W2 organs. *D*, Face view of a flower of *Aconitum napellus* L. showing the dorsal W2 organs concealed in the dorsal W1 organ. *E*, Inflorescence of *Aconitum gymnandrum* Maxim. (Sichuan, China). *F*, Lateral view of a dissected flower of *D. elatum* with the dorsal W1 organ isolated, one lateral and one ventral W1 organ removed to reveal the two spurred dorsal and two flat lateral W2 organs. *G*, Lateral view of a dissected flower of *Aconitum lycoctonum* subsp. *moldavicum* (Hacq.) Jalas with the dorsal W1 organ isolated to reveal the two dorsal W2 organs. *H*, Inflorescence of *Aconitum*

Table 1

Diversity of Terms Used to Name the W1 and W2 Organs in Delphinieae

W1 organs	W2 organs	References
Perianth leaves	Honey leaves	Prantl 1887
Tepals	Nectaries and staminoides	Nevskii 1937
Perianth leaves	Nectar leaves	Janchen 1949
Sepals	Petals or nectar leaves	Leppik 1964
Sepals	Petals or staminodia	Hoot 1991
Tepals	Nectary organs	Erbar et al. 1998; Leins and Erbar 2010
Sepals	Petals	Huth 1895; Kosuge and Tamura 1988, 1989; Blanché 1990; Trifonova 1990; Tamura 1993; Kosuge 1994; Bosch et al. 1997; Fukuda et al. 2001; Endress and Matthews 2006; Jabbour et al. 2009; Rasmussen et al. 2009; Flora of China and Flora of North America (http://www.efloras.org)
Tepals	Petals or nectar leaves	Ronse de Craene 2010
Whorl one-type petaloid organs	Whorl two-type petaloid organs	E. Kramer, personal communication
W1 organs	W2 organs	This article

2007; our fig. 1C, 1J, 1K, 1M, 1N), which their body weight displaces downward, bringing abdomen and legs in contact with dehiscing anthers (Macior 1975). In *Aconitum*, bumblebees must insert their bodies into the hood in order to reach the nectar spurs (Fukuda et al. 2001), bringing their abdomen and legs in contact with dehiscing anthers.

Surprisingly, before this study the number of primordia forming the developed W2 organs in *Delphinium* flowers was not clear, with some workers assuming that two primordia were involved in the unicarpellate subclade (Payer 1857; Kosuge and Tamura 1989), others four or five (Kemularia-Nathadze 1940). To determine the number of participating primordia, we selected five species of *Delphinium* that, together with previously investigated species, represent the major clades in the genus as revealed in a molecular phylogeny (Jabbour and Renner 2012). We also wanted to determine the evolutionary trajectories of hood-shaped (fig. 1D, 1H, 1O) and nightcap-shaped (fig. 1G, 1I, 1W–1X) flowers and nectar spur length and how these traits might vary with pollinator type or species altitudinal ranges. Delphinieae are most diverse in the Himalayan Mountains, and molecular clock dating suggests Late Miocene bursts of diversification in the long-lived high-altitude species (Jabbour and Renner 2012). To answer these questions

we analyzed 11 traits in species representing the geographic and phylogenetic ranges of Delphinieae. Pollinator nectar-foraging behavior was assessed on the basis of literature and a film clip showing a bumblebee visiting Delphinieae flowers (video 1).

Material and Methods

Species Sampling and SEM Study

In June 2011, flower buds of wild *Delphinium anthoroideum* Boiss. (*Consolida anthoroidea* [Boiss.] Schrödinger = *Aconitella anthoroidea* [Boiss.] Soják; fig. 1V–1W), *D. hohenackeri* Boiss. (*Consolida hohenackeri* [Boiss.] Grossh. = *Aconitella hohenackeri* [Boiss.] Sójak; fig. 1X), *D. orientale* J. Gay (=*Consolida orientalis* [J. Gay] Schrödinger; fig. 1T), *D. persicum* Boiss. (=*Consolida persica* [Boiss.] Schrödinger; fig. 1U), and *D. consolida* L. (=*Consolida regalis* Gray; fig. 1S) were harvested in Iran. Herbarium vouchers are kept in the herbaria of Tehran University and Munich University (S. Zarre et al. 42188, 42191, 42187, 42186, and 42192). Buds from different individuals covering the entire developmental sequence were fixed in FAA. Buds were dissected with a Wild M75 stereomicroscope (Heerbrugg, Switzerland), dehy-

(**Fig. 1,** *continued*) sp. *I*, Flower of *A. lycoctonum* subsp. *moldavicum* with a visitor. *J*, Upper view of a flower of *D. grandiflorum* L. showing the five petaloid W1 organs (the dorsal one being spurred) and the four W2 organs (two dorsal spurred and two lateral flat). *K*, Androecium concealed beneath the two lateral W2 organs of a flower of *D. grandiflorum* (view from below). *L*, Androecium and labia of the two dorsal W2 organs revealed by removing the two lateral W2 organs of a flower of *D. grandiflorum* (view from below). Stamens with dehiscing anthers are in dorsal position, whereas immature stamens are decurved and occupy the lowest part of the androecium. *M*, Isolated lateral W2 organ (the left one in a face view of the flower) of a flower of *D. grandiflorum* showing a short stalk and a labium with a patch of yellow hairs. *N*, Isolated lateral W2 organ (the right one in a face view of the flower) of a flower of *D. grandiflorum* showing a short stalk and a labium with a patch of yellow hairs. *O*, Dissected outer whorl of a flower of *A. napellus* showing the five W1 organs (two ventral, two lateral, and one dorsal). *P*, Side view of the flower of *A. napellus* with the W1 removed showing the two dorsal W2 organs and some lateral and ventral staminoid W2 organs (violet slender stalks) surrounding the compact androecium. *Q*, Inflorescence of *D. ajacis*. *R*, Lateral view of a dissected flower of *D. ajacis* showing the single W2 organ incised between the three-lobed labium (upper lobe bifid) and the nectariferous spur in order to flatten the labium. *S*, Side view of a young flower of *D. consolida*. *T*, Population of *D. orientale* in its natural habitat in Iran. *U*, Inflorescence of *D. persicum*. *V*, Inflorescence of *D. anthoroideum* in its natural habitat in Iran. *W*, Inflorescence of *D. anthoroideum*. *X*, Flower of *D. hohenackeri*. *Y*, Flower of *D. teheranicum* Boiss. (=*Consolida teheranica* [Boiss.] Rech.f. = *Aconitella teheranica* [Boiss.] Sójak). Note the membrane attaching the spur tip to the spur body in *W–Y*. Photograph credits: *A–D, F, G, I, J–R*: Florian Jabbour; *E*: Gu Lei; *H*: Jared Lockwood; *S–Y*: Shahin Zarre. Scale bars: *A*, 8 cm; *B, D, E, G–J, O, Q–S, U, W, X*, 1 cm; *C, P, Y*, 6 mm; *F*, 2 cm; *K, L*, 4 mm; *M, N*, 2 mm.

Table 2

Length of Outer and Inner Spur(s) and Altitudinal Range for Species of *Delphinium* and *Staphisagria*

Genus, species	Mean length of outer spur (mm)	Mean length of inner spur (mm)	Spur length category	Altitude (m)	References
Delphinium:					
carduchorum	13.5	11	2	2250–2850	Davis 1965; Munz 1967c
tuberosum	13.5	13	2	~2000	Munz 1967c
cyphoplectrum	10	10	2	300–2700	Huth 1895; Davis 1965; Munz 1967c; http://coldb.mnhn.fr/ScientificName/delphinium/cyphoplectrum
aquilegifolium	11.5	12	2	1900–2500	Munz 1967c
szowitsianum	12.5	12.5	2	1800–2000	Huth 1895; Nevskii 1937; Davis 1965; Munz 1967c
dasycaulon	6.5	4.5	1	1400–3000	Huth 1895; Munz 1967a
macrocentron	24	20	4	1500–3500	Huth 1895; Munz 1967a
leroyi	37.5	37.5	5	1500–3500	Huth 1895; Munz 1967a
kohatense	6	5	1	<3000	Munz 1967c
suave	12.5	12	2	2600–3800	Huth 1895; Munz 1967c
denudatum	14.5	14	3	1300–2600	Huth 1895; Munz 1967d
saniculifolium	10	9	2	900–3000	Huth 1895; Munz 1967c, 1968a
griseum	10	10	2	<3000	Munz 1967c
semibarbatum	9.5	8	2	<2000	Huth 1895; Nevskii 1937; Munz 1967c
uendelboi	10.5	10	2	700–1600	Iranshahr 1992
biternatum	15.5	11	3	1700–2500	Huth 1895; Nevskii 1937; Munz 1967c
albocoeruleum	20	17.5	3	1500–4900	Huth 1895; Munz 1967d, 1968a; Flora of China
fissum	15.5	15.5	3	1000–1650	Davis 1965; Munz 1967c; Bosch 1997; Ilarlsan et al. 1997
macrostachyum	10	9	2	1200–2000	Huth 1895; Davis 1965; Munz 1967c; Ilarlsan et al. 1997
freynii	14	...	2	2100–2300	Huth 1895; Nevskii 1937; Davis 1965
kurdicum	10.5	10	2	1000–1400	Huth 1895; Davis 1965; Munz 1967c; Ilarlsan et al. 1997
incisum	17	16	3	...	Munz 1967d, 1968a
pentagynum	14	10	2	<1500	Huth 1895; Munz 1967a
nevadense	17.5	16	3	1700–2000	Huth 1895; Bosch 1997; herbarium material (M, MSB)
emarginatum	15	14.5	3	...	Huth 1895; Munz 1967a; Bosch 1997
sylvaticum	16	15	3	...	Huth 1895; Munz 1967a; Bosch 1997
ochotense	14.5	16.5	3	...	Munz 1968a
bonvalotii	23.5	...	4	1100–3800	Huth 1895; Flora of China
maackianum	17.5	15.5	3	600–900	Huth 1895; Nevskii 1937; Munz 1968a; Flora of China
crassifolium	15.5	14	3	1600–2300	Huth 1895; Nevskii 1937; Munz 1968a
delavayi	23.5	17.5	4	~3200	Huth 1895; Munz 1968a
pylzowii	26	25	4	3000–4500	Huth 1895; Munz 1967d
potaninii	25	21.5	4	3000–4200	Huth 1895; Munz 1968b
beesianum	22.5	21	4	4000–5000	Munz 1967d
gyalanum	20	15.5	3	~2700	Munz 1968a
glaciale	15	13	3	3000–6500	Huth 1895; Munz 1967d
tangkulaense	11.5	...	2	4700–5500	Flora of China
caeruleum	21	14.5	3	3000–5800	Munz 1967d
muscosum	14	16	2	4500–5500	Munz 1967d
pyramidale	13.5	14	2	2000–3500	Munz 1968a
flexuosum	16.5	15	3	~2000	Nevskii 1937; Davis 1965; Munz 1968a; Ilarlsan et al. 1997

