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Table des matières

Introduction

1.La fleur : une structure reproductrice complexe et spécialisée

1.1. Définition

Linné définit la fleur comme une structure faisant partie de la fructification d'un végétal et comme étant l'ensemble formé par le calice, la corolle, les étamines et le pistil. Dans sa définition il donna une importance particulière au nombre et à la disposition des différentes parties qui la composent afin de les utiliser postérieurement dans sa classification végétale [1]. Saint-Hilaire la définit comme étant une structure composée d'un ou plusieurs organes sexuels entourés ou non d'une enveloppe, ou, dans le cas contraire, d'une ou plusieurs enveloppes mais sans organes sexuels. Il décompose la fleur en réceptacle, calice, corolle, étamines, disque, pistils et ovules [2]. Le glossaire des plantes de Kew la définit comme étant un axe portant un ou plusieurs pistils et/ou une ou plusieurs étamines accompagnés généralement par d'autres structures afin de la rendre plus fonctionnelle ou plus attractive pour les pollinisateurs [3].

Dans ce travail, nous allons considérer la fleur comme étant formée d'un axe principal (l'axe floral) à croissance déterminée, composé de nombreuses structures qui accomplissent diverses fonctions et qui sont réparties de façon condensée le long de cet axe, sur des zones concentriques précises. Sur cet axe sont présents les organes reproducteurs de la plante, généralement entourés par des organes foliaires modifiés, facilitant, entre autres, l'attraction des pollinisateurs et la protection du bouton floral [4–10]. Ainsi, de la base à l'apex de l'axe floral (et de l'extérieur vers l'intérieur), les organes composant une fleur complète forment 1) le périanthe, composé de sépales et pétales, 2) l'androcée, composé des organes mâles de la fleur, les étamines et 3) le gynécée composé des organes femelles de la fleur, les carpelles [4,7].

1.2. Le périanthe : une remarquable diversité

Le périanthe est formé par les organes périphériques et stériles entourant les organes sexuels sur l'axe floral [4]. D'un point de vue évolutif, ils correspondent à des organes foliaires dérivés [9] et ont pour principales fonctions: 1) de recouvrir et protéger les organes sexuels de la fleur avant son ouverture et 2) d'attirer les pollinisateurs afin de faciliter le phénomène de pollinisation, après l'anthèse [4].

Dans plus de 75% des espèces de plantes à fleurs, le périanthe est composé de deux séries d'organes distincts : le calice, composé de l'ensemble des sépales, et la corolle, composée de l'ensemble des pétales.

1.2.1. Le calice

Linné définit le calice de la fleur comme étant l'écorce végétale présente chez les fruits [1]. Quelques années plus tard, Necker [11] introduit les termes "sépale", du grec σκέπη (*skepê*: couverture), pour faire référence au organes foliaires sur l'axe floral.
En effet, le calice est composé de l'ensemble des sépales qui correspondent aux organes, généralement robustes et foliacés, ayant une fonction protectrice avant l'anthèse [4,9].

1.2.2. La corolle

La corolle est l'ensemble des pétales de la fleur. Linné définit les pétales comme étant le couvercle de la fleur, et les décompose en un onglet (partie inférieure du pétale), une lame (partie supérieure) et un nectaire (partie mellifère) [1].
De nos jours, les pétales sont considérés comme les structures de la fleur, généralement colorées, participant à l'attraction des pollinisateurs. Ce sont des organes foliaires modifiés présentant une texture généralement plus délicate que celle des sépales, ainsi qu'une surface plus grande que ces derniers [4,9].

1.3. Les organes sexuels de la plante à fleurs : l'androcée et le gynécée

Les étamines, organes mâles de la fleur qui composent l'androcée, sont des structures formées par des anthères, structures de production et de presentation du pollen, et par un filet reliant l'anthère au réceptacle floral [9,12].
Les carpelles correspondent à la structure femelle de la plante et forment le gynécée. Même s'ils correspondent à des structures présentes chez toutes les plantes à fleurs et sont indispensables pour leur reproduction, leurs homologies et origines sont encore ambigües et restent toujours débattus [9,13–16].
Toutefois, cette description ne correspond qu'à une description généraliste de la fleur typique et vise à donner uniquement des repères du plan d'organisation de la structure

florale. Au-delà de ce cadre général, de nombreuses variations peuvent être identifiés particulièrement pour les organes du périanthe [4,9,17–21].

1.4. Des variations phénotypiques à l'origine de la diversité morphologique des fleurs

Dans certains cas, les deux verticilles (enssemble d'organes floraux rattachés au même niveau de la tige) formant le périanthe peuvent être composés d'organes très ressemblants qui permettront de renforcer les fonctions de protection ou d'attraction du périanthe. Dans le cas où les organes du périanthe ne peuvent pas être différenciés, on parle de tépales et le périanthe est appelé "périgone" [4]. Dans d'autres cas, les sépales peuvent prendre certaines caractéristiques des pétales typiques et vice versa. Pour ces cas précis on parle respectivement de sépales pétaloïdes ou de pétales sépaloïdes.

La diversité observée au sein des fleurs et concernant le plan de base de celles-ci (*bauplan*) est due à une accumulation de variations dans la morphologie des organes floraux. Ces variations peuvent être classées en : 1) variations de forme, 2) variations de taille, 3) variations du type d'organes présents dans la fleur, 4) variations du nombre d'organes et 5) variations dans la position des organes sur l'axe floral. A cette classification on peut ajouter les variations issues de la connexion structurale intime (avec ou sans fusion) entre organes, appelée synorganisation [22–25], et créant des structures plus complexes telles que des calices ou corolles gamosépales ou gamopétales respectivement, ou des structures nectarifères formée par la soudure post-génitale de deux pétales (comme c'est le cas dans les fleurs de *Consolida* (DC.) Gray [Ranunculaceae]) [26]. Un bel exemple de la diversité des plantes à fleurs est présenté dans le résume visuel de la diversité florale de Byng et al. (2018) (Figure 1) [17].

La fleur, avec le carpelle, est la structure la plus caractéristique des angiospermes. Elle participe à la diversité du groupe et à son succès évolutif [19,20,27].

Figure 1. Diversité florale au sein des Angiospermes. Chaque cadre présente quelques exemples de fleurs des familles composant les grands clades au sein des Angiospermes (selon APG IV). Figure modifiée de Byng et al. (2018).

1.5. Origine de la fleur : une structure bisexuée

Avant de traiter de l'apparition du groupe des angiospermes, il m'a paru nécessaire de dater et d'expliquer l'apparition de la fleur, structure emblématique du groupe (ou au moins, l'apparition la structure bisexuée chez les plantes à graines, à l'origine de la fleur).

Les premiers fossiles de structures bisexuées correspondant à une structure florale datent du Crétacé inférieur ou du Jurassique (129-113 Ma) [28–30]. Un exemple de ces fossiles sont ceux d' *Archaefructus liaoningen* Sun, Dilcher, Zheng & Zhou datant du Jurassique supérieur et montrant des axes d'Angiospermes fructifiés, composés de petits follicules avec une structure foliaire à la base [28]. Un autre exemple est celui de l'espèce aquatique *Montsechia vidalii* (Zeiller) Texeira datant du Crétacé inférieur et représentée par plus de 1 000 spécimens qui ont permis la réalisation d'une étude complète de la morphologie, de l'anatomie et des aspects reproductifs de l'espèce [30]. Toutefois, des recherches récentes ont examiné des fossiles de structures florales datant du Jurassique supérieur (145-160 Ma) [31] ; ces derniers résultats sont encore débattus.

Parmi toutes les hypothèses permettent d'expliquer l'origine de la bisexualité sur un même axe, j'ai choisi d'en aborder deux en particulier. D'un côté, la "*male theory*" (« théorie mâle ») [25,32–34] propose que cette organisation des sexes est apparue à travers la perte d'un gène de la famille *LEAFY* (le gène *NEEDLY*), ayant comme conséquence le développement ectopique d'ovules sur la face supérieure d'une structure laminaire mâle portant des sacs polliniques sur sa face inférieure (aussi appelée microsporophylle). D'un autre côté, la "*out-of-male theory*" [25,35] (« à partir du masculin ») propose la modification d'un cône mâle après avoir subi une réduction dans l'expression des gènes participant à l'établissement de l'identité des organes mâles au sommet de celui-ci et donnant origine à des structures femelles.

2. Les angiospermes : un groupe récent dans l'histoire des plantes, à diversification rapide et grand succès évolutif

2.1. Histoire du terme "angiosperme" : des graines encapsulées aux plantes à fleurs

Le terme angiosperme résulte de la combinaison des mots grecs ἀγγεῖον (*angeîon*: vase, réceptacle) et σπέρμα (*spérma*: semence, graine) [36,37] et désigne le groupe de plantes présentant des "*graines dans un réceptacle"*. Déjà proposé par Paul Hermann en 1690, pour désigner les plantes "*seminibus capsulis inclusis*", c'est à dire les plantes dont les graines sont encapsulées [38]; en opposition aux gymnospermes ou plantes à graines nues [37]. Le nom est repris par Linné afin de définir une structure présentant un péricarpe très apparent et constituant une capsule à la maturité [37,39].

Finalement, ce n'est que dans la première moitié du 19ème siècle que le terme prend son sens actuel après que les études de Robert Brown ont mis en évidence la différence entre la réception directe du pollen par les ovules nus des Cycadophytes, conifères et Gnetales, et la réception indirecte, à travers une structure spécialisée (le stigmate) du pollen par les ovules entourés d'un carpelle. Cependant, et dû au fait que dans ses travaux l'auteur ne fait pas référence aux graines mais aux ovules des plantes, celui-ci n'utilisa pas expressément le terme "angiosperme" [40]. Postérieurement, ces observations seront enrichies par les études de Hofmeister sur le développement du péricarpe et sur l'origine de l'embryon des plantes à fleur jusqu'alors considéré comme une simple excroissance du tube pollinique [41] et non pas le résultat de la fécondation de l'ovule permise grâce au développement du tube pollinique à travers le stigmate et le style [42–44]. C'est ainsi que le terme sera utilisé dans un sens élargi, jusqu'à l'heure actuelle, pour définir le clade des plantes à fleurs. De nos jours, les deux termes (Angiospermes et plantes à fleurs) sont utilisés de façon indistincte pour faire référence à tous les végétaux présentant une structure florale, avec un carpelle, telle qu'on la rencontre chez les Angiospermes [45].

2.2. Généralités sur les angiospermes : un groupe diversifié avec un grand succès écologique

Les Angiospermes forment le groupe de plantes le plus diversifié du monde végétal et représente à lui seul 90 % des espèces de plantes terrestres actuelles (ca. 300 000)

[18,27,46]. Cette diversité spécifique se voit aussi reflétée dans la diversité des caractéristiques végétatives, les Angiospermes s'adaptant à presque tous les écosystèmes terrestres [45,47,48]. En effet, le groupe présente une répartition cosmopolite allant du cercle arctique jusqu'aux côtes antarctiques, présentant une richesse en familles particulièrement élevée à l'équateur des continents américains et asiatiques [49,50]. Il a été montré que les conditions climatiques sont un des facteurs déterminants pour la richesse spécifique chez les angiospermes [51]. L'équateur américain présente la plus grande richesse en familles herbacées tandis que l'équateur asiatique est particulièrement riche en familles d'arbres [49,50]. Ce succès évolutif est à relier à la diversité morphologique végétative, mais aussi à la diversité de la structure florale responsable de la reproduction.

2.3. Historique sur les classifications des angiospermes : de la médecine au rôle prépondérant de la morphologie florale

Différents systèmes de classifications des plantes à fleur se sont succédés en fonction des intérêts de chaque époque. Ainsi, jusqu'à la fin du Moyen Âge, la plupart des ouvrages correspondent à des compilations des usages des plantes en médecine. Ces ouvrages fournissent une description sommaire des plantes en utilisant leurs noms vernaculaires. Rares sont ceux qui présentent des illustrations en tant qu'indication pour faciliter la détermination des espèces. Un bel exemple de ces œuvres est le traité *De materia medica* de Dioscorides (1er siècle après J.-C). Pendant tout le Moyen Âge, l'Europe occidentale eut accès à ce texte de référence grâce à des copies manuscrites de sa traduction latine. Un exemplaire de ce manuscrit est conservé à la Bibliothèque nationale de France sous la cote "Latin 12995". Au 16ème siècle, et grâce aux avancés techniques, apparaissent les premières impressions à large diffusion, parmi lesquelles on trouve les versions latines de Matthioli complétées par des dessins de l'auteur [52,53], la traduction espagnole de Laguna [54] ou la traduction française de Mathée [55]. Toutefois ces ouvrages restent très sommaires et correspondent plus à des inventaires qu'à de véritables classifications. Ainsi la détermination des plantes reste encore difficile à l'époque et les classifications rudimentaires.

A partir de la Renaissance, les progrès scientifiques amènent des études plus rigoureuses des plantes, de leur morphologie et, plus particulièrement, de leur morphologie florale.

Preuve en est l'impressionnante œuvre de Fuchsio [56] composée de planches dessinées très détaillées des caractéristiques morphologiques de nombreuses plantes afin de faciliter leur identification.

Au cours des 17ème et 18ème siècles, et grâce aux connaissances issues des premiers auteurs de la renaissance sur la morphologie, les modes de vie et la reproduction végétale, un changement de paradigme a lieu : on ne s'intéresse plus aux seules vertus médicinales des plantes, on se focalise plutôt sur la compréhension de l'organisation de la nature. Il faut avoir en tête que la vision fixiste de l'époque oriente l'élaboration de classifications basées sur la ressemblance entre plantes, la notion d'évolution n'étant pas reconnue. C'est ainsi qu'apparaissent les premières classifications modernes des plantes. Un exemple de classification moderne serait celle de Bauhin [57] qui considère plusieurs caractères afin de définir ce qu'il établit déjà comme genre et espèce et propose un système qui sera systématisé et repris par Linné [39,58]. Ces classifications visent l'établissement de groupes que Linné qualifiera de "naturels", tels la classe des "*systématiques orthodoxes universels"* au sens de Linné [1].
Désormais, l'étude approfondie de la fleur ainsi que des éléments qui la composent devient une étape fondamentale de la classification des plantes. Les caractères de la corolle [59,60], du calice [61] ou sexuels [39] sont donc utilisés dans diverses classifications proposées à l'époque. Vers la fin du 18ème siècle et avec les avancées techniques des moyens d'observation, les classifications botaniques commencent à s'enrichir d'autres types de caractères tels que la vascularisation des organes ou ceux relatifs aux propriétés chimiques des parties de la plante. Toutefois, les caractéristiques florales restent des éléments majeurs dans les classifications des plantes à fleur. En 1789, Jussieu publie son *Genera Plantarum* [62]. Celui-ci sera considéré plus tard par le *Code international de la nomenclature botanique* [63] comme le point de départ de la nomenclature des familles végétales. S'inspirant de quelques idées de Jussieu, de Candolle propose en 1824 une classification utilisant des caractères anatomiques et des cotylédons. Sa classification restera toutefois fondée principalement sur les caractères morphologiques floraux [58,64].

La plupart des classifications du 19ème siècle utiliseront aussi de façon prépondérante les caractères floraux. Bentham et Hooker, par exemple, proposent trois grands groupes

au sein des angiospermes: les *Polypetaleae* (à pétales libres), les *Gamopetaleae* (à pétales soudés) et les *Monochlamydeae* (sans pétales) [65]. C'est ainsi que grâce à l'importance accordée aux caractères floraux principalement, et grