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► **To cite this version:**

Julie Zalko, Samuel Frachon, Antoine Morel, Thierry Deroin, Felipe Espinosa, et al.. Floral organogenesis and morphogenesis of [i]Staphisagria[/i] (Ranunculaceae): Implications for the evolution of synorganized floral structures in Delphinieae. International Journal of Plant Sciences, 2020, 10.1086/711471 . hal-02975177

HAL Id: hal-02975177

<https://hal.science/hal-02975177>

Submitted on 22 Oct 2020

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Floral organogenesis and morphogenesis of *Staphisagria* (Ranunculaceae): Implications for the evolution of synorganized floral structures in Delphinieae

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Shortened title: The hyperorgan of Delphinieae

Keywords: Delphinieae, nectar spur, perianth, petal fusion, *Staphisagria*, synorganization

Abstract

Premise of the research. Floral synorganization is a structural feature of many speciose angiosperm taxa, and is considered as a morphological innovation paving the way for evolutionary diversification. *Staphisagria* is sister to the remaining Delphinieae, the only lineage of Ranunculaceae characterized by zygomorphic flowers. We aim at providing a description of floral organogenesis and morphogenesis in both *Staphisagria* species, presenting the disparity of Delphinieae hyperorgan in a phylogenetic framework, and proposing a scenario of likely developmental pathways underlying the different types of hyperorgans in Delphinieae.

Methodology. We carried out morphological, anatomical, and developmental studies on flowers of *Staphisagria macrosperma* and *S. picta*.

Pivotal results. Synorganization is complex in *Staphisagria* and Delphinieae as a whole, and involves flower dorsoventralization, nesting of spurs, postgenital fusion of petals, and the formation of a shared cavity. From a choripetalous ancestor, late and partial postgenital fusion among dorsal petals evolved once or twice in the tribe.

Conclusions. Delphinieae flower includes nested spurs and nested floral parlors. These key innovations, unique in angiosperms, probably led to the diversification of this species-rich tribe in the Northern Hemisphere. The length of the inner (nectariferous) spurs and the nested floral parlors determine the range of pollinators able to collect nectar. These traits could be used to revise the circumscription of taxonomic groups within the tribe, and should be taken into account when examining the possible coevolution between Delphinieae flowers and their pollinators. Integrating this new knowledge about the hyperorgan will be essential for future research in taxonomy, evo-devo and pollination ecology in Delphinieae.

Introduction

Floral synorganization is a structural feature of many speciose angiosperm taxa, and is considered as a morphological innovation paving the way for evolutionary diversification (Endress 2011). Although the term synorganization was coined by a zoologist in 1952 (Remane 1952), the concept did not spread much within this community (but see Salvini-Plawen and Splechtna 1979; Wagner 1989). However, it thrived quite early in botany (Vogel 1959, 1969), and its definition has been successively amended by floral morphologists (Endress 1990; Erbar 1991; Verbeke 1992; Specht and Bartlett 2009; Endress 2010a; Ronse De Craene 2010; Ley and Claßen-Bockhoff 2012; Endress 2016; Sokoloff et al. 2018). Building on these works, we would define the concept as follows:

Synorganization is the macroscopic integration and close spatial association of two or several neighboring structural elements or ancestrally independent organs, with or without fusion. Regarding function, the product of synorganization is not simply the sum of the parts, rather the elaboration of a complex functional unit or hyperorgan. Regarding evolution, a hyperorgan is a morphological – sometimes key – innovation. Structures can only be considered as synorganized in a taxon comparatively with non-synorganized homologous structures in the sister taxon.

Synorganized floral parts have been described in a wide range of angiosperm lineages (Endress 1990, 2010b). In Ranunculales, the earliest-diverging order of eudicots (APG IV 2016), synorganization was briefly reported in the flowers of the tribe Delphinieae, in which the nectar spurs of the dorsal petals are nested within the hollow dorsal sepal (Chartier et al. 2016; Chen et al. 2018; Jabbour et al. in press). The phylogenetic position of Delphinieae within Ranunculaceae, and the floral morphological evolution in the family were both

clarified recently (Zhai et al. 2019; Carrive et al. 2020). Phylogenetic relationships within Delphinieae have been largely clarified as well in the last ten years (Jabbour and Renner 2011b, 2012b; Wang et al. 2013; Espinosa et al. 2017; Hong et al. 2017; Xiang et al. 2017).

The Delphinieae calyx is zygomorphic and consists of one dorsal, two lateral, and two ventral sepals. The corolla is dorsoventralized (hence zygomorphic) and generally comprises four developed petals in dorsal position. The two dorsalmost are spurred and nectariferous, and the pair of petal spurs is nested within the spur or hood of the dorsal sepal (for more details about Delphinieae floral organization, see Jabbour and Renner 2012a). Floral morphology in Delphinieae has been the object of numerous studies, mostly focusing on spurred petal morphology and development (Payer 1857; Kosuge and Tamura 1988, 1989; Erbar et al. 1998; Jabbour et al. 2009; Jabbour and Renner 2012a; W. Zhang et al. unpublished manuscript). According to Endress (2001) a spur is a hollow outgrowth of a laminar organ. Anatomical aspects of nectar production in Delphinieae start to be better understood (Antoń and Kamińska 2015). In order to describe the nectariferous hyperorgan development and structure, we focus on the genus *Staphisagria* Hill, which is sister to all the other Delphinieae. The name *Staphisagria* (Hill 1756) refers to the similarity of the leaves with leaves of wild vine (from the Greek σταφίς [staphis] = grape, and άγριος [agrios] = wild). This genus of Mediterranean plants consists of a pair of species, *S. macrosperma* Spach and *S. picta* (Willd.) Jabbour, according to Aboucaya (1989) and Léotard (2002; this MSc thesis became available to us in 2018), the latter having synonymized *S. requienii* (DC.) Spach with *S. picta*, based on multivariate analyses of morphological traits. *Staphisagria macrosperma* is widely distributed in the Mediterranean Basin, except in Libya and Egypt. In contrast, *S. picta* is endemic to Corsica, Sardinia, Mallorca and Hyères islands (Léotard 2002; Orellana 2008; Tison and de Foucault 2014). The traditional use of *S. macrosperma* seeds as a cure for diverse ailments

(Bruneton 1995) might explain its widespread occurrence, and the very contrasted distribution areas of both species called for investigations of their respective genetic diversity (Orellana et al. 2009a, 2009b).

Our objective is two-fold:

- 1) Provide a thorough description of floral organogenesis and morphogenesis in both *Staphisagria* species, focusing on the development of the hyperorgan.
- 2) In a recently updated phylogenetic framework (Espinosa et al. 2017; Hong et al. 2017; Xiang et al. 2017), and for each of the constitutive genera of Delphinieae, namely *Staphisagria* Hill, *Aconitum* L., *Gymnaconitum* (Stapf) Wei Wang & Z.D.Chen and *Delphinium* L., present the morphological diversity of the hyperorgan, and propose a scenario of likely developmental pathways and molecular bases possibly underlying the different types of hyperorgans in Delphinieae.

Material and Methods

Sampling

Young inflorescences and flower buds from both *Staphisagria* species were sampled from living plants, and samples were fixed with FAA (90% ethanol 70%, 5% formalin, 5% acetic acid) for 24 hours, and stored in a mixture of water, ethanol and glycerol in equal volumes. Plants were grown in the Mediterranean greenhouse (four individuals of *S. macrosperma* sampled in April-May 2019) and in the garden of the MNHN seed bank, and in the garden of the MNHN École de Systématique (many individuals of *S. picta* sampled in May 2015, June 2016, and May 2017). Seeds were provided by the MNHN seed bank (*S. picta*, # accession 08-38, collected in 1986 in Porquerolles (France)), or were sent by the Conservatoire botanique national méditerranéen de Porquerolles (*S. macrosperma*, # accession M 7145 – Lyo 3141*, collected in 1992 in Flassans-sur-Issole (Var, France); *S. picta*, # accession M

16358 – 1992 B7, collected in 1992 in Calenzana (Corsica, France); # accession M 6531 – Lyo 1017, collected in 1991 in Le Brégançonnet (Porquerolles island, France)).

Plants from both species are kept as herbarium specimens at Paris Herbarium (P); barcodes for *S. macrosperma* are P04023428 and P04023429, and for *S. picta* are P04023155 and P04023156. High-resolution images can be downloaded from P online public database (<https://science.mnhn.fr/institution/mnhn/collection/p/item/search/form>).

For the anatomical study, flowers were taken from the following herbarium specimens: P02345181 (*S. macrosperma*, one flower) and P00249055 (*S. picta*, two flowers). As the anatomical study was carried out long before the flowering of *S. macrosperma* plants, we could not rely on fresh material for both species, hence we had to sample buds from herbarium specimens.

To investigate the evolution and development of the Delphinieae hyperorgan in a phylogenetic framework, we chose the well-studied genus *Nigella* as a representative of Nigelleae, the sister group of Delphinieae (Zhai et al. 2019). *Nigella*, and especially *Nigella damascena*, has been intensively studied regarding the development and evolution of its perianth (Zhao et al. 2011; Gonçalves et al. 2013; Zhang et al. 2013; Derooin et al. 2015; Jabbour et al. 2015; Wang et al. 2015; Damerval and Becker 2017; Damerval et al. 2018; Yao et al. 2019; Liao et al. 2020; F. Jabbour et al. unpublished manuscript). Because nectariferous petals originated once within Ranunculaceae (Carrive et al. 2020), petals in Nigelleae and Delphinieae are homologous.

Scanning electron microscopy observations

A total of about 200 floral buds for each of the *Staphisagria* species were dissected using a stereoscope (Nikon SMZ 745T). They were dehydrated in an ethanol series (70%, 80%, absolute alcohol) and dried using an Emitech K850 critical point dryer (Quorum

Technologies), then mounted on aluminum stubs with colloidal graphite, sputter coated with carbon (60 seconds of metallization) using a EM ACE600 fine coater (Leica), and observed using a SU3500 scanning electron microscope (Hitachi).

Restoration of herbarium material and anatomical sections

One preanthetic floral bud was sampled from a herbarium specimen for each of the species *S. macrosperma* and *S. picta*. They were restored in an ammoniacal 10% aq. solution at 60°C during 24 hours, then fixed with FAA for 24 hours, and finally stored in a mixture of water, ethanol and glycerol in equal volumes.

Buds were dehydrated through a t-butyl series and embedded in paraffin (melting point: 58–60 °C) (Gerlach 1984). Serial transverse sections were cut at a thickness of 12 µm using a rotary microtome (Leitz 1512, Germany), then stained with AstraBlue 0.5% aq. (Chroma® 1B 163) and Ziehl's Fuchsine (RAL® 320490-1000) 10% aq. and mounted in the mounting medium Eukitt (O.Kindler GmbH® E0214). Mounted material was observed using Zeiss Axioskop microscope. Samples are kept at the plant histology micro-slide library at Paris Herbarium (P; <https://www.mnhn.fr/en/collections/collection-groups/botany/histotheque-histology-library>).

Results

Floral organogenesis and outer/inner spur length ratio

In both *Staphisagria* species, floral organs initiate centripetally following an ontogenic spiral. Spiral phyllotaxis is most obvious in the calyx (quincuncial aestivation of the five petaloid sepals; fig. 1A) and in the androecium (c. 40 stamen primordia organized in parastichies; fig. 1B, 1C, 1E, 1F). Petal primordia (most generally eight) initiate after sepals and before stamens. Their development stops soon after initiation, and continues again once carpel

primordia initiate (fig. 1B, 1D–1F). Floral organogenesis is completed when the three free carpels (fig. 1C, 1E–1H) are initiated. Zygomorphy is established after floral organogenesis is completed, through the dorsoventralization of the corolla (fig. 1D): the four dorsal petals develop and differentiate (the two dorsalmost petals get spurred) while the four ventral ones remain at a rudimentary developmental stage, and are very often not noticeable at anthesis. Unicellular trichomes with swollen base are formed on the abaxial side of sepals when stamen primordia initiate (fig. 1B, 1C).

Flowers open and mature on the raceme in an acropetal sequence. In the *S. macrosperma* and *S. picta* plants we cultivated, outer spur (borne on the dorsal sepal) length is c. 2.3 mm and 9 mm, respectively, and inner spur (borne on each of the dorsalmost petals) length is c. 5.8 mm and 7 mm, respectively (fig. 1I–1L). The ratios outer/inner spur length are hence 0.4 and 1.3, respectively, and while the outer spur of *S. macrosperma* is four times shorter than that of *S. picta*, inner spurs are of similar lengths in both species. The outer spur is generally bifid in both species (fig. 1K, 1L), but this feature is mostly visible in *S. picta* flowers (fig. 1L, and P04023155 herbarium specimen). Lateral petals have a narrow claw and a dilated lamina.

Petal morphogenesis: late partial postgenital fusion and parallel formation of notch and spur

The corolla of *Staphisagria* consists of four dorsal petals and four ventral petals (fig. 2A–2J). Front, lateral and upside-down views of the corolla show that the four dorsal petals are the only ones to develop and grow. All four dorsal petals are bilobed (fig. 2B–2D, 2G–2J) and are characterized by the formation of a basal ridge, the height of which corresponds to the height of the fusion among the four petals (fig. 2G–2J). Each of the two dorsalmost petals is basally and laterally fused to the other dorsalmost petal and to the adjacent dorsolateral petal (fig. 2G–2J), and fusion occurs at a late developmental stage, i.e. long after organogenesis is completed and during carpel morphogenesis. Ventral petals tend to become triangular in

shape as development proceeds (fig. 2B, 2D, 2E–2J). In teratological cases in *S. picta* (c. 2% of the dissected floral buds), the two dorsalmost petal primordia fuse at a very early developmental stage, generating a single dorsalmost organ, flanked by dorsolateral petals (fig. 2K, 2L). In rare cases, we observed partial fusions among adjacent ventral petals, and noted that ventral primordia developed into a thin stalk of petaloid tissue.

Hollow outgrowths are formed abaxially in the two dorsalmost petals (fig. 2G–2J). These outgrowths are not located at the exact middle of the dorsal petal limbs. Rather, they are displaced towards the dorsal pole of the flower (fig. 2G–2I). This is especially visible on the two dorsalmost petals (fig. 2I). The dorsolateral petals remain relatively flat, in spite of developing also a ridge (fig. 2G–2J), while the dorsalmost petals get spurred (fig. 2I). In the latter petals, the formation of the spur parallels that of the notch, and the pair of dorsalmost petals with deep notches facing each other creates a shared cavity (fig. 3A–3G). At the stage when hairs develop on the upper limit of the notch, the opposite side of the petal limb becomes winged (fig. 3F). Spur formation in the petals precedes that in the dorsal sepal (fig. 3H–3J), and both inner spurs grow equally (fig. 3K).

All dorsal petals are nectariferous

The anatomical sections of the flower of *S. macrosperma* show that the tip of the outer spur is bifid (fig. 4A), and that the spurs of the two dorsalmost petals are nested within the spur of the sepal (fig. 4B). Results for *S. picta* (not shown) are identical. The basal union of the four dorsal petals is shown in fig. 4C–4E. The four dorsalmost petals are nectariferous (fig. 4B, 4G–4H). The inner epidermis of the spur is covered with large cells colored in red (fig. 4B). Nectariferous tissue was found as well in the dorsalmost petals at the floor of the shared cavity (fig. 4G), hence relatively close to spur entrance, and at the inner epidermis of the pits of the dorsolateral petals (fig. 4H).

Discussion

The complexity of Staphisagria hyperorgan

Staphisagria hyperorgan consists of a pair of spurs nested in a third one. The spurred dorsal petals are nectariferous, basally fused, and share a common cavity at spur entrance. The investigation of flower development was key to identify the different structures involved in the formation of the hyperorgan. Flower development in *Staphisagria* is similar to that in *Delphinium* (Jabbour et al. 2009; Chang et al. 2019; W. Zhang et al. unpublished manuscript). Five sepal quincuncially initiate, and the development of eight petal primordia (exceptionally nine, fig. 2F) stops soon after initiation. This developmental delay observed in petals is common in Ranunculaceae (Payer 1857; Jabbour et al. 2009; Zhao et al. 2011, 2012) and is also a feature of other plant families (Payer 1857; Sattler 1973).

In a first stage, the four dorsal petals develop in a similar way, and exhibit an adaxial basal ridge (growing into a scale). A small hollow depression is delimited adaxially by this scale and abaxially by the petal labium. In the postgenitally and basally fused dorsal petals, the height of the fused tissue does not exceed the position of the scales. Basal connation is always observed between the two dorsalmost petals, and often observed between three out of the four dorsal petals, the unfused one being the eighth initiated petal (not the seventh), so the farthest spatially to the dorsalmost ones on the ontogenic spiral. In rare cases, only the pair of dorsalmost petals were basally fused.

In a second stage, the hollow depression between the scale and the labium gets deeper in the two dorsalmost petals, leading to the formation of spurs on the abaxial side of the labium.

In a third stage, the dorsal sepal gets spurred, and this outer spur includes the pair of inner spurs. Our measurements for outer spur length (*S. macrosperma*: 2.3 mm, and *S. picta*: 9 mm) are in agreement with those of Léotard (2002) who reported, based on a much larger

sampling, that outer spur length was on average 3.3 mm ([2; 4.5], standard deviation (s.d.) = 0.8) and 7.7 mm ([4.5; 11.3], s.d. = 1.1), for *S. macrosperma* and *S. picta*, respectively.

The fourth and last stage corresponds to the inner and outer spur growth and to the resupination of the dorsolateral petals (fig. 1I, and fig. 2D in Jabbour and Renner 2011a).

During spur development, each of the two dorsalmost petals becomes notched, and both notches facing each other form a cavity shared by both petals. This observation was already illustrated by Payer (1857), analyzing floral development in *S. picta* (<https://digi.ub.uni-heidelberg.de/diglit/payer1857bd2/0067/image>).

Anatomical investigation of preanthetic *Staphisagria* flowers revealed that nectariferous tissues were present on the inner epidermis of the inner spurs as shown in other Delphinieae taxa (Kosuge and Tamura 1988; Erbar et al. 1998; Antoń and Kamińska 2015), but secretory tissues were also found at the floor of the shared cavity (hence at the entrance of the spur) and on the inner epidermis of the small hollow depression in each of the dorsolateral petals. In the three flowers investigated, the four dorsal petals contain secretory tissues. This is an interesting finding as dorsolateral petals are usually regarded in the Delphinieae as having a role in attracting the pollinators and acting as a landing platform. Kosuge and Tamura (1988) already noted that in Ranunculaceae, nectar could be secreted and stored in small and not-so-deep cavities at the adaxial surface of the petal labium.

Hyperorgan organization in Delphinieae is unique among angiosperms, as it possesses a pair of nectariferous spurs included in a third hollow structure. Other flowering plant taxa present pairs of spur, but they are not nested within an outer spur (e.g. *Satyrion* Sw., Orchidaceae), or not nectariferous (*Diascia* Link & Otto, Scrophulariaceae).

Hyperorgan disparity in Delphinieae

This is our second attempt to categorize Delphinieae hyperorgan. Compared with the first one (Jabbour and Renner 2012a), in addition to the number, arrangement (e.g. nested spurs) and broad shape of organs involved, we consider here the basal fusion among the dorsal petals and the presence of notches on the dorsalmost petals. This high level of synorganization is uncommon in angiosperms, where it is thought to be low in flowers with spiral phyllotaxis (Endress 1990).

Three categories of hyperorgan can be distinguished in Delphinieae when considering the morphology and arrangement of the pair of dorsalmost petals and of the dorsal sepal (fig. 5).

We here define three hyperorgan categories:

(1) the “Delphinium” type – c. 300 species – two notched, basally-fused nectar spurs included in an outer spur. This type is found in *Staphisagria* and *Delphinium* subg. *Delphinium*, *D.* subg. *Anthriscifolium* (W.T.Wang) Wei Wang, *D.* subg. *Delphinastrum* (DC.) Peterm., and *D.* subg. *Oligophyllum* Dimitrova (following the classifications of Malyutin (1987) and Xiang et al. (2017)). Basal fusion of the two inner spurs was observed on many type specimens (suppl. fig. 1, note 1). Taxa with the shortest outer spur (*Staphisagria*), and the longest (*D. leroyi* Franch. ex Huth, suppl. fig. 2), are included in this category. More investigations are needed to determine the proportion of taxa from the “Delphinium” type for which dorsolateral petals are nectariferous and/or basally postgenitally fused (note 1, suppl. fig. 1) and/or resupinated (W. Zhang et al. unpublished manuscript).

(2) the “Consolida” type – c. 60 species – a single nectar spur included in an outer spur or in a hood-shaped dorsal sepal. The inner spur results from the total postgenital fusion of the two dorsalmost petal primordia. This type is found in *D.* subg. *Consolida* (DC.) Gray (P.-E. Du Pasquier et al. unpublished manuscript).

(3) the “Aconitum” type – c. 300 species – two nectar spurs borne on long stalks included in a hood-shaped dorsal sepal, which can be more (*A.* subg. *Lycocotnum* (DC.) Peterm.) or less (*A.*

subg. *Aconitum*, A. subg. *Galeata* (Rapaics) Y.Hong & Q.E.Yang, A. subg. *Fletcherum* (Tamura) Y.Hong & Q.E.Yang) basally constricted, and more (the subgenera just cited hereabove) or less (*Gymnaconitum*) covering the inner structures.

The shared cavity of Delphinieae hyperorgan could be considered as a floral parlor *sensu* Leppik (1964). According to Leppik, a parlor “is a reception room for floral visitors in a zygomorphic flower. It has a landing place, entrance, guiding signs to nectar deposits and other accommodations for welcomed visitors. But it is frequently closed for unwanted visitors”. Regarding *Aconitum* flower, he adds: “Although the monkshood flower is open to all insects, not all visitors in the parlor can locate and reach the nectar deposits hidden in coiled nectaries”. We foresee that the lateral and ventral sepals, and the dorsolateral petals all participate in shaping the entrance of the flower, being a broader floral parlor leading to the parlor of the dorsal petals (suppl. fig. 3).

Flowers of Delphinieae basically consist of nested spurs (spur(s) of the dorsal petal(s) included in the spur or hood of the dorsal sepal) and nested floral parlors. The first, and most obvious floral parlor, is the one that is created by the notches of the dorsalmost petals facing each other in the flowers from the “Delphinium” type. Both dorsal spurs seem there to create a single hollow structure. In the “Consolida” type, the dorsal spur is indeed a single hollow structure, which does not result from a notching that would extend to the tip of the spur, rather from the postgenital fusion of the pair of dorsal petal primordia. The second floral parlor is the one generated by the ventral and lateral sepals, and by the dorsolateral petals, arranged in such a way that they form a cup imposing a certain movement to the visitor, and this in melittophilous, sphingophilous and ornithophilous flowers.

Delphinieae flowers, especially those from the “Aconitum” and “Consolida” types, show a tubular organization, due to this second floral parlor. However, and in contrast with

sympetalous flowers (Endress 2001), the sexual organs in Delphinieae are not located down the tube, but at the entrance of the tube. In addition, floral tubes in sympetalous flowers end in the floral base, which has a robust architecture, while floral spurs usually have no reinforced end (Endress 2001). This is not the case in Delphinieae, as the inner spurs might be protected by the outer spur or hood from herbivores or from large variations in temperature and humidity.

Characterizing in further details the disparity of Delphinieae hyperorgan would require to consider the shape of all perianth parts, the relative proportions of the notch length, the inner and outer spur length, and the position of the nectariferous tissue. The limit is now of taxonomic nature, and although small subclades of Delphinieae were recently revised (*Staphisagria* (Jabbour and Renner 2011a), *A.* subg. *Lycotconum* (Hong et al. 2017), *Gymnaconitum* (Wang et al. 2013), *D.* subg. *Delphinium* (Du Pasquier et al. study under way), *D.* subg. *Anthriscifolium* (W. Zhang et al. unpublished manuscript), *D.* subg. *Consolida* (P.-E. Du Pasquier et al. unpublished manuscript)), the species-rich *A.* subg. *Aconitum*, *D.* subg. *Delphinastrum* and *D.* subg. *Oligophyllon* are still in need of important taxonomic (and nomenclatural) reevaluation. This taxonomic work that is still to be undertaken will necessarily have to characterize traits that have never been included in descriptions and keys so far (Huth 1895; Davis 1965; Munz 1967), namely, the length of inner spurs and the length of the notch on the inner spurs. *Staphisagria macrosperma* is known as the species with the shortest outer spur in the tribe Delphinieae (*D. leroyi* is the one with the longest outer spur, suppl. fig. 2, Johnson 2001; Chartier et al. 2016). While this is true for the outer spur, inner spurs are not exceptionally short in this species. The inner spurs are the structures that likely coevolve with the pollinators and that should be considered as being highly taxonomically discriminant.

The function of Delphinieae hyperorgan is likely a combination of attraction, rewarding, and protection of visitors and pollinators. It appeared possible that the cavity shared by the spurred petals (the floral parlor) could serve as a shelter for small insects (FJ, pers. obs.; suppl. fig. 2), probably also feeding on nectar. Studies about the pollination ecology of Delphinieae flowers are not scarce (e.g. Macior 1975; Bosch et al. 1997; Johnson 2001; Liao et al. 2007; Wang et al. 2017; Jacquemart et al. 2019), and provide solid bases for future investigations that will have to integrate the morphological features analyzed here above.

Possible evolutionary trends and developmental pathways

We here discuss the evolution of Delphinieae hyperorgan, and propose a developmental scenario leading to the diversity of floral organizations observed in this tribe. The evolutionary and developmental scenarios presented here are updated versions of those presented by Jabbour and Renner (2012a) and Jabbour et al. (2009), respectively.

The hypothetical common ancestor of Nigelleae and Delphinieae likely had a bipartite perianth consisting of petaloid sepals and c. 8 nectariferous and free petals, and its floral organs were arranged following a spiral phyllotaxis. In the lineage leading to Delphinieae, a major developmental change occurred, viz. calyx and corolla dorsoventralization, leading to zygomorphic flowers. All flowers of Delphinieae lack ventral petals at the adult stage (although four petal primordia are initiated ventrally). Around half of the species within the tribe have four dorsal petals (two dorsalmost spurred, and two dorsolateral), the other half of the species have only one pair of developed petals, in dorsal position. Due to the topology of the tree resulting from the most recent phylogenetic studies, the ancestor of Delphinieae could have had two or four dorsal petals (both options equally parsimonious).

In few cases (c. 60 species in *D.* subg. *Consolida* (P.-E. Du Pasquier et al. unpublished manuscript)), flowers are tremendously reduced in size and organ number, and present only a

single petal, resulting from the postgenital fusion of the two dorsalmost petal primordia (Jabbour and Renner 2012a; Chang et al. 2019). In marginal cases, flowers are peloric and lack a corolla at the adult stage (*D. turcicum*, in *D. subg. Delphinium*: Espinosa et al. 2017; *D. ecalcaratum*, in *D. subg. Anthriscifolium*: Ding et al. 1981; W. Zhang et al. unpublished manuscript).

The evolutionary trend towards petal fusion (generally basal, but total in the subgenus *Consolida*) in Delphinieae is to be highlighted, as fusion among floral organs is often linked with the presence of whorls (Endress 2001; Endress and Doyle 2009).

Flowers in Ranunculales show no or partial postgenital fusion (Endress and Doyle 2009). In Ranunculaceae, fusion among floral organs is recorded mostly in gynoeceium (Hoot 1991; Endress and Doyle 2009). Based on the most recent phylogenetic framework available for Delphinieae, late and partial postgenital fusion evolved once (with a reversion to choripetaly in each of the lineages leading to *Aconitum* and *Gymnaconitum*) or twice (in each of the lineages leading to *Staphisagria* and *Delphinium*) in the tribe, and both scenarios are equally parsimonious. In *Delphinium*, postgenital fusion even became total (and early) in the lineage leading to *D. subg. Consolida* (fig. 5). This is not the first report of fusion among the dorsal petals in *Staphisagria* and *Delphinium* (Verlaque and Aboucaya 2001; Jabbour and Renner 2012a), and a study on an exhaustive sampling of Delphinieae is required to investigate the degree and timing of petal fusion (e.g. postgenital early in *D. subg. Consolida*, postgenital late in *Staphisagria*).

Additionally, anatomical investigations are required to document the diversity of nectariferous tissue location within the Delphinieae corolla, and to check whether secretory tissues are found not only in the inner epidermis of the spurs, but also at the floor of the shared cavity and in the small hollow cavity at the base of the dorsolateral petals. These findings will be pivotal to revise our ideas about pollinators' behavior when foraging for

nectar in a Delphinieae flower. If spur length coevolves with the pollinators, this coevolution may not be as tight as expected (see Whittall and Hodges 2007; Vlašánková et al. 2017), as the inner spurs would not filter as strongly for the pollinators able to reach the nectar. In addition, in Delphinieae, the developmental background of the coordinated dorsal petal and dorsal sepal deformation still has to be investigated, using for instance floral mutants lacking corolla.

Genes involved in floral zygomorphy, petal fusion, and nectar spur development have been characterized in some species, that could fuel a candidate gene approach for deciphering the genetic origin of the nectariferous hyperorgan of Delphinieae. As for zygomorphy, the recurrent recruitment of *CYCLOIDEA*-like genes in various taxa has been observed (for review, Hileman 2014). In Ranunculaceae, *CYC*-like genes have been characterized in various species including many Delphinieae (Jabbour et al. 2014). Two paralogous gene lineages have been found in the family with an additional duplication in each *CYC*-like lineage in the Delphinieae ancestor. The expression of *CYC*-like genes during floral development of selected Delphinieae species reveals species- and organ/stage-specific patterns, suggesting post-duplication species-specific subfunctionalisation (Jabbour et al. 2014). Functional analyses are required to assess the morphogenetic roles of these paralogs. Boundary genes that establish delimitation between meristems and organs and between organs by repressing growth can be considered good candidates for the evolution of petal fusion. The NAM/CUC3 subfamily of the NAC-domain transcription factor family has been especially investigated but with variable results in the different eudicot species studied to date (Rebocho et al. 2017; Preston et al. 2019; Phillips et al. 2020). The involvement of *KNOX* genes in the formation of ectopic outgrowths on the corolla tube has been demonstrated in *Antirrhinum majus* L. (Plantaginaceae), making these genes possible candidates for spur formation (Golz et al. 2002). Indeed, *KNOX* genes appear involved in spur formation in the related species *Linaria*

vulgaris Mill. (Box et al. 2011). In contrast, there is no evidence of a role of *KNOX* genes in the development of the spurs in the Ranunculaceae genus *Aquilegia* L. that has evolved spurs independently from the Plantaginaceae (Yant et al. 2015). In the latter, the role of a member of the TCP transcription factor family, TCP4 and of auxin-related genes have been strongly suspected (Yant et al. 2015). Indeed, two *AUXIN RESPONSIVE FACTOR* genes, *ARF6* and *ARF8* have recently been shown to promote cell elongation and the maturation of the nectary during spur formation in *Aquilegia coerulea* E.James (Zhang et al. 2020). In this species and in contrast with core eudicots where a role of the *YABBY* gene *CRABS CLAW* has been found (Lee et al. 2005), nectary formation seems to involve genes of the *STYLISH* transcription factor gene family (Min et al. 2019). Interestingly expression of *STY1* has been observed in the spurred petals of *Delphinium exaltatum* Aiton and of the Berberidaceae *Epimedium grandiflorum* C.Morren, that are independent cases of nectariferous spur evolution in the Ranunculales (Min et al. 2019).

Zygomorphy, partial petal fusion, presence of spurs and nectaries are all floral traits that are highly homoplastic among angiosperms, and it is worth underlining that candidate approaches addresses the concepts of deep homology and/or of evolutionary tinkering from a toolkit of transcription factors and pathways. Increasing knowledge about the origin of these homoplasious traits are of great interest for understanding constraints and prospects in floral evolution.

Conclusions

Floral synorganization is highly complex in *Staphisagria*, and involves flower dorsoventralization, nesting of spurs, postgenital fusion of petals, and the formation of a shared cavity possibly disrupting the tight coevolutionary relationship between these spurred flowers and their pollinators that has been so far hypothesized. The disparity of the

Delphinieae hyperorgan was discussed in a phylogenetic framework, and likely developmental pathways leading to the diversity of floral phenotypes were proposed. The flower of Delphinieae includes nested spurs and nested floral parlors, and these key innovations, unique in angiosperms, probably led to the diversification of this species-rich tribe in the Northern Hemisphere. We expect our study to inspire research in the fields of taxonomy, evo-devo, and pollination ecology.

Note 1 Species of *Delphinium* for which there is evidence that dorsalmost petals are notched and basally fused, and that a scale and a hollow depression can be found at the base of dorsolateral petals. Species name, barcode of the type specimen kept at P Herbarium, specification whether the notch and the basal fusion (indicated by a scar) of dorsalmost petals (^a), and/or the scale of dorsolateral petals (^b) are visible on the illustration found on the herbarium sheet (suppl. fig. 1). High-resolution images of the specimens can be accessed using the link: <https://science.mnhn.fr/institution/mnhn/collection/p/item/search>.

D. albiflorum, P00195724^a; *D. albocoeruleum*, P00195731^b; *D. altissimum*, P00195749^b; *D. batangense*, P00195918^{ab}; *D. caeruleum*, P00197011^{ab}; *D. ceratophorum*, P00197081^b; *D. chrysotricum*, P00197116^b; *D. hirticaule* var. *coelestinum*, P00197486^b; *D. dasystachyon*, P00197161^a; *D. davidi*, P00197163^b; *D. fargesii*, P00197304^{ab}; *D. hirticaule* var. *hirticaule*, P00197478^{ab}; *D. incisum*, P00198623^{ab}; *D. lacostei*, P00198649^{ab}; *D. lanigerum*, P00198661^a; *D. lankongense*, P00198659^b; *D. maackianum*, P00198688^b; *D. maximoviczii*, P00198693^b; *D. mosoynense*, P00198714^{ab}; *D. oliganthum* (determined as *D. likiangense*), P00198675^{ab}; *D. orthocentrum*, P00198833^a; *D. pachycentrum* var. *pachycentrum*, P00198837^{ab}; *D. penicillatum*, P00198846^{ab}; *D. ponticum*, P00195723^a; *D. pylzowii*, P00198967^{ab}; *D. quercetorum*, P00198968^a; *D. siwanense*, P00198553^{ab}; *D. souliei*, P00201009^{ab}; *D.*

tatsienense, P00201068^b; *D. thibeticum* var. *subintegrum*, P00201112^b; *D. trochophorum*, P00201173^a; *D. trifoliolatum*, P00201192^{ab}; *D. tuberosum*, P00201214^{ab}; *D. yunnanense*, P00201265^{ab}.

Acknowledgements

We acknowledge the MNHN seed bank and the MNHN Ecole de Systématique garden, the Plateau technique de microscopie électronique (UMR 7245 CNRS/MNHN; Géraldine Toutirais), and the Conservatoire botanique national méditerranéen de Porquerolles. The MNHN gives access to the collections in the framework of the RECOLNAT national Research Infrastructure. We thank Catherine Damerval for critical input on an earlier draft of the manuscript, Guillaume Léotard for providing his MSc thesis, Sonia Yakovlev for confirming the diploidy of *S. picta*, Andriy Novikov for taking the pictures of the trans-illuminated herbarium specimens, and Eugénie Carnero-Diaz and Isabel Le Disquet for taking pictures of the anatomical sections. We also thank Julie Kang (Editor) and two anonymous reviewers for their critical and helpful comments. This work was funded by the MNHN ATM (Action Transversale du Muséum) Blanche and by the Institute of Systematics, Evolution, Biodiversity (ISYEB) at the National Museum of Natural History, Paris. The field trip to Malawi was funded by the National Geographic Society (grant #9989-16 to FJ).

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Figure captions

Fig. 1 Developmental sequence of the floral meristem of *Staphisagria*. Sequences of both species are identical. SEM pictures with a black and white background were taken from *S. macrosperma* and *S. picta* buds, respectively. *A*, Floral meristem showing the quincuncial organization of the five sepals, at the stage of stamen initiation. The second (hence dorsal) sepal in the initiation sequence is at the bottom. *B*, Floral meristem at an equivalent developmental stage with the second, fourth and fifth sepals sectioned, showing the four dorsal petals (asterisks) and the first two primordia of each stamen parastichy. *C*, Floral meristem at the completion of organogenesis. Three carpel primordia initiate at the top of the meristem. *D, E, F*, Upside-down and two lateral views of the floral bud at successive developmental stages, showing the dorsal petals (asterisks) which development is delayed compared with the development of stamens and carpels. Ventral petals are indicated with arrowheads. *G*, Three free carpels. *H*, Longitudinal section of a carpel showing ovule placentation. *I*, Flower of *S. macrosperma*, with five petaloid sepals, two resupinated dorsolateral petals, the limb of which partly covers the sexual organs, and two dorsalmost petals. The tips of the spurs of the latter petals are nested in the spur of the dorsal sepal. *J, K*, Lateral and back view of a *S. macrosperma* flower, showing the tips of the spurs of the dorsalmost petals pointing upwards (arrows) in the bifid spur of the dorsal sepal. *L*, Flower of *S. picta*. Pet.: petal. Scale bars: *A*, 150 μm ; *F, H*, 200 μm ; *B, C, D, E*, 250 μm ; *G*, 1 mm; *I, J, K, L*, 1 cm.

Fig. 2 Developmental sequence of the corolla of *Staphisagria*. SEM pictures with a black and white background were taken from *S. macrosperma* and *S. picta* buds, respectively. *A, B, C, D*, upside-down and lateral views of successive developmental stages. *E, F, G, H, I, J*, Front views of successive developmental stages. *A, B*, Upside-down views of the floral meristem

showing the dorsal (asterisks) and ventral (arrowheads) petals at successive developmental stages. Dorsal petals develop while ventral ones remain in a rudimentary developmental stage. *C*, lateral view showing four bilobed basally fused dorsal petals. Only two out of the four ventral petals are visible. *D*, Upside-down view of the floral meristem showing the dorsal (asterisks) and ventral (arrowheads) petals, at a stage when the dorsalmost petals get spurred. *E*, *F*, Front views of the corolla (all other floral organs removed) at successive developmental stages, showing the developing four dorsal petals. Note the five ventral petals (arrowheads) in *F*. *G*, *H*, *I*, *J*, Successive stages of the developing corolla. The bilobed, basally fused dorsal petals present a basal ridge. The limb of all of them shows a basal depression, which develops into a spur in the pair of dorsalmost petals. *K*, *S. picta* floral bud showing the pair of dorsalmost petal primordia getting fused. *L*, At a later stage, both dorsal petals are totally fused into a single four-lobed spade-like structure. Scale bars: *E*, *K*, *L*, 200 μm ; *A*, *C*, *F*, 250 μm ; *B*, *D*, *G*, *H*, 500 μm ; *I*, *J*, 1 mm.

Fig. 3 Developmental sequence of one of the two dorsalmost petals and of the dorsal sepal of *Staphisagria*. SEM pictures with a black and white background were taken from *S. macrosperma* and *S. picta* buds, respectively. *A*, *B*, *C*, *D*, *E*, *F*, Successive developmental stages of one of the two dorsalmost petals getting notched and spurred. Note the scars on both sides of the short stalk indicating basal fusion with the adjacent petals. In *F*, the arrowhead points at the hairs developping on the upper limit of the notch. *G*, View on the abaxial side of the pair of dorsalmost petals. The black and white arrows indicate the fusion of the stalks, and the width of the shared cavity, respectively. *H*, *I*, *J*, *K*, Successive developmental stages of one of the two dorsalmost petals in *S. picta* (*H*, *I*, *J*; including the dorsal sepal getting spurred) and *S. macrosperma* (*K*). In *K*, although underdeveloped, one ventral petal is visible (arrowhead). Scale bars: *A*, *B*, *C*, 250 μm ; *D*, *E*, *H*, *I*, 500 μm ; *F*, *G*, *J*, 1 mm; *K*, 2 mm.

Fig. 4 Transverse sections of a preanthetic floral bud of *Staphisagria macrosperma*, showing the anatomy of the floral structures. *A*, Transverse section of the bifid tip of the dorsal sepal spur. *B*, Transverse section of the dorsal sepal spur, closer to the floral receptacle, showing the pair of inner spurs. *C, D, E, F*, Transverse sections of the floral bud at four ascending levels. *S*₂ indicates the dorsal sepal, second in the initiation sequence. Green arrowheads indicate the fusion between adjacent dorsal petals. Note the inner spurs (*C, D*), the shared cavity at the entrance of the spurs and the hollow depression on the dorsolateral petals (*E, F*). *G, H*, Magnified views of one dorsal and one dorsolateral petal. Secretory tissues (large cells colored in red) are located at the floor of the shared cavity and on the walls of the hollow depression, respectively. Scale bars: *F*, 50 μm ; *E*, 100 μm ; *A, B*, 200 μm ; *C, D, G, H*, 500 μm .

Fig. 5 Disparity of the Delphinieae hyperorgan in a phylogenetic context and developmental pathway possibly explaining the morphogenesis of each of the types of hyperorgan. The well-supported topology synthesizes our current knowledge about phylogenetic relationships in Delphinieae (Jabbour and Renner 2011*b*, 2012*b*; Wang et al. 2013; Espinosa et al. 2017; Hong et al. 2017; Xiang et al. 2017; Zhai et al. 2019). The type of hyperorgan is indicated in the black boxes. Genera and subgenera are specified in the grey text boxes. Within the spurred or hooded dorsal sepal (purple), the dorsalmost petals (green) present a shared cavity, or are borne on long stalks, or are totally fused. Color codes for the developmental pathway: Calyx: light blue: undifferentiated petaloid sepal, purple: dorsal sepal, light green: lateral sepal, mauve: ventral sepal. Corolla: orange: underdeveloped petal, red: developing petal, blue: undifferentiated nectariferous petal, light green: dorsolateral petal, dark green: spurred

and nectariferous dorsal petal. Basally (and facultative) and totally fused organs are joined with thin and thick links, respectively. Yellow: androecium. Pink: gynoecium.

Supplementary figures

Suppl. Fig. 1 Herbarium specimens of *Delphinium* including an illustration showing that dorsalmost petals are notched and basally fused (scar indicated by the green arrow), and that a scale can be found at the base of dorsolateral petals (red arrow). *A*, specimen of *D. likiangense* Franch., barcode P00198675. *B*, type specimen of *D. souliei* Franch., P00201009. *C*, *D*, Magnification of a portion of the illustration from *A* and *B*, respectively. High-resolution images of the specimens can be accessed using the link:

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Suppl. Fig. 2 Flower of *D. leroyi* photographed on the Nyika Plateau, Malawi, in May 2019. *A*, Lateral view of the flower. The inner spurs can be distinguished by holding the outer spur against the light. *B*, Same flower, with all perianth organs removed, except one dorsal petal. Two beetles from the same species were found at the bottom of the shared cavity. *C*, Folding and compressing the inner spur results in a droplet of nectar forming at the bottom of the shared cavity. One beetle is in contact with this droplet of nectar. Scale bars: *A*, *B*, *C*, 1 cm. Photograph credits: Florian Jabbour.

Suppl. Fig. 3 The floral parlor of Delphinieae flowers, composed of the cup-like shaped calyx, from the widest to the narrowest. Herbarium specimens were trans-illuminated to reveal the structures hidden in the dorsal sepal. *A*, *Aconitum ambiguum* Rchb., barcode P00601133. *B*, *A. macrorhynchum* Turcz., P00201813. *C*, *A. sinomontanum* Nakai, P00201961. *D*, *Delphinium anthoroideum* Boiss., P00195782. *E*, *D. macrocentrum* Oliv., P02379750. *F*, *D. nanum* DC., P02819166. High-resolution images of the specimens can be accessed using the link: <https://science.mnhn.fr/institution/mnhn/collection/p/item/search>.

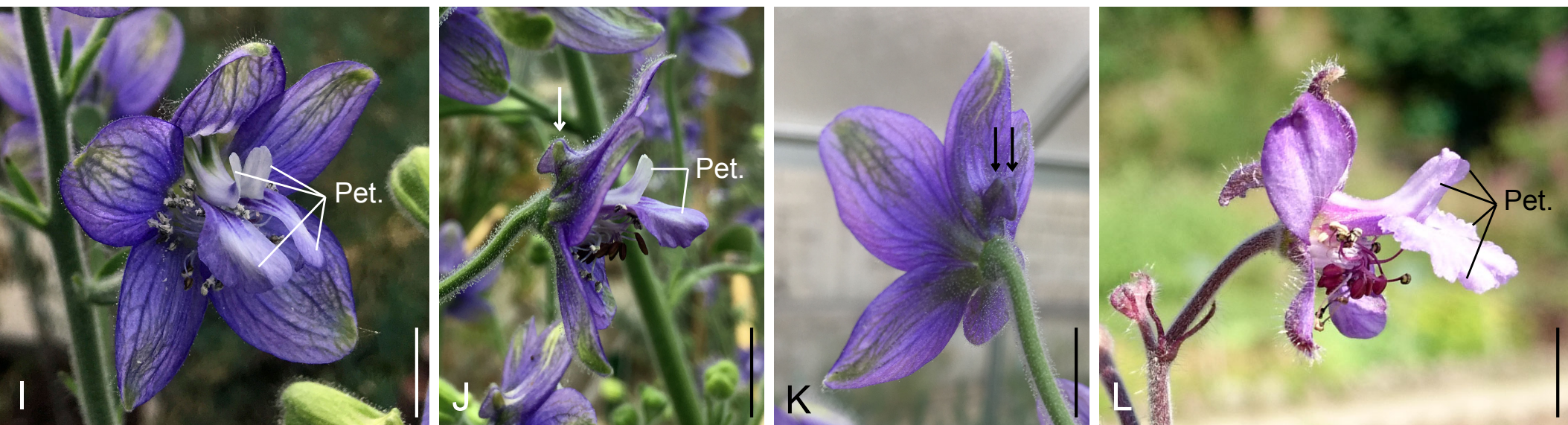
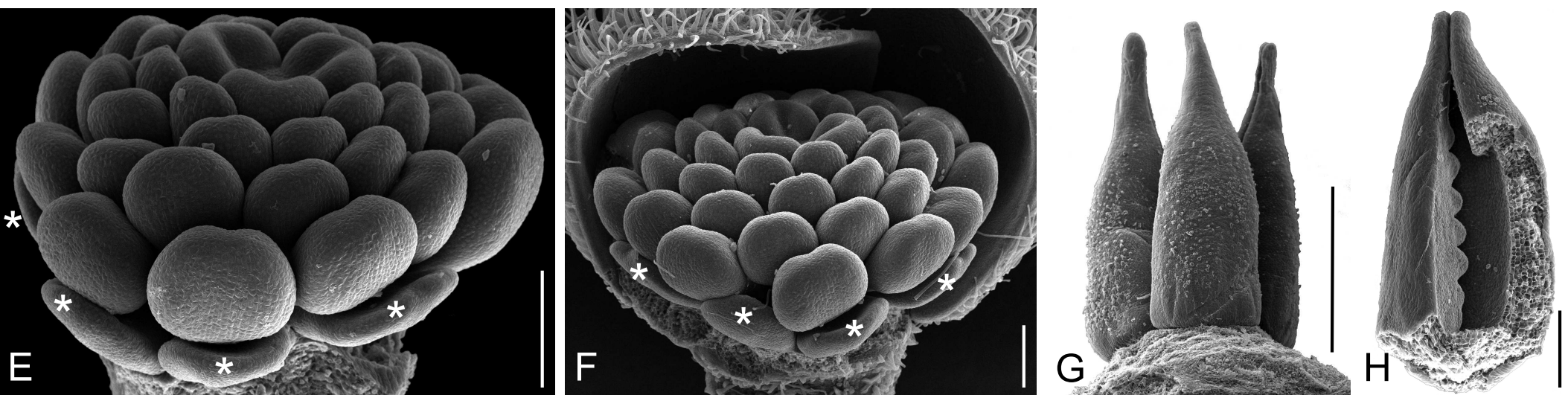
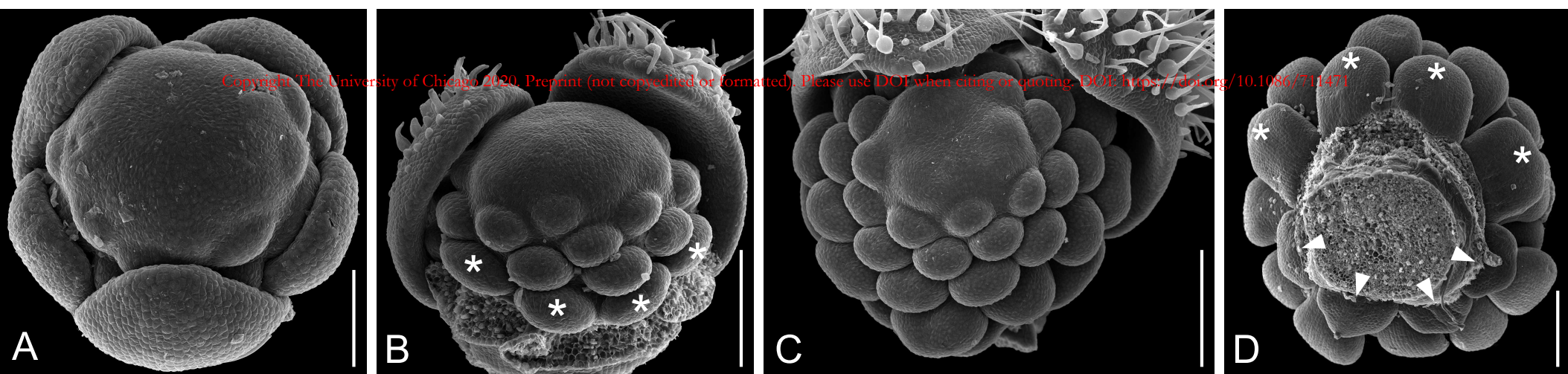


Figure 2

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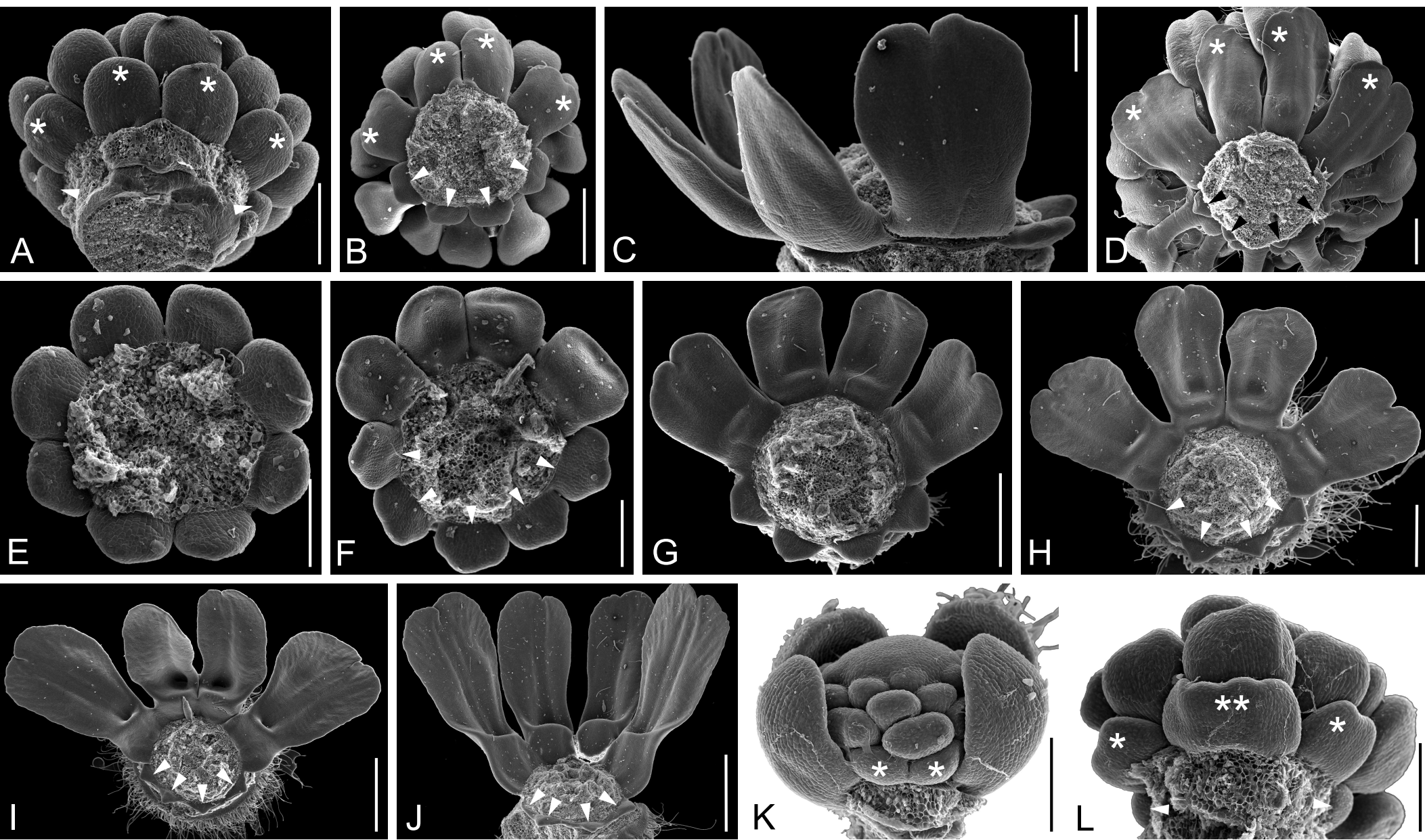


Figure 3

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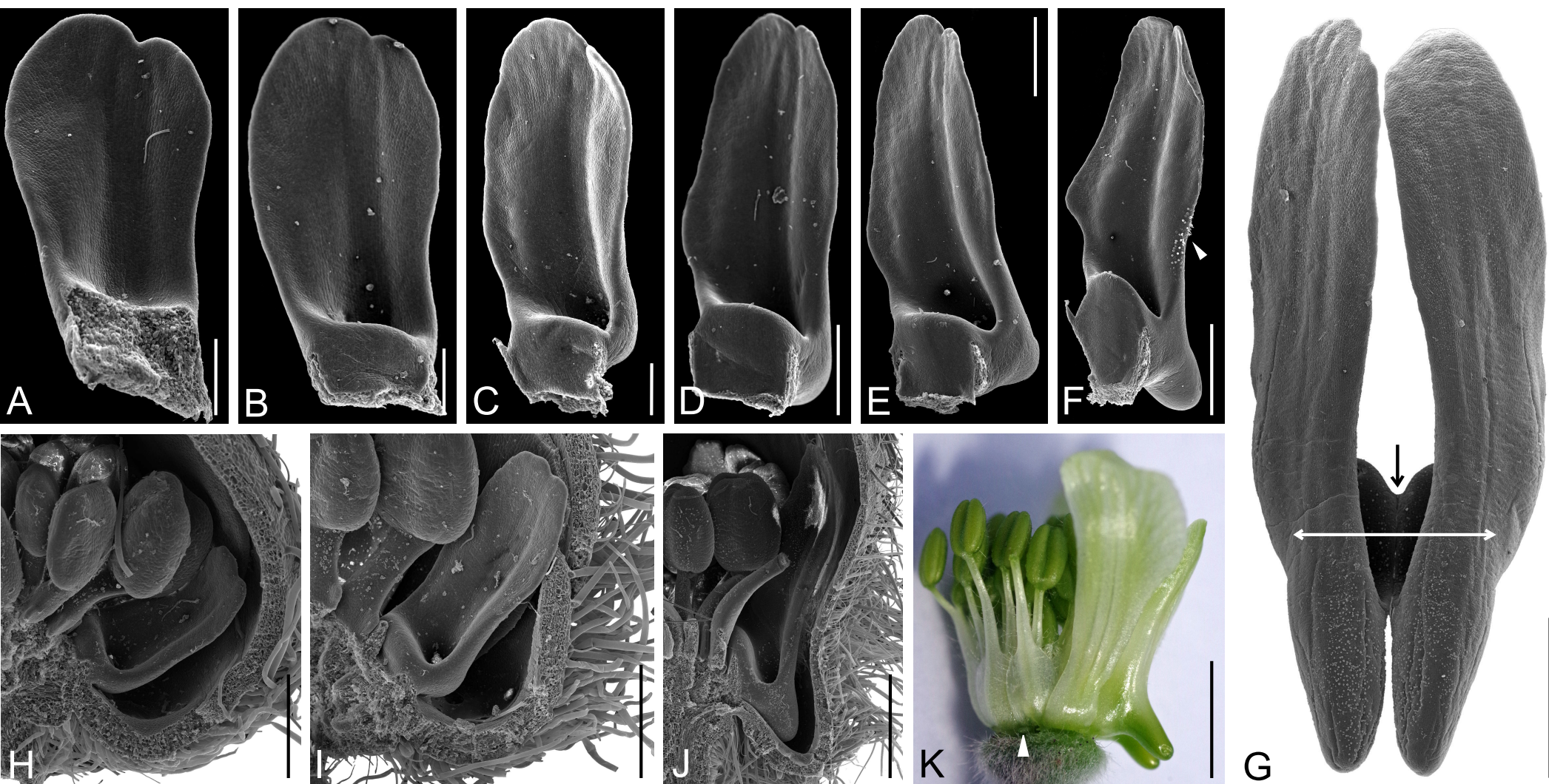


Figure 4

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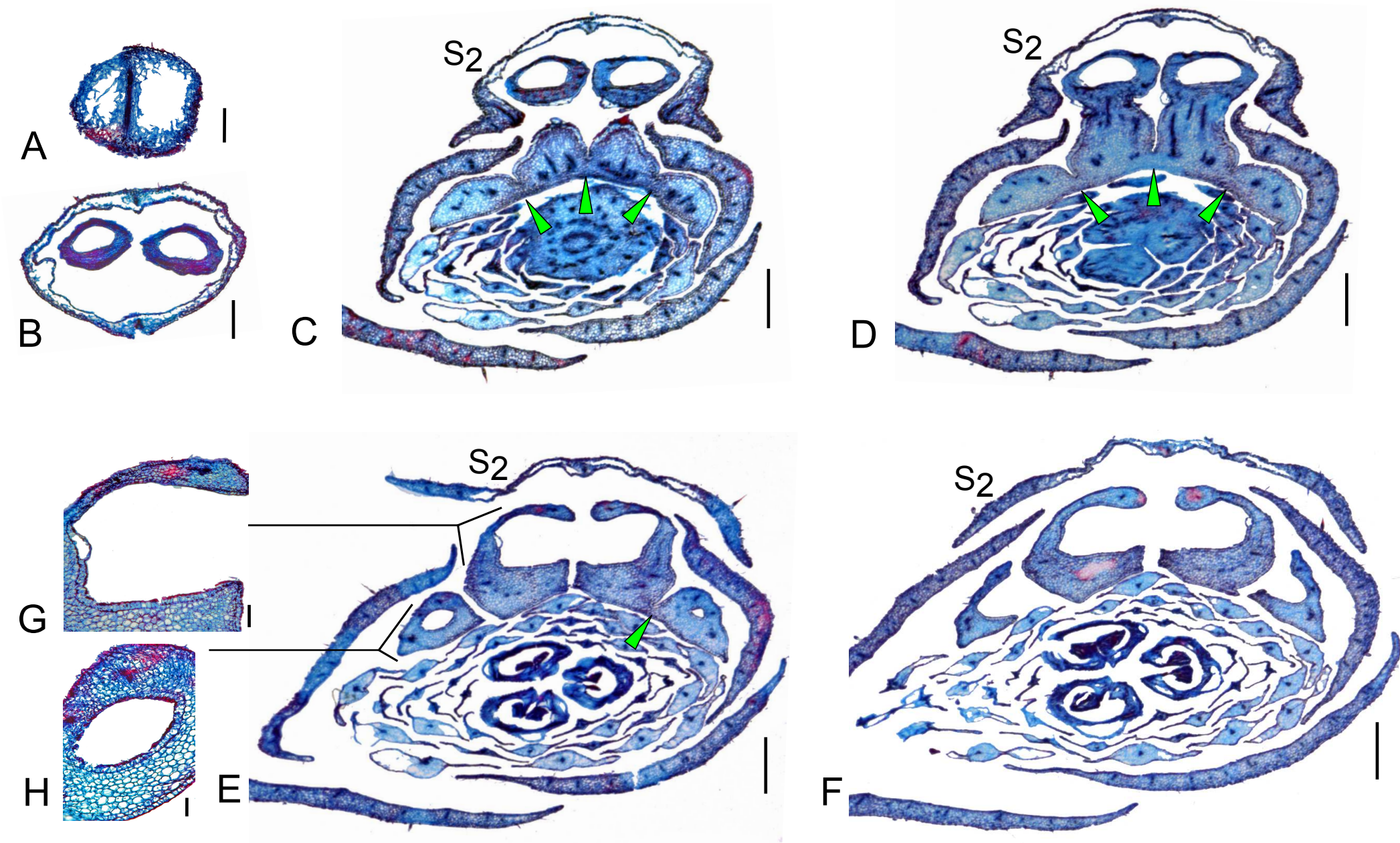
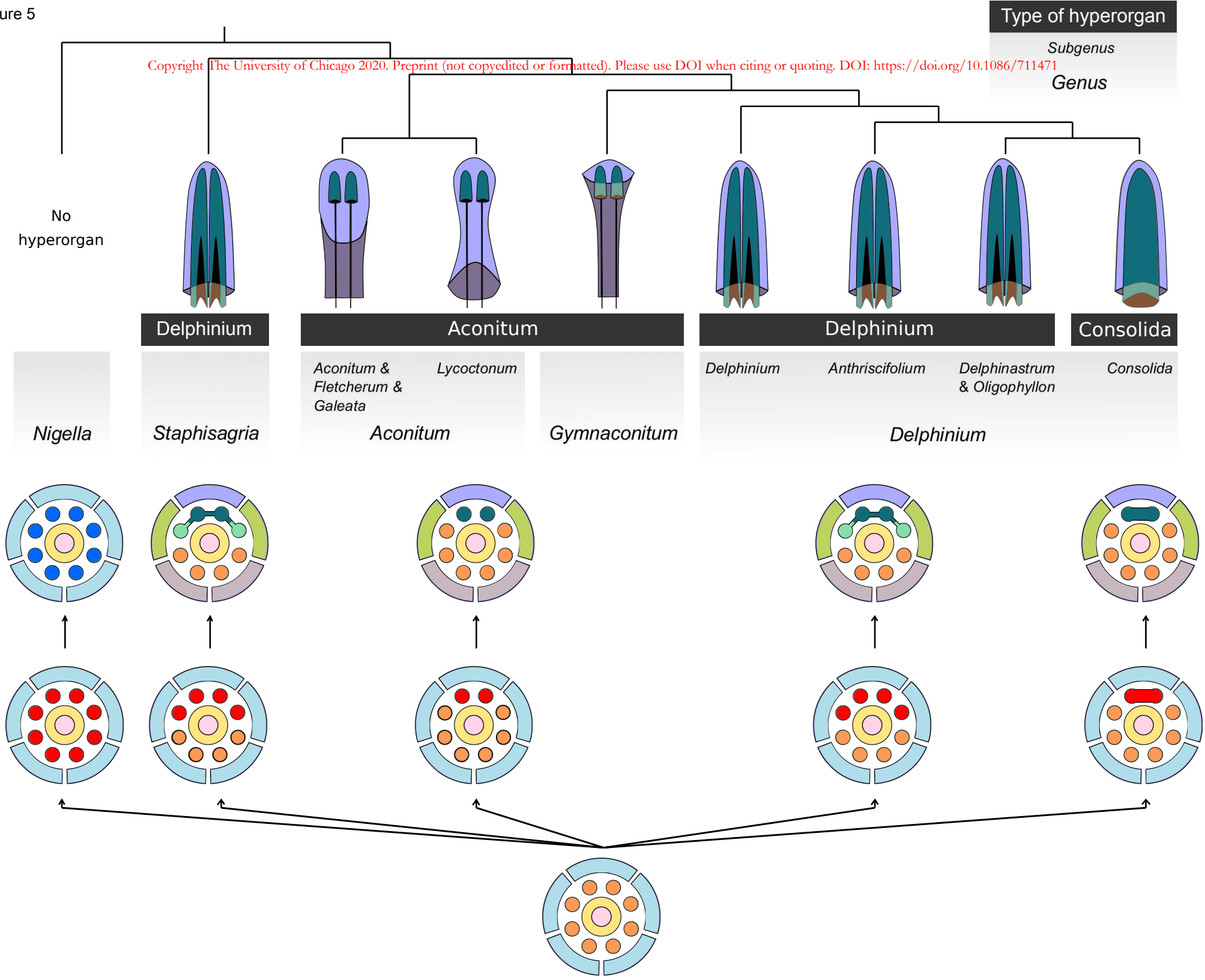
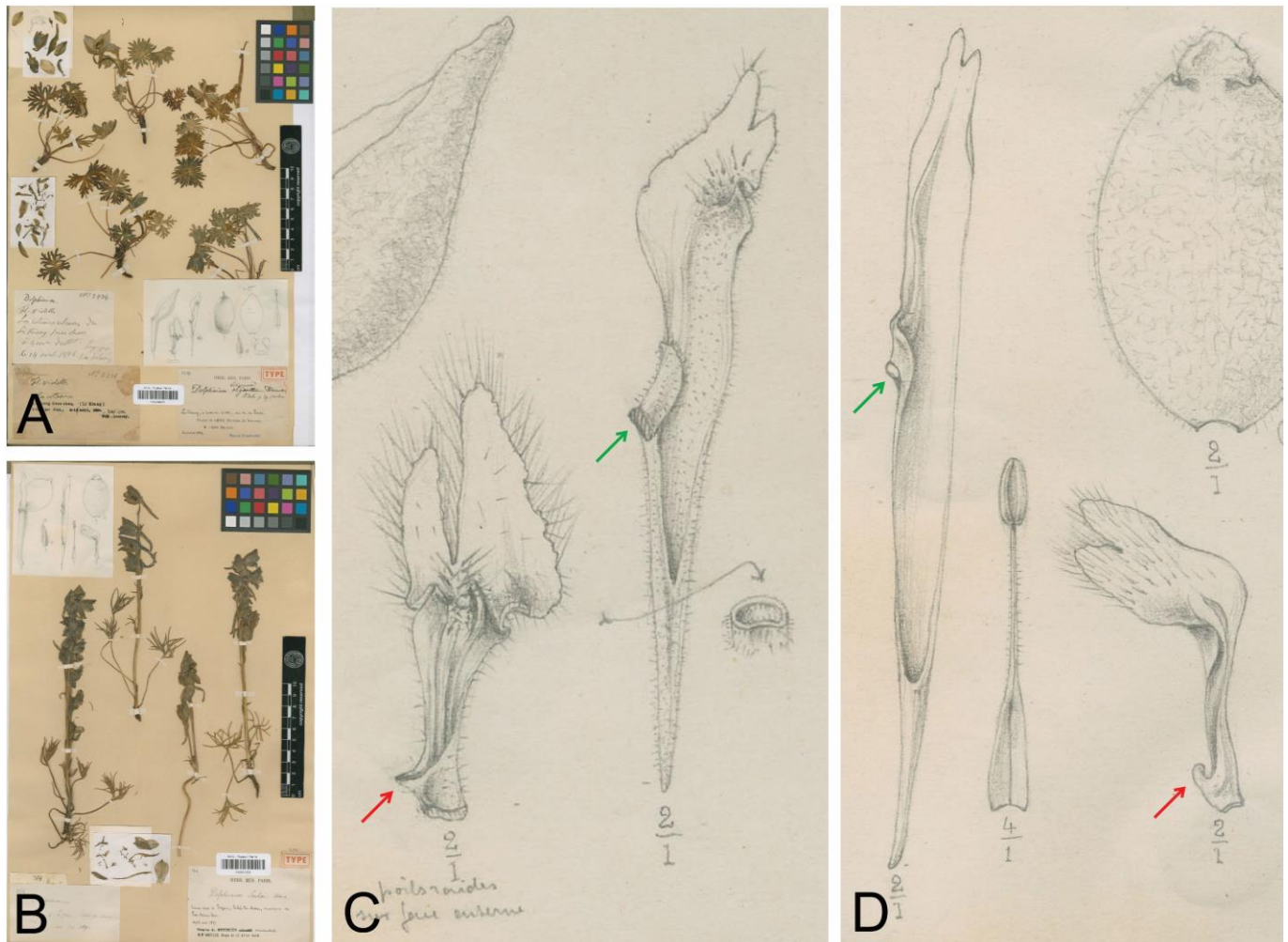


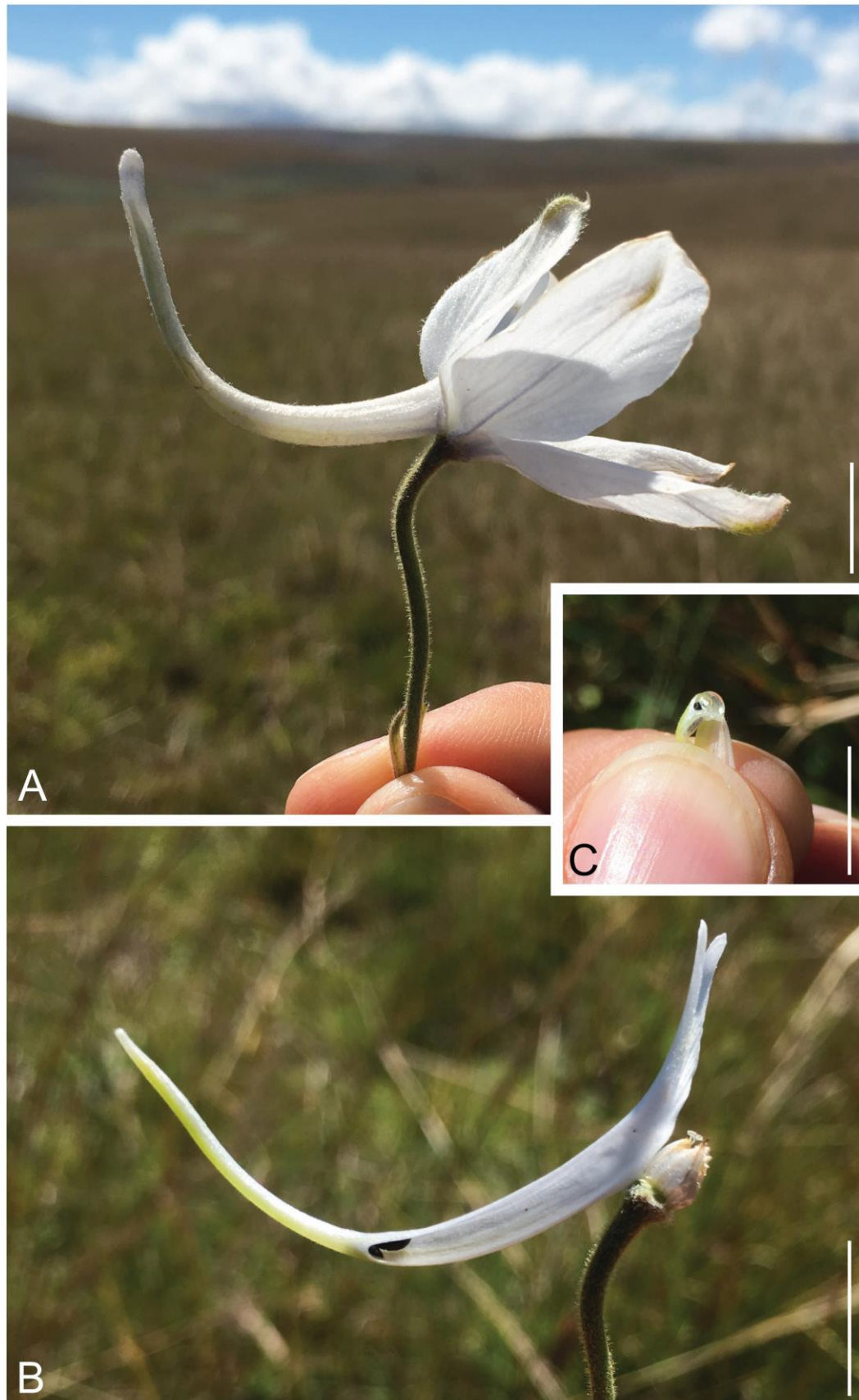
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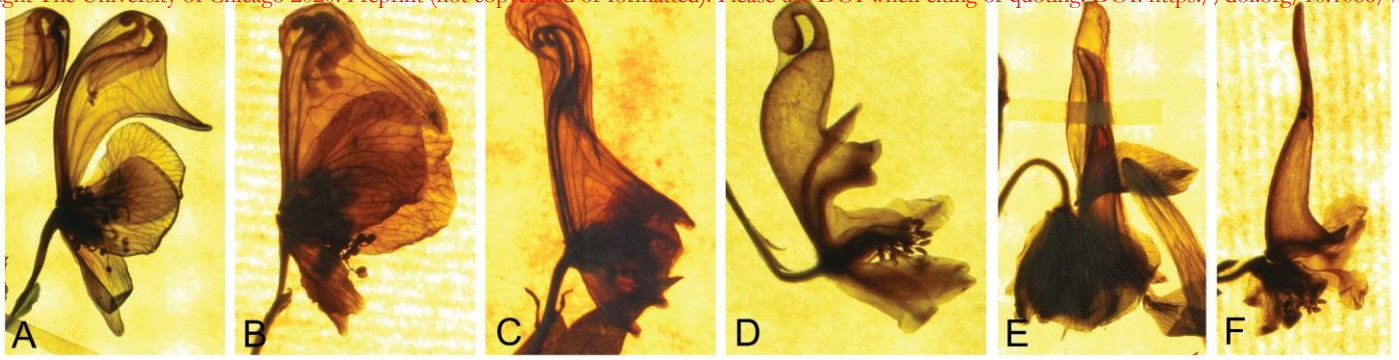
Supplementary figures



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