Table 2

(Continued)

Genus, species	Mean length of outer spur (mm)	Mean length of inner spur (mm)	Spur length category	Altitude (m)	References
dolichostachyum	10.5	10	2	~2200	Davis 1965; Munz 1967*c*; Ilarlsan et al. 1997
minjanense	6.5	8.5	1	~3650	Munz 1967*d*
cashmerianum	13.5	9.5	2	3000–5000	Huth 1895; Munz 1967*d*
oreophilum	12.5	11	2	3000–3600	Huth 1895; Nevskii 1937; Munz 1968*a*
afghanicum	7.5	9.5	2	~3800	Munz 1968*a*
crispulum	14	12	2	1000–3000	Nevskii 1937; Munz 1967*d*, 1968*a*; Ilarlsan et al. 1997
elbursense	19	11.5	3	2400–2600	Munz 1968*a*
cuneatum	13.5	...	2	...	Nevskii 1937
elatum	14	10	2	800–1800	Huth 1895; Munz 1968*a*
oxysepalum	20	13.5	3	1200–1700	Huth 1895; Pax 1982
dubium	15	...	3	1000–2600	Herbarium material (M, MSB)
montanum	18.5	16.5	3	~2000	Huth 1895; Bosch 1997
bulleyanum	20	19	3	2800–5100	Munz 1968*b*
multiplex	14	...	2	1500–1800	Flora of North America
viride	19	...	3	~2200	Huth 1895; herbarium material (M, MSB)
bicornutum	13	...	2	...	Huth 1895; holotype of *Delphinium bicornutum* Hemsl. subsp. *oaxacanum* MJ Warnock[a]
hesperium	13.5	...	2	10–1500	Flora of North America
hansenii	11	...	2	60–3000	Flora of North America
gypsophilum	11	...	2	150–1200	Flora of North America
parryi	12.5	...	2	0–1700	Huth 1895; Flora of North America
cardinale	20	...	3	50–1500	Huth 1895; Flora of North America
parishii	11	...	2	200–3900	Huth 1895; Flora of North America
polycladon	16.5	...	3	2200–3600	Flora of North America
luteum	15.5	...	3	0–50	Flora of North America
recurvatum	12.5	...	2	30–600	Huth 1895; Flora of North America
decorum	16	...	3	0–2300	Huth 1895; Flora of North America
patens	11.5	...	2	80–2800	Huth 1895; Flora of North America
bakeri	11	...	2	100–300	Flora of North America
nudicaule	18.5	...	3	0–2600	Huth 1895; Flora of North America
trolliifolium	17.5	...	3	30–3600	Huth 1895; Flora of North America
barbeyi	17	...	3	2500–4100	Huth 1895; Flora of North America
exaltatum	10.5	...	2	150–2000	Flora of North America
gracilentum	10	...	2	150–2700	Flora of North America
bicolor	18	...	3	70–3100	Huth 1895; Flora of North America
nuttallianum	15.5	...	3	300–3500	Huth 1895; Flora of North America
depauperatum	14	...	2	1800–2600	Huth 1895; Flora of North America
tricorne	16	...	3	0–2300	Huth 1895; Flora of North America
kamaonense	14.5	14	3	3000–4600	Huth 1895; Munz 1967*d*
brachycentrum	16	14	3	0–1600	Huth 1895; Nevskii 1937; Munz 1968*a*; Flora of North America
tatsienense	30	30	5	2500–3000	Huth 1895; Munz 1967*d*
middendorffii	21.5	...	4	...	Huth 1895
sparsiflorum	7	7.5	1	~2600	Huth 1895; Munz 1968*a*
kansuense	18	...	3	~3000	Flora of China

cheilanthum	17.5	15.5	3	700–800	Huth 1895; Nevskii 1937; Munz 1967d; Flora of China
grandiflorum	19	17	3	100–3500	Huth 1895; Nevskii 1937; Munz 1967d; Flora of China
pachycentrum	15	19	3	3000–4500	Huth 1895; Munz 1968a
tsarongense	19	22	3	4000–5000	Munz 1967d
orthocentrum	12	11	2	~3500	Huth 1895; Munz 1968a; Flora of China
trichophorum	19.5	20	3	3700–5200	Huth 1895; Munz 1967d
sutchuenense	15.5	15	3	~2800	Huth 1895; Munz 1967d, 1968a; Flora of China
thibeticum	21	19.5	3	2800–3800	Munz 1968a; Flora of China
oxycentrum	34	...	5	~4000	Munz 1968a; Flora of China
viscosum	18	13.5	3	3000–5300	Huth 1895; Munz 1967d
glandulosum	20	22	3	800–1600	Huth 1895; Davis 1965; Munz 1967b
mauritanicum	17	17	3	1400–2100	Huth 1895; Munz 1967a; Bosch 1997; herbarium material (M, MSB)
axilliflorum	7.5	5.5	2	<1800	Huth 1895; Davis 1965; Munz 1967b
ajacis	16	15.5	3	500–3000	Huth 1895; Nevskii 1937; Munz 1967a, 1967b; Bosch 1997
incanum	6	...	1	0–2100	Huth 1895; Munz 1967b
orientale	10	8.5	2	0–2500	Nevskii 1937; Davis 1965; Munz 1967a, 1967b
divaricatum	20.5	...	3	0–1000	Huth 1895; Nevskii 1937; Munz 1967b
tenuissimum	8.5	...	2	500–1200	Huth 1895; Soó 1922
consolida	18.5	16.5	3	0–1000	Huth 1895; Nevskii 1937; Munz 1967a, 1967b
stocksianum	19	17	3	1000–2000	Nevskii 1937; Munz 1967b
flavum	5	3.5	1	<1000	Huth 1895; Munz 1967b
persicum	20	...	3	1000–2000	Nevskii 1937; Munz 1967b
barbatum	7	...	1	700–2000	Huth 1895; Nevskii 1937; Munz 1967b
saccatum	4.5	5	1	...	Huth 1895; Davis 1965; Munz 1967b
aconiti	4	4	1	<1100	Huth 1895; Davis 1965; Munz 1967b
olopetalum	17.5	16	3	~100	Huth 1895; Davis 1965; Munz 1967b
venulosum	15	...	3	400–1200	Huth 1895; Davis 1965; Munz 1967c; Ilarlsan et al. 1997
virgatum	16	14.5	3	<1000	Munz 1967c; Ilarlsan et al. 1997
hirschfeldianum	12.5	...	2	...	Herbarium material (M, MSB)
balcanicum	15	...	3	150–900	Pawlowski 1963
balteratum	15	19	3	400–5200	Huth 1895; Munz 1967a
peregrinum	17.5	13	3	<2000	Huth 1895; Munz 1967a, 1967c; Ilarlsan et al. 1997
obcordatum	16	15.5	3	<1100	Munz 1967a; Bosch 1997
nanum	19	14	3	0–30	Huth 1895; Munz 1967a, 1967c; herbarium material (M, MSB)
verdunense	16	15.5	3	200–1500	Munz 1967a; Bosch 1997; herbarium material (M, MSB)
cossonianum	13.5	14.5	2	0–500	Munz 1967a; Bosch 1997; herbarium material (M, MSB)
favargeri	19	17.5	3	~1400	Bosch 1997; herbarium material (M, MSB)
gracile	17.5	16	3	<1200	Munz 1967a; Bosch 1997
balansae	14.5	14	3	<2500	Huth 1895; Munz 1967a; Bosch 1997
macropetalum	11.5	11	2	1800–2300	Huth 1895; Bosch 1997; herbarium material (M, MSB)
anthriscifolium	16	15	3	0–1700	Huth 1895; Munz 1967c; Flora of China
Staphisagria:					
macrosperma	3.5	6	1	70–100	Huth 1895; Munz 1967a; Bosch 1997; Ilarlsan et al. 1997
requienii	7	...	1	30–60	Huth 1895; Pawlowski 1993
pictum	7	6.5	1	0–100	Pawlowski 1993; Bosch 1997; herbarium material (M, MSB)

Note. All the species included in this table were also included in the phylogenetic study of Jabbour and Renner (2012), and the species list follows the order of appearance in the phylogenetic tree. Flora of North America and Flora of China were consulted online (http://www.efloras.org).
[a] #2536, TEX00370790, collection date Oct. 19, 1981 (http://plants.jstor.org/specimen/tex00370790).

Table 3

Recorded Pollinators for Species of Delphinieae

Genus, species	Included in Jabbour and Renner (2011a, 2012)	Bumblebees[a]	Diptera	Hawkmoths[b]	Hummingbirds	References
Aconitum:						
anthora	Yes	Yes	Yes			Bosch et al. 1997
columbianum	Yes	Yes				Brink 1980; Brink and deWet 1980; Pleasants and Zimmerman 1980; Bosch and Waser 1999: Dukas 2005
delphinifolium	Yes	Yes				Harder 1990
gymnandrum	Yes	Yes[c]				Zhang et al. 2006; Duan et al. 2009
japonicum		Yes				Fukuda et al. 2001
kusnezoffii	Yes	Yes				Liao et al. 2007, 2009
lycoctonum	Yes	Yes				Bosch et al. 1997; Utelli and Roy 2000, 2001
napellus	Yes	Yes	Yes			Bosch et al. 1997
noveboracense		Yes				Kuchenreuther 1996
septentrionale	Yes	Yes				Thøstesen and Olesen 1996
Delphinium:						
ajacis	Yes	Yes	Yes			Bosch et al. 1997
balansae	Yes	Yes				Bosch et al. 1997
barbeyi	Yes	Yes			Yes	Williams et al. 2001
bicolor	Yes	Yes				Bauer 1983; Ishii and Harder 2006
bolosii		Yes	Yes			Bosch et al. 1998
cardinale	Yes				Yes	Grant 1966; Schlising and Turpin 1971; Cronk and Ojeda 2008
consolida	Yes	Yes				De-yuan 1986; Bosch et al. 1997
dasycaulon	Yes	Yes				Johnson 2001
decorum	Yes	Yes				Guerrant 1982
favargeri	Yes	Yes	Yes			Bosch et al. 1997
glaucum		Yes				Ishii and Harder 2006
gracile	Yes	Yes				Bosch et al. 1997
leroyi	Yes			Yes		Johnson 2001
mauritanicum	Yes	Yes				Bosch et al. 1997
montanum	Yes	Yes	Yes			Bosch et al. 1997
nelsonii (syn. *D. bicolor*)	Yes	Yes			Yes	Waser 1978; Price and Waser 1979; Pleasants and Zimmerman 1980; Waser and Price 1980, 1981
nudicaule	Yes				Yes	Guerrant 1982; Cronk and Ojeda 2008
nuttallianum	Yes	Yes			Yes	Bosch and Waser 1999; Schulke and Waser 2001; Williams et al. 2001
obcordatum	Yes	Yes		Yes		Bosch et al. 1997
parryi	Yes	Yes				Powell and Jones 1983
pubescens	Yes	Yes	Yes			Bosch et al. 1997
sclerocladum	Yes	Yes				De-yuan 1986
stenocarpum	Yes	Yes				De-yuan 1986
tricorne	Yes	Yes			Yes	Leppik 1964; Macior 1975
verdunense	Yes	Yes		Yes		Bosch et al. 1997
virescens		Yes				Waddington 1981
Staphisagria:						
macrosperma	Yes	Yes	Yes	Yes		Bosch et al. 1997
picta	Yes	Yes	Yes	Yes		Bosch et al. 1997

[a] Including other bees (Apoidea).
[b] Including other Lepidoptera.
[c] Wind pollination recorded.
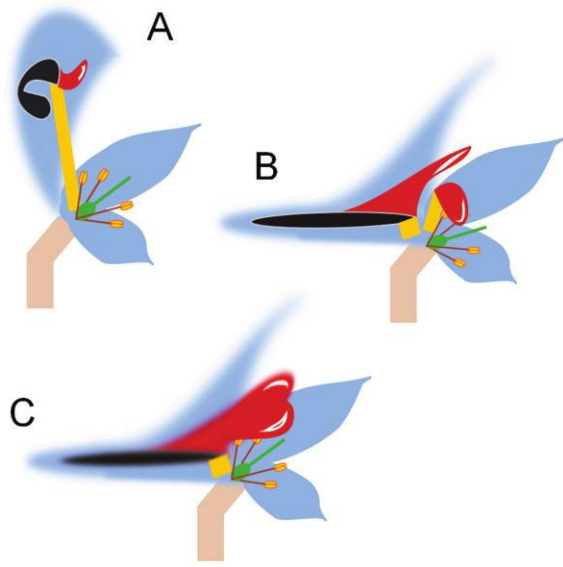
Fig. 2 Schematic longitudinal sections of the three types of perianth organization in the tribe Delphinieae. *A*, Hooded type (found in *Aconitum*). *B*, Spurred type (found in *Delphinium* and *Staphisagria*). *C*, Spurred type with fused W2 organs (found in *Delphinium* subclade *Consolida*). W1 organs are shown in blue. The stalk, labium, and spur of W2 organs are yellow, red, and black, respectively. Half organs are drawn with faded lines. The gynoecium and the androecium are indicated by a green structure surrounded by four stamens (not the actual number of male and female units). In *B* and *C*, part of the androecium is concealed beneath the lateral W2 organ and the lateral lobe of the W2 organ.

drated in an ethanol-acetone series, and dried with a Bio-Rad E3000 critical-point dryer (Watford, UK). Dried floral structures were mounted on aluminum stubs with colloidal graphite and coated with platinum using a Bal-Tec SCD 050 sputter-coater (Witten, Germany) and observed with a Supra 55VP scanning electron microscope (LEO 438 VP; Cambridge, UK). Pickled reference material and platinum-coated material are kept at the Institute of Systematic Botany and Mycology, University of Munich (LMU).

Inference of Floral Trait Changes

Two databases were compiled, using relevant literature cited in tables 2 and 3. In the first, the 125 species of *Delphinium* and three species of *Staphisagria* included in the phylogenetic tree of Jabbour and Renner (2012) were scored for length of outer nectar spur, length of inner nectar spur(s), and altitudinal range (table 2). The second database contains information on pollinator types (bumblebees and other bees, hummingbirds, hawkmoths) based on the studies cited in table 3. We wanted to test for correlations between pollinator type and altitude because Delphinieae are most diverse in Southeast Asian mountains and the Rocky Mountains, where hummingbirds or bees might replace moths (Arroyo et al. 1982). For one species of *Aconitum*, a close-up movie (video 1, available in the online edition of *International Journal of Plant Sciences*) provided information on bumblebee movement during nectar

foraging. Phylogenies were rooted based on the more comprehensively sampled Delphinieae phylogeny of Jabbour and Renner (2012). *Aconitum* species were not scored for nectary length because their nectaries are curved inside their hood-shaped flowers. Third, we analyzed 11 floral traits, presented in "Results," on a simplified phylogeny of the Delphinieae.

In table 2, the continuous trait "spur length" was coded as an ordered multistate character with five states: 1: [0–7] (i.e., from 0 to 7 mm, excluding 0 and including 7); 2:]7–14]; 3: [14–21]; 4: [21–28]; and 5: 28+. Inner spur lengths are interesting from an evolutionary point of view because they determine the nectar reward, but more measurements are available for the outer spur length because that is what botanists in the field and herbarium report (table 2). Seven-millimeter intervals were the best compromise to group both measurements, which do not always covary.

Trait reconstruction relied on maximum likelihood (ML) as implemented in Mesquite, version 2.75 (Maddison and Maddison 2011), and the Markov k-state one-parameter model, which assumes a single rate for all transitions between character states. Analyses were carried out on the cladogram obtained by Jabbour and Renner (2012), with the transition parameters estimated based on the tip trait states (i.e., mean spur length category).

Results

Development of the W2 Organs in the Five Newly Investigated Species of Delphinium

The five species newly investigated here all have a single W2 organ resulting from the postgenital fusion of the dorsal W2 primordia. Early development is common to all five (figs. 3A–3D, 4A–4D, 5A–5G, 6A–6H, 7A–7D), whereas late development is species specific.

At first, seven or eight W2 primordia are initiated, the two dorsal ones (fig. 3A, 3B) facing the dorsal W1 organ (the second in the initiation sequence of W1 organs; figs. 3A, 3B,


Video 1 Still photograph from a video (available in the online edition of *International Journal of Plant Sciences*) showing a bee foraging for nectar in *Aconitum* flowers. In the video, the repeated "in and out" of the bee's body can be seen during each flower visit. Video courtesy of Katharine Tatum. Location: Wales, UK.
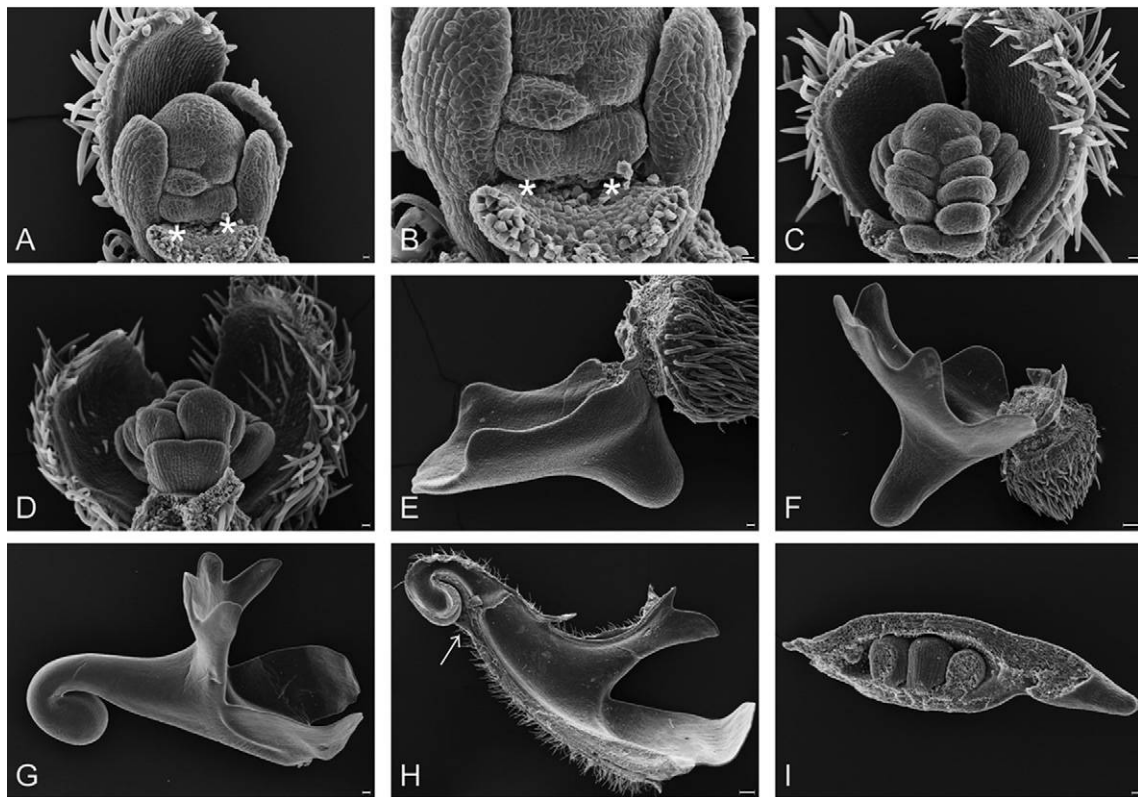
Fig. 3 Developmental sequence of the W2 organ in *Delphinium anthoroideum*. *A*, Floral bud with the dorsal W1 organ (second in the initiation sequence) sectioned to show the two W2 primordia (above the asterisks). Stamen primordia are initiating on the meristematic dome. *B*, Closer view of the W2 primordia (up the asterisks) shown in *A*. *C, D*, Later stages of bud development showing the fused W2 organs and the developing stamens. In *C*, the single carpel is not yet initiated. *E–G*, Successive stages of the five-lobed W2 organ development. The upper lobe is bifid. In *G*, the three lateral and upper lobes are grouped together, and the lower ones are wing shaped. *H*, Longitudinal section of the dorsal W1 organ and of the tightly enclosed W2 organ. A membrane (arrow) links the decurved tip of the spur to the main body of the spur. *I*, Longitudinal section of the single carpel. Scale bars: *A, B*, 10 μm; *C, D*, 20 μm; *E, I*, 30 μm; *F, G*, 100 μm; *H*, 200 μm.
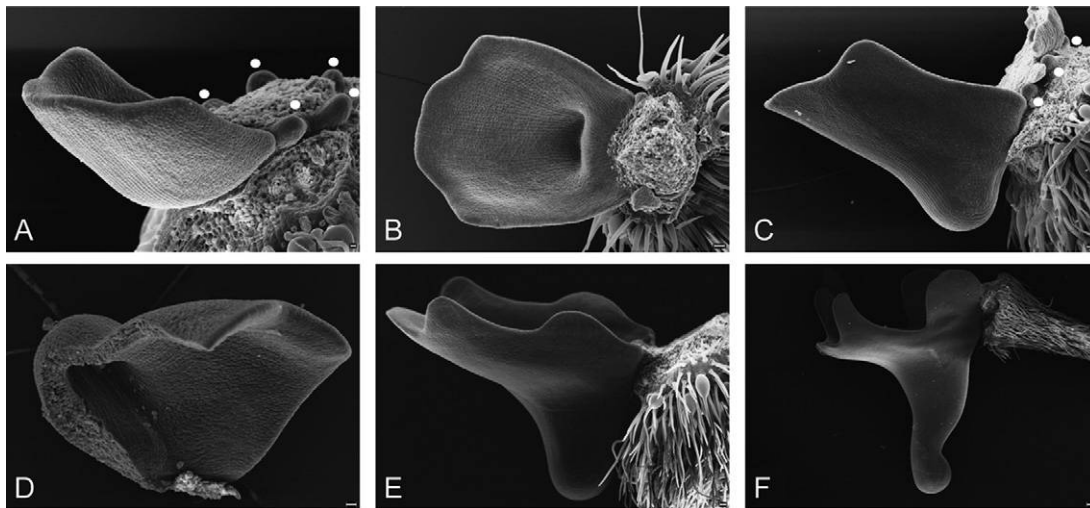
Fig. 4 Developmental sequence of the W2 organ in *Delphinium hohenackeri*. *A*, Bud with all organs removed except the dorsal organ and the five nondeveloped lateral and ventral organs of W2 (dots). *B*, Face view of the dorsal W2 organ with the spur being formed. At this stage, the W2 organ is three lobed. *C*, Lateral view of the dorsal W2 organ. At this stage, it is five lobed. Three nondeveloped W2 organs are visible (dots). *D*, Longitudinal section of the W2 organ showing the hollow spur concealing the nectariferous cells. *E*, Late developmental stage of the W2 organ. *F*, W2 organ in a preanthetic flower. The three lateral and upper lobes are in a dorsal position, and the lower ones are wing shaped. A constriction is visible below the tip of the elongating spur. Scale bars: *A*, 10 μm; *B, C, E*, 30 μm; *D*, 20 μm; *F*, 100 μm.
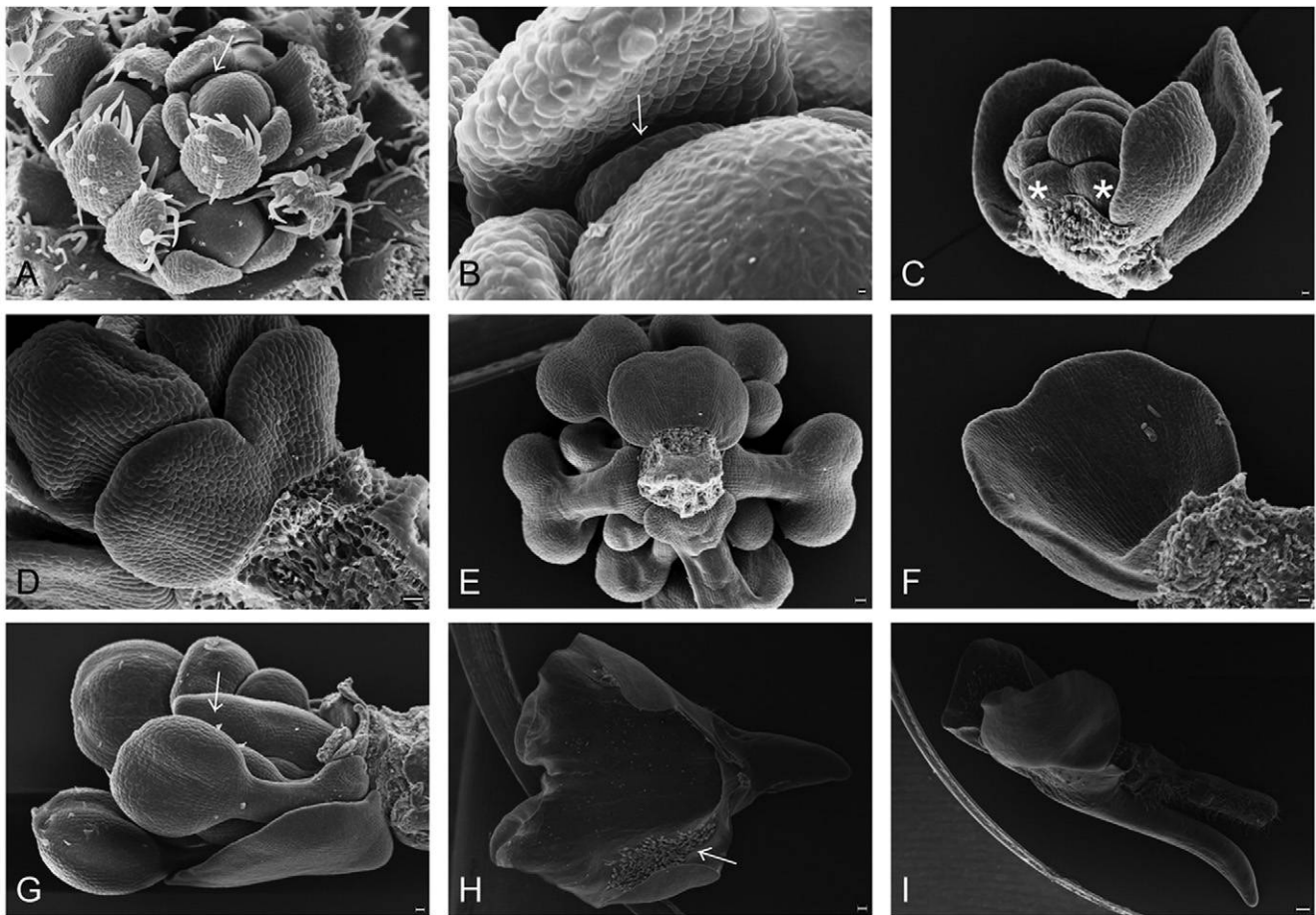
Fig. 5 Developmental sequence of the W2 organ in *Delphinium orientale*. *A*, Young inflorescence. The W2 organ (arrow) is facing the second W1 organ in the initiation sequence. *B*, Magnified portion of *A* showing the W2 organ (arrow). *C*, Bilobed (asterisks) W2 organ, revealed by sectioning the dorsal W1 organ. At this stage, stamen primordia are initiating. *D*, Heart-shaped W2 organ. *E*, Upside-down view of the developing bud with the W1 organs removed, showing the dorsal W2 organ. *F*, W2 organ with the spur being formed. At this stage, it is very slightly three lobed (upper one bifid). *G*, Side view of the bud with the W1 organs and ventral stamens removed. A single carpel is shown by the arrow. *H, I*, W2 organ at successive late developmental stages with the spur elongating. The labium is three lobed (upper lobe bifid). The lateral lobes are hairy (arrow in *H*). Scale bars: *A, D, F*, 20 µm; *B*, 3 µm; *C*, 10 µm; *E, G*, 30 µm; *H*, 100 µm; *I*, 200 µm.

5*A*, 5*B*, 7*A*). Very shortly after initiation, the five/six ventral and lateral primordia stop developing (figs. 4*A*, 6*D*–6*F*), while the pair of dorsal ones fuse (figs. 3*C*, 5*B*, 5*C*, 6*A*, 7*A*, 7*B*) and form a bilobed heart-shaped organ (figs. 3*C*, 5*C*, 5*D*, 6*C*, 7*C*). In both genera, floral organs are initiated on a spiral (figs. 3*A*, 5*A*), and hence the pair of dorsal W2 organs that fuse are not of the same age. During development, the W2 organ grows and differentiates into a three-lobed labium (figs. 4*B*, 4*C*, 6*F*) and a hollow invagination (figs. 4*B*, 5*F*, 6*F*, 6*G*). The development of this single W2 organ is delayed compared to stamen development (figs. 3*C*, 3*D*, 5*D*, 5*E*, 5*G*, 6*B*, 7*C*).

The late development of the W2 organ in preanthetic flowers follows species-specific trajectories. In *Delphinium anthoroideum* and *D. hohenackeri*, the W2 labium becomes five lobed (figs. 3*E*, 3*F*, 4*E*), while in the other three species it remains three lobed (figs. 5*H*, 6*H*, 7*E*, 7*F*), with the lateral lobes being very slightly bilobed (figs. 6*I*, 6*K*, 7*E*). The upper lobe is always bifid (figs. 3*F*, 3*H*, 5*H*, 6*H*, 6*I*, 7*E*, 7*F*). In *D.*

anthoroideum and *D. hohenackeri* the spur becomes constricted shortly before its tip, and the lower lobes of the labium become wing shaped (figs. 3*G*, 4*F*), while in *D. orientale*, *D. persicum*, and *D. consolida* the spur elongates parallel to the pedicel (figs. 5*I*, 7*E*, 7*F*) or perpendicular to it (fig. 6*I*–6*K*). In some species, the lateral lobes of the labium become hairy (figs. 5*H*, 7*F*). Eventually, the spur lifts up (figs. 3*E*–3*G*, 4*E*, 4*F*), and its tip can become decurved (fig. 3*G*, 3*H*). Sometimes, the decurved tip attaches to the main body of the spur via a membrane (figs. 1*W*–1*Y*, 3*H*).

Floral Trait Evolution in Delphinieae

The legend of figure 8 lists the discrete states of the 11 floral traits (nine focusing on the perianth and two on stamens and carpels) that we analyze below. All flowers of Delphinieae are zygomorphic (fig. 8*A*–8*C*, trait 1) although zygomorphy is less pronounced in *Staphisagria* (fig. 8*B*, 8*C*, trait 2; W2 with very short spurs and W1 organs almost all equal
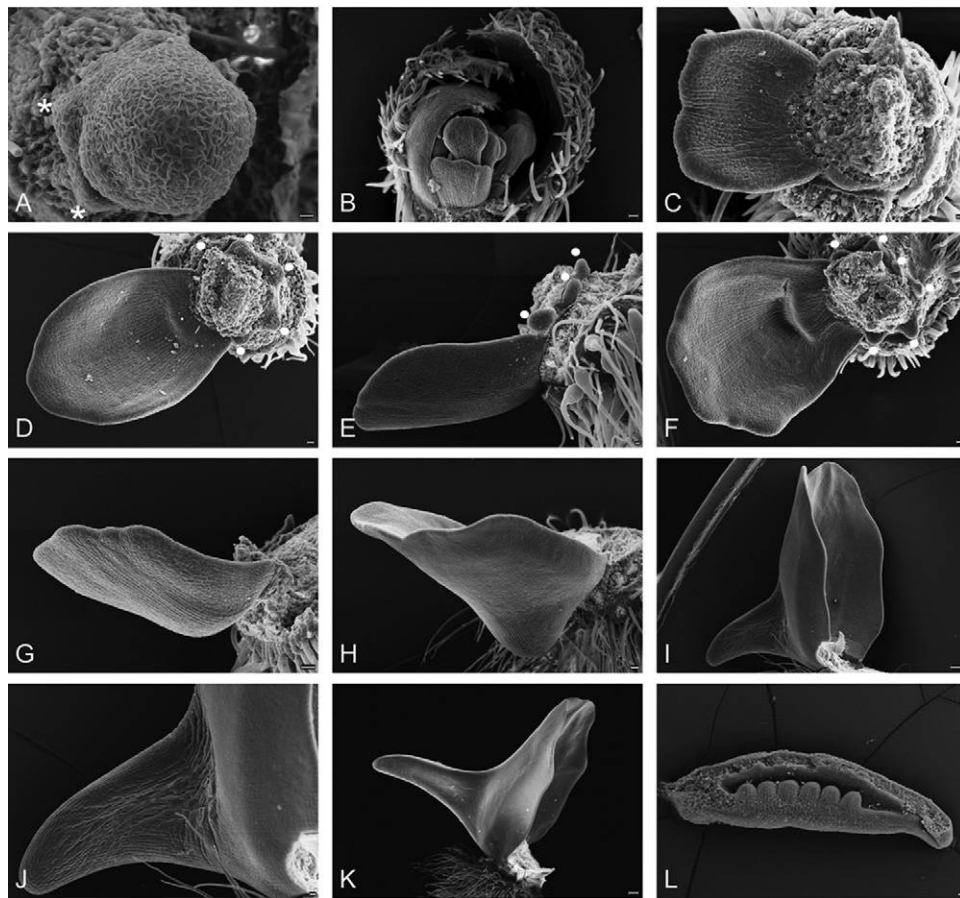
Fig. 6 Developmental sequence of the W2 organ in *Delphinium persicum*. *A*, Young bud at stamen initiation stage with W1 organs removed. Asterisks indicate fused W2 dorsal primordia. *B*, Bud with dorsal W1 organ sectioned revealing the dorsal W2 organ. *C–I*, Upper (*C*, *D*, *F*) and lateral (*E*, *G–I*) views of the W2 organ at successive developmental stages with the spur elongating. Other floral organs removed. In *D–F*, dots indicate nondeveloped W2 organs. *J*, Magnified view of *I* showing the spur of the W2 organ. *K*, Mature stage of a W2 organ with other floral organs removed. *L*, Longitudinal section of the single carpel. Scale bars: *A*, *C*, *E*, 10 μm; *B*, *G*, *H*, 30 μm; *D*, *F*, *J*, *L*, 20 μm; *I*, *K*, 100 μm.
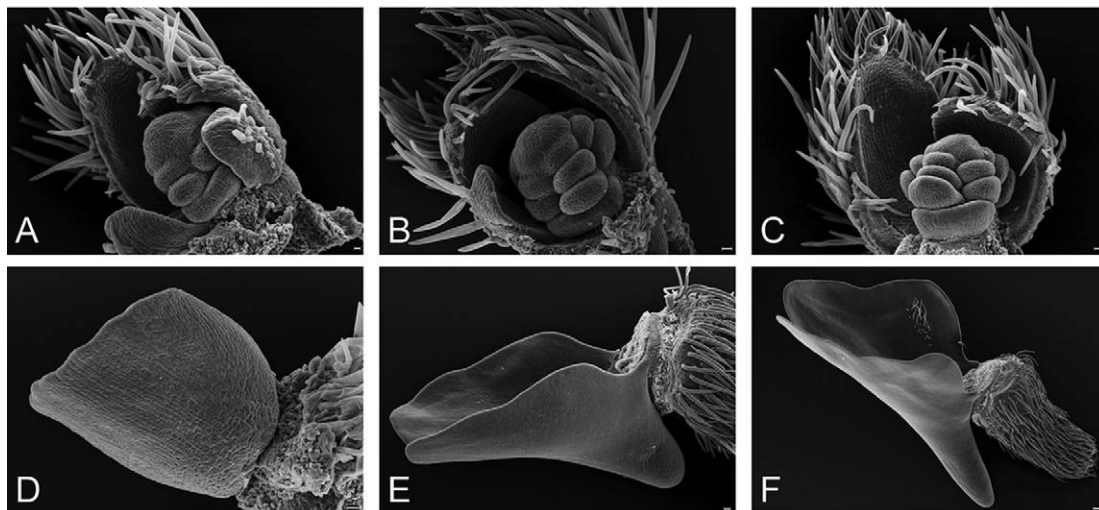
Fig. 7 Developmental sequence of the W2 organ in *Delphinium consolida*. *A–C*, Successive developmental stages of a bud with the dorsal W1 removed, revealing the W2 organ and the initiating stamens. *D–F*, Successive stages of the elongating spur of the W2 organ. Other floral organs removed. In *F*, the labium is three lobed (upper lobe bifid). Scale bars: *A*, 10 μm; *B–D*, 20 μm; *E*, 30 μm; *F*, 100 μm.
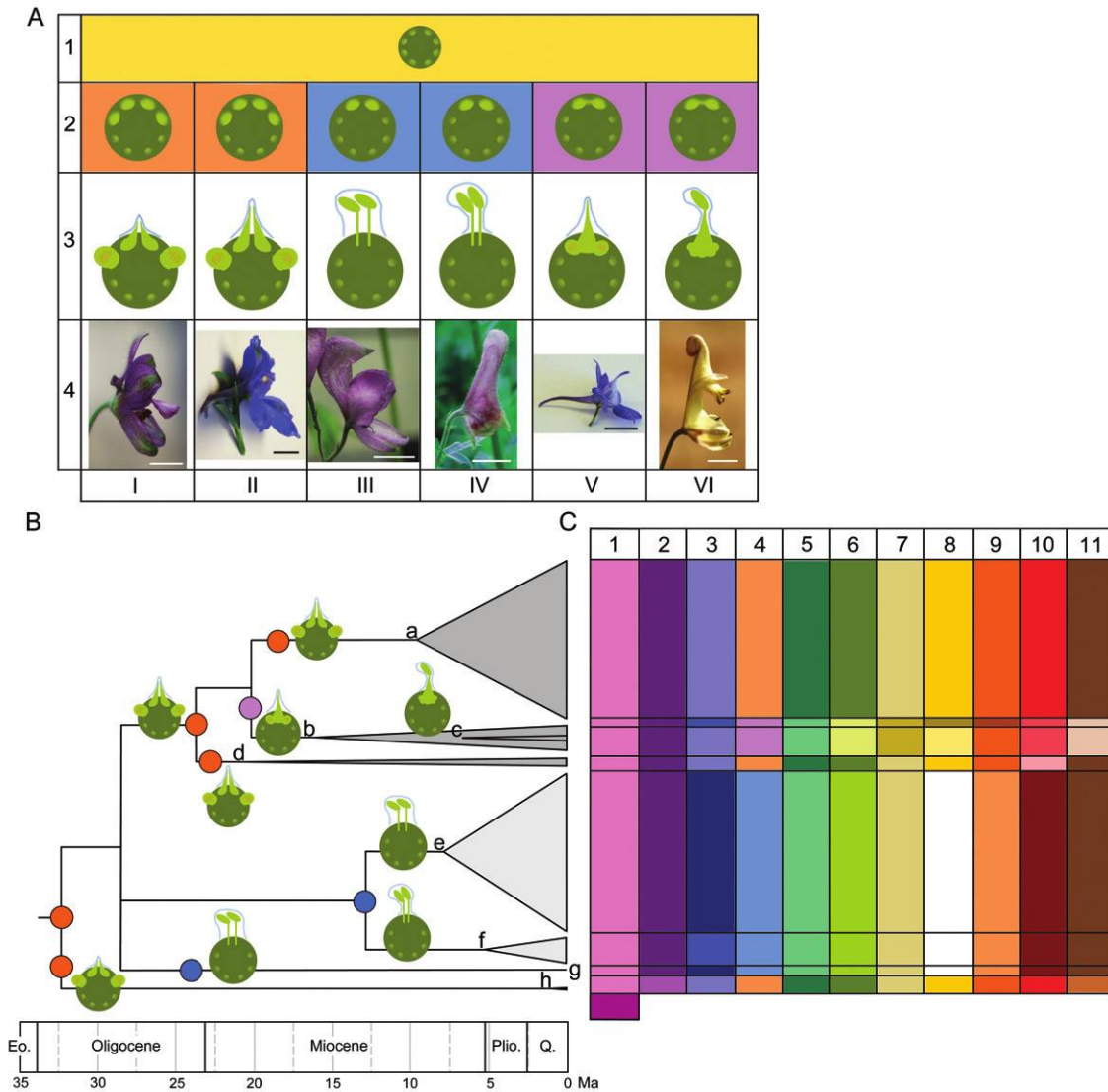
Fig. 8 *A*, Six types (I–VI) of W2 organization in Delphinieae and three stages of their respective developmental sequence. The development of W2 primordia (light green) is shown on the floral meristem (dark green disk). The blue line represents the shape of the dorsal W1 organ in the adult flower. Type I: *Staphisagria*; type II: *Delphinium*; type III: *Aconitum* subg. *Aconitum* and *Gymnaconitum*; type IV: *Aconitum* subg. *Lycoctonum*; type V: *Delphinium* subclade *Consolida*; type VI: species from the *Delphinium* subclade *Consolida* formerly treated as *Aconitella*. Photographs from left to right: *Staphisagria macrosperma*, *Delphinium grandiflorum*, *Aconitum napellus*, *Aconitum lycoctonum* subsp. *moldavicum*, *D. ajacis*, *D. anthoroideum* (all photos by F. Jabbour, except the last one, by S. Zarre). Scale bars: 4–I to 4–V, 1 cm; 4–VI, 4 mm. *B*, Perianth types in Delphinieae and the most parsimonious reconstruction of changes in the second developmental stage of W2. The phylogeny and molecular clock dating are from Jabbour and Renner (2012). a = *Delphinium* subg. *Delphinastrum* and *Oligophyllon*; b = *Consolida* s.l.; c = *Aconitella*; d = *Delphinium* subg. *Delphinium*; e = *Aconitum* subg. *Aconitum*; f = *Aconitum* subg. *Lycoctonum*; g = *Aconitum* subg. *Gymnaconitum*; h = *Staphisagria*. *C*, Character states matrix for the Delphinieae. The different characters and relative states are as follows: 1 = floral symmetry: actinomorphy (dark pink), zygomorphy (light pink); 2 = degree of zygomorphy: strong (dark purple), slight (light purple); 3 = shape of dorsal W1 organ: hood (dark blue), nightcap (medium blue), spur (light blue); 4 = category of perianth at the second developmental stage: four developed W2 organs (orange), two developed organs (blue), two fused developed W2 organs (purple); 5 = number of developing W2 primordia: four (dark green), two (light green); 6 = number of W2 organs at adult stage: four (dark green), two (medium green), one (light green); 7 = fusion of the two dorsal W2 organs: yes (dark beige), no (light beige); 8 = number of lobes, plainly visible from the outside of the flower, in the set of W2 organs: five (brown), four (yellow), three (light yellow); 9 = shape of the nectariferous organs: short stalk and long limb (dark orange), long stalk and short limb (light orange), group in which both phenotypes occur (brown); 10 = mean number of stamens: ~15 (very light red), 15–25 (light red), 20–40 (red), 25–50 (dark red; sources: Bosch et al. 2001; Duan et al. 2009); 11 = number of carpels: three or more (dark brown), three (medium brown), one (light brown).
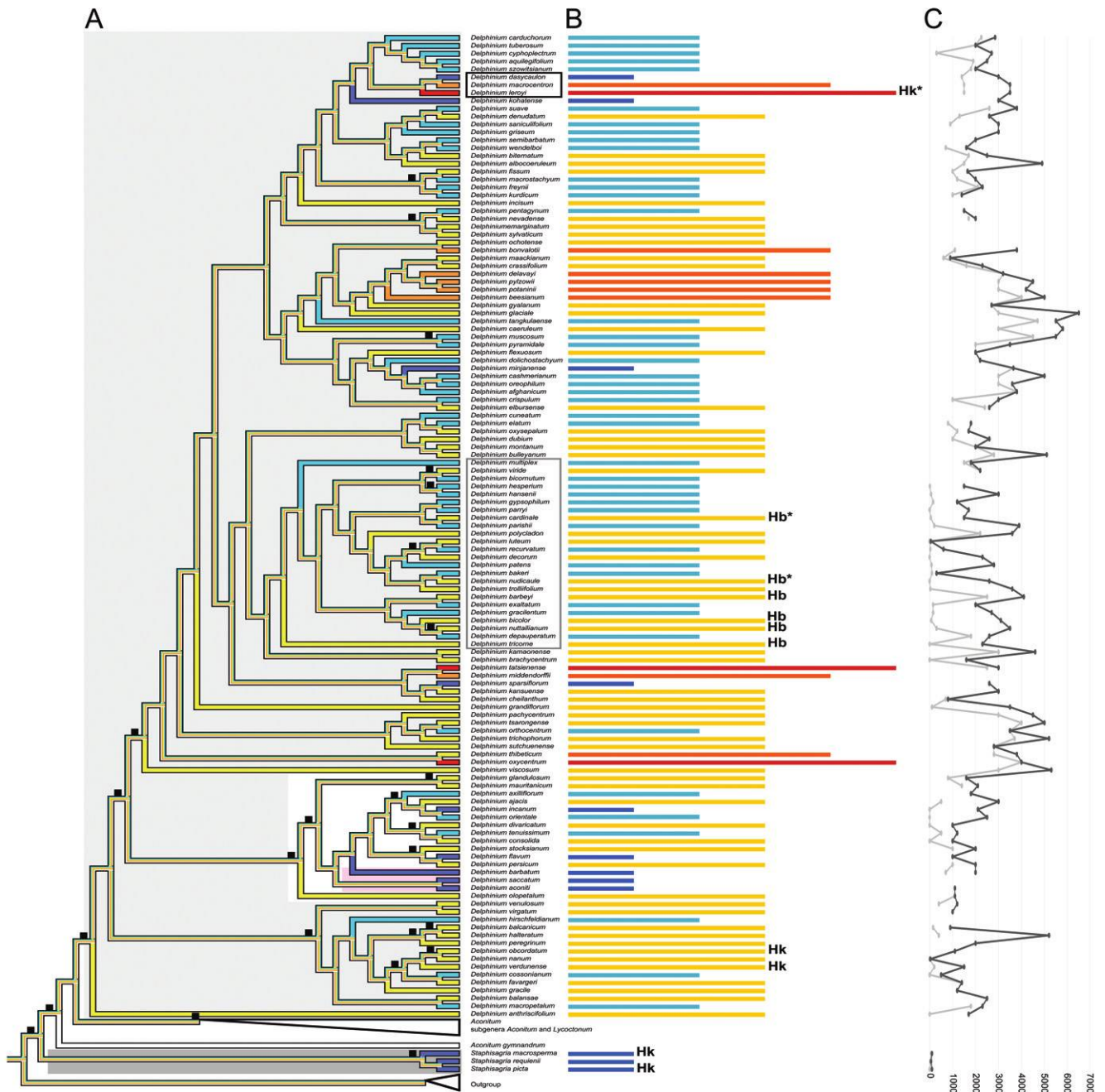
Fig. 9 *A*, Spur length categories plotted on a plastid and nuclear phylogeny for the Delphinieae (Jabbour and Renner 2012). Spur length is recorded for *Delphinium* (gray shading), *Staphisagria* (dark gray shading), the *Delphinium* subclade *Consolida* (white area within the gray shading), and the species formerly treated as *Aconitella* (pink shading). Black squares at nodes indicate maximum likelihood bootstrap support >70%. Branches are colored by spur length category (for absolute lengths, see "Material and Methods"; table 2). Dark gray rectangle framing species names indicates species with a tropical African distribution. Light gray rectangle framing species names indicates species with a North American distribution. *B*, Spur length category shown for each species of *Delphinium* and *Staphisagria*. Bars are colored according to the five spur length categories (see "Material and Methods"; table 2). Hk = hawkmoth pollination; Hk* = exclusive hawkmoth pollination; Hb = hummingbird pollination; Hb* = exclusive hummingbird pollination. *C*, Altitudinal range shown for each species of *Delphinium* and *Staphisagria* (see table 2). Gray and black lines indicate the lowest and highest altitude (m), respectively, at which populations were recorded.

Table 4

Floral Formulas of the Four Floral Types of Delphinieae

Taxonomic group	Floral formula
Aconitum	‡1.2.2 W1 + 2.0.6 W2 + 25–50 A + 3–5 G
Formerly *Aconitella*	‡1.2.2 W1 + (2)^5.0.6 W2 + 15–25 A + 1 G
Delphinium subclade	
Consolida	‡1.2.2 W1 + (2)^3.0.6 W2 + 15–25 A + 1 G
Staphisagria and	
other *Delphinium*	‡1.2.2 W1 + 2.2.4 W2 + 15–40 A + 3–5 G

Note. The numbers recorded for W1 refer to the dorsal, lateral, and ventral organs, respectively. The two first numbers recorded for W2 refer to the spurred and flat lateral organs, respectively. The third number refers to the remaining primordia. In the *Delphinium* subclade *Consolida* and the group formerly treated as *Aconitella*, the exponents refer to the number of lobes exhibited by the W2 organ labium. A double dagger indicates zygomorphy. Sources: Trifonova 1990; Ronse De Craene 2010; Flora of North America and Flora of China (http://www.efloras.org).

in size and shape; fig. 1C). The nightcap shape evolved twice, once in *Aconitum* subg. *Lycoctonum* (figs. 1G, 1I, 8A–8C, trait 3) and again in the *Delphinium* subgroup *Aconitella*, 10 species initially described in *Delphinium*, then moved to *Consolida*, and finally considered a separate genus, *Aconitella*, by Spach (1838), because of the *Lycoctonum*-like shape of its flowers (fig. 1W, 1X; eight of the 10 *Aconitella* species were sampled and formed a clade; Jabbour and Renner 2011a).

The reduction from four dorsal W2 organs to two also evolved twice, once in *Aconitum* and again in *Delphinium* subg. *Consolida/Aconitella* (fig. 8A–8C, trait 5), but fusion of the two dorsal W2 primordia occurred only in the *Consolida* subclade (fig. 8A–8C, traits 4, 7). The outer perianth whorl in mature Delphinieae flowers thus can have (i) four developed organs (hence four labia; fig. 8C, trait 8), the two dorsalmost being nectariferous; (ii) two developed organs (*Aconitum*); or (iii) just a single organ (*Consolida* subclade; fig. 8B, 8C, trait 6). The labium of the W2 organ is dissected into three or five lobes in the *Delphinium* subclades *Consolida* and *Aconitella* (fig. 8B, 8C, trait 8). The limb is longer than the stalk in most but not all *Staphisagria* and *Delphinium*, while the opposite is true in *Aconitum* (fig. 8B, 8C, trait 9).

The number of stamens is higher in *Aconitum* and the high-altitude (>1000 m; fig. 9C) perennial *Delphinium* than in the annual *Delphinium* (fig. 8B, 8C, trait 10; table 4). *Aconitum gymnandrum* (fig. 1E), *Staphisagria macrosperma* (fig. 1C), *D. mauritanicum* Coss. (=*Consolida mauritanica* [Coss.] Munz), and *D. cossonianum* Batt., with ~70, 40, 25, and 16 stamens, respectively, produce ~9140, 4560, 1490, and 3750 pollen grains/stamen, respectively (Bosch et al. 2001; Duan et al. 2009), a large number compared to the typical 10–20 ovules/carpel. Carpel number is lowest in the *Consolida* subclade (figs. 3I, 6L, 8B, 8C, trait 11; table 4).

Nectar Spur Length, Pollinators, and Altitude

Nectar spur length, pollinator type, and altitude are shown on the phylogeny in figure 9A–9C, and the underlying infor-

mation is summarized in tables 2 and 3. Most Delphinieae are bumblebee pollinated, although a few North American *Delphinium* species rely on hummingbirds for pollination and an African species is hawkmoth pollinated. Spur length shows no strong phylogenetic signal, with sister species often having markedly different spur lengths (fig. 9). The longest spurs are found in bee-pollinated species in high-altitude areas in southeast China (where 60% of *Delphinium* species occur) and in a hawkmoth-pollinated species in tropical Africa (where a clade of three species occurs); no correlation was detected between spur length (see "Material and Methods" for length categories) and mean altitude recorded for a species (fig. 10).

Discussion

Development of the Paired Nectar Spurs in Delphinium

This study broadens the database for floral development in *Delphinium*, a genus of ~350 species, including 65 species previously sometimes placed in *Consolida* or *Aconitella* (only 12 species names from these latter genera have never been placed in *Delphinium*, and based on the molecular tree and morphology, they all belong in *Delphinium*; Jabbour and Renner 2011a, 2012). Floral development in the five newly studied species is similar to that in the four species of *Delphinium* studied previously (*Delphinium divaricatum* Ledeb. [=*Consolida divaricata* (Ledeb.) Schrödinger], *D. consolida* [Payer 1857], *D. grandiflorum* L., *D. ajacis* L. [=*Consolida ajacis* (L.) Schur; Kosuge and Tamura 1989], *D. grandiflorum* [Jabbour et al. 2009]), although the W2 development is species specific. Of the initial seven or eight W2 primordia, only two develop (as indicated in Payer 1857; Kosuge and
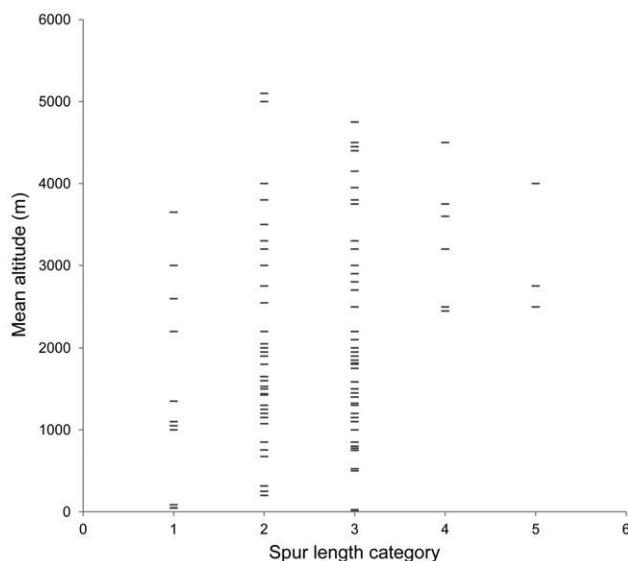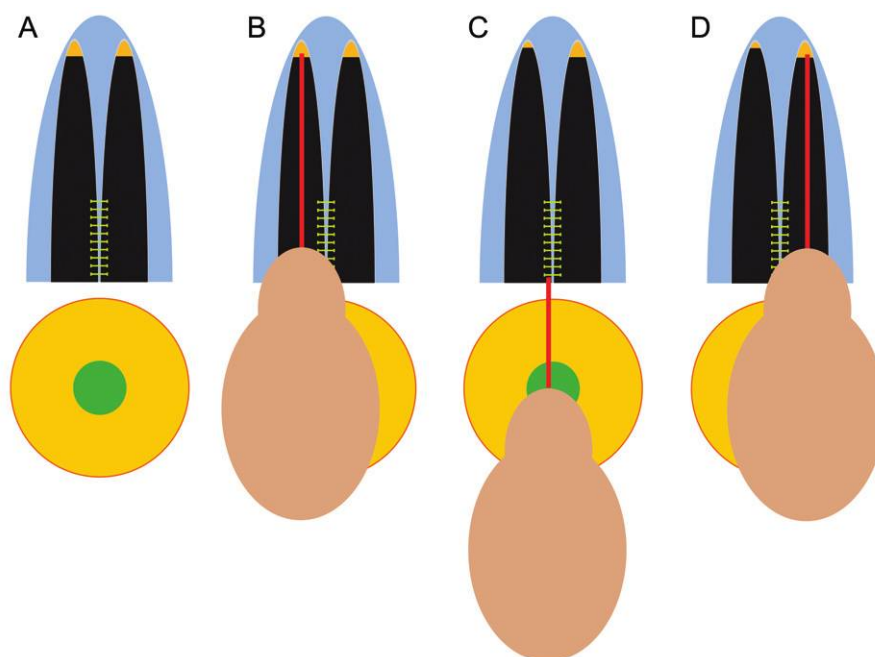

Fig. 10 Relationship between the mean altitudes recorded for the species of *Delphinium* and *Staphisagria* included in the study of Jabbour and Renner (2012) and spur length category (see "Material and Methods"; table 2).

Fig. 11 Schematic sequence of a pollinator foraging for nectar in a flower of Delphinieae (see video 1). *A*, From the bottom to the top: androecium (yellow disk) and gynoecium (green disk); the pair of W2 spurs (black) concealing nectar (orange) and enclosed in the W1 spur (blue). The green zip linking the sides of the internal spurs symbolizes the more or less deep antechamber shared by the W2 organs. *B*, Pollinator (brown) inserts its tongue/proboscis (red) into one of the internal spurs for reaching nectar. Its body is in contact with the sexual organs of the flower. *C*, After removing nectar from the first spur tip, the pollinator has to retract its tongue/proboscis and fly backward to position its body in front of the second spur. To restrict the energy costs, the insect limits its movements, and its body therefore stays in contact with the sexual organs. *D*, Pollinator flies inward again and inserts its tongue/proboscis in the second spur.

Tamura 1989; but contra Kemularia-Nathadze 1940) and then fuse postgenitally (figs. 3*A*, 3*B*, 5*A*–5*C*, 7*A*, 7*A*). Our illustrations show clearly that the young W2 organ is heart shaped, the two lobes corresponding to initially free primordia. Then, an invagination is formed at the base of the organ, which is the precursor of the nectar spur. Later on, the spur tip can become constricted and the spur is elongated or lifted up.

Perianth Evolution in Delphinieae Revisited

As long as *Delphinium* was considered derived from *Aconitum* (Trifonova 1990; Bosch 1997), it was logical for au-

thors to assume an evolutionary transition from two to four developing W2 organs. Alternatively, when it was hypothesized that *Delphinium* and *Aconitum* were sister genera, with their closest outgroup(s) unclear (Trifonova 1990; Wang et al. 2009), the ancestral W2 organization could not be inferred. However, with the molecular phylogeny now showing that *Delphinium* is not nested in *Aconitum* and that *Staphisagria* is sister to all other Delphinieae (Jabbour and Renner 2011*b*), a new reconstruction of Delphinieae flower evolution is in order. A remaining handicap is that the sister group of Delphinieae is not firmly known. Based on the phylogeny of Wang et al. (2009), the Delphinieae sister group may be either
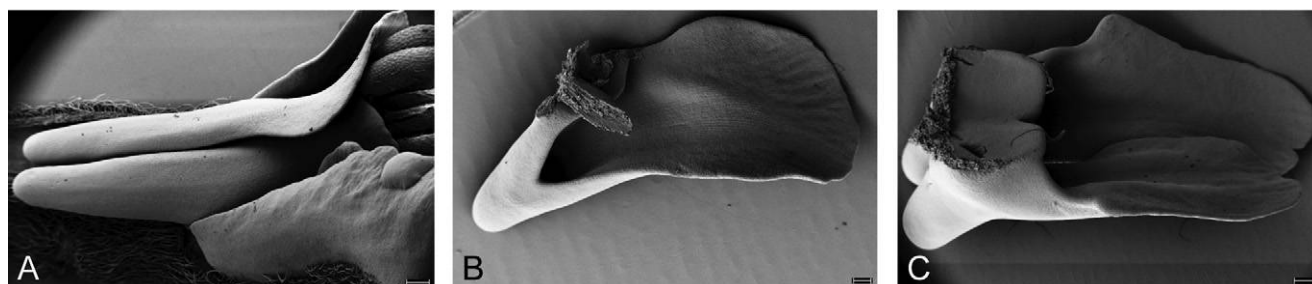
Fig. 12 Sharing of an antechamber without or with fusion of the two dorsalmost W2 organs in *Delphinium* and *Staphisagria*. *A*, Spurs in *Delphinium grandiflorum* flanked by the two flat dorsolateral W2 organs. *B*, Close-up of one of the two spurs shown in *A*. The notched face of the spur is pressed against the notched face of the other spur. *C*, Preanthetic flower of *Staphisagria macrosperma*. Both labia and spurs are free, while the two adjacent sides of the stalks are fused. These three SEM photographs were obtained during the developmental study of Jabbour et al. (2009). Scale bars: *A*, *C*, 200 μm; *B*, 100 μm.
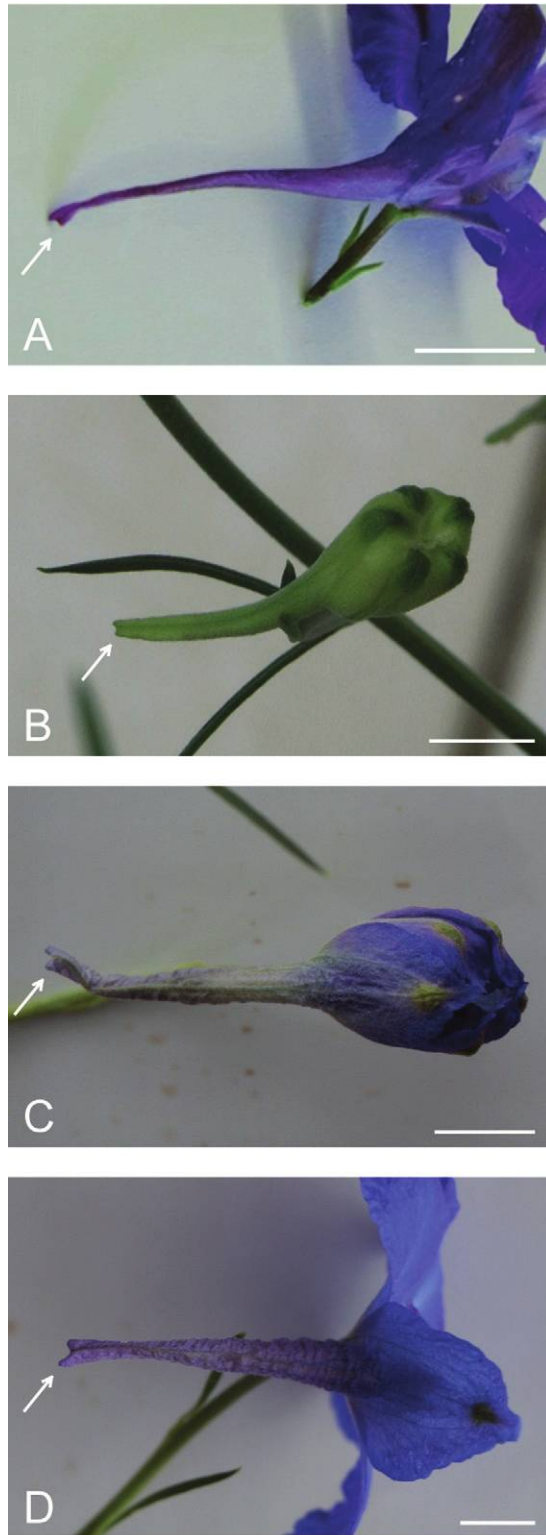
Fig. 13 *A*, Adult flower of *Delphinium ajacis* showing the bifid tip of the spurred dorsal W1 organ. *B–D*, Successive ontogenic stages of a flower of *D. grandiflorum* showing the bifid tip of the spurred dorsal W1 organ. *B*, Floral bud. *C*, Late floral bud. *D*, Open flower. Scale bars: 6 mm.

Nigella or an *Adonis/Megaleranthis/Trollius* clade or a clade including 22 genera (among them *Helleborus*; clade F in fig. 3 of Wang et al. 2009).

To synthesize the diversity in perianth shapes and floral organization in Delphinieae, we use a combination of floral formulas (table 4), schemes of the six types of perianth organizations and their developmental sequence (fig. 8*A*), and schematic longitudinal sections of flowers (fig. 2*A*–2*C*). Homoplasy is surprisingly limited. The nightcap shape of the dorsal W1 organ evolved twice, once in a *Delphinium* subgroup formerly treated as *Aconitella* and once in *Aconitum* subg. *Lycoctonum*. The reduction from four to two developing primordia also evolved twice, once in *Aconitum* and once in *Delphinium* (fig. 8*B*). In *Aconitum*, W2 organ number reduction is accompanied by a change in the relative proportions of the labium, spur, and stalk and the replacement of the outer spur by a hood (figs. 2, 8*B*), while in *Delphinium* subg. *Aconitella/Consolida* it is accompanied by the fusion of the W2 dorsal primordia and the growth of basal wings on the sides of the adult W2 organ. Overall, *Delphinium* flowers have very similar W2 labium shapes, probably to provide a landing place for their bee pollinators.

Stamen number is lowest in annual *Delphinium* (subg. *Delphinium*, except *D. anthriscifolium* Hance, *Consolida* subclade); it is highest in perennial high-altitude Delphinieae (see fig. 8*C*, traits 10, 11). High stamen numbers and abundant pollen production may be advantageous at high altitudes with adversarial climates, where pollinators may sometimes be limiting. The combination of low floral organ numbers and fusion of perianth parts characterizing the *Consolida/Aconitella* clade of *Delphinium* is a unique case among angiosperms with spiral phyllotaxis (Endress 1990).

Delphinieae Nectar Spurs in Relation to Pollinators

The paired nectar spurs of Delphinieae are almost unique among angiosperms. Depending on the size of the nectar antechamber, or "floral parlor" (Leppik 1964; figs. 11, 12*B*), a pollinator will have little or almost no space to move its body for inserting, retracting, and inserting again its tongue to take up the nectar from both spur tips (fig. 11*A*–11*D*; video 1; the repeated "in and out" of the bee's body at each flower can be seen clearly in the video). Pooling the nectariferous structures by reducing the length of the free spur tips and increasing the length of the fused spur section may be advantageous for attracting large bees. From a pollinator's point of view, insertion and retraction of the tongue should be easier in flowers with a large antechamber and partial fusion of W2 organ stalks. Such partial fusion (with spur tips still free) is found in *D. grandiflorum* (fig. 12*A*, 12*B*; also in Kosuge and Tamura 1989), *D. macrocentron* Oliv., *D. kingianum* Brühl ex Huth (Huth 1895), and *S. macrosperma* Spach (fig. 12*C*). Keeping a pollinator inside the flower longer (because it needs to move back and forth in order to exploit both spur tips) probably increases pollen export and uptake, fitting with the thousands of pollen grains produced by each flower ("Results").

Bumblebees pollinate 99% of the species for which we have data (table 3; fig. 1*B*, 1*I*), and if we judge from the blue flower color (see fig. 1*A*, 1*B*, 1*F*, 1*J*–1*N*, 1*Q*–1*S*) and hidden

nectar, most remaining Delphinieae (~650 species) also are bee pollinated. A similar example of diffuse coevolution between numerous species of bumblebees and a clade of angiosperms is *Pedicularis*, with roughly 600 species (Macior 1984). The three Mediterranean species in the genus *Staphisagria*, the sister clade to the remaining Delphinieae, have short-spurred, mostly bee-pollinated flowers. Of the 10 North American species with pollination data, six have bluish purple to red flowers that are hummingbird pollinated (table 3; all are included in the phylogeny; fig. 9). Since their phylogenetic relationships are not clear (Koontz et al. 2004; Jabbour and Renner 2012), the number of transitions from bee to hummingbird pollination cannot be inferred. All bird-pollinated species have spurs of intermediate lengths ("Material and Methods"; fig. 9) with a relatively small antechamber, forcing birds to insert their tongue twice to reach the separate internal spurs.

The species with the longest spurs, *D. leroyi* Franch. ex Huth from tropical Africa (table 3; fig. 9*A*, 9*B*), is hawkmoth pollinated. However, four Mediterranean species with middle-sized and short spurs also are visited by hawkmoths besides bees (fig. 9*A*, 9*B*; table 3), possibly without the moths being pollinators, because a hawkmoth's weight is insufficient to depress the lateral W2 organs (Macior 1975).

From these data it appears that coevolutionary relationships between spur lengths and pollinator tongue lengths is short-circuited in Delphinieae by the paired nectar spurs that force pollinators to move back and forth to explore both spurs (as clearly visible in video 1). In other Ranunculaceae, for example, *Aquilegia*, flowers with different spur lengths are adapted to pollinators with different proboscis lengths (hawkmoths, bees, hummingbirds; Whittall and Hodges 2007). Delphinieae, however, have undergone very few pollinator switches (from bees to hummingbirds in North America and from bees to hawkmoths in one African species; the exact number of switches is not yet clear) and instead have continued to rely on bees. Bees may not have selected for extremely long nectar spurs, given that they need to move their proboscis and body back and forth inside the flower in order to explore both spurs.

Evolutionary theory suggests that Delphinieae flowers might be partially rewardless, that is, that the two spurs may not contain the same amount of nectar, because once a bee has landed, it is committed to exploring both spurs; exploring one spur would constitute a potential loss of food acquisition. Partially rewardless mutants are extremely common across angiosperms (Renner 2006) and should be selectively favored as long as nectar production is costly. However, we know of no data on this point. We did not find any relation between spur length and the mean altitude at which a species occurs. The evolution of spur length in Delphinieae, however, is likely influenced by many other factors, including inflorescence size (and hence flower number) and mean pollinator weight (and hence calorie needs).

Open Questions regarding Delphinieae Spurs and Main Conclusions

The external hood or spur that envelopes the inner nectar spurs is molded on their shape (fig. 1*F*, 1*G*), suggesting possible mechanical and developmental constraints exerted by the W2 organs on the W1 organ. Flowers of *D. ajacis* L. (*Consolida ajacis* [L.] Schur.; fig. 13*A*), *D. grandiflorum* (fig. 13*B*–13*D*), and *Staphisagria picta* (Willd.) F.Jabbour (Bosch 1997) sometimes have outer spurs with a bifid tip (even if, in the case of *D. ajacis*, the two initiated dorsal W2 primordia fuse into a single inner spur), hinting at a structural connection between inner and outer spur development. Mutants lacking W2 organs could show whether the molecular determinism of both W1 and W2 spurs is linked or whether the inner nectar spurs exert a mechanical constraint on the outer envelope. Dissections at early developmental stages to remove the dorsal W1 organ could also help detect changes in W2 organ development. The removal of W2 organs, which tightly protect the W1 organs in bud, seems less feasible and would almost certainly affect the development of the outer perianth whorl.

A main conclusion of this study concerns the adaptive value of the paired nectar spurs of most Delphinieae, which are hidden inside an envelope formed by the outer whorl: separate spur tips force pollinators to insert their tongues twice, resulting in a more or less pronounced back-and-forth movement that must enhance pollen uptake and deposition.

Acknowledgments

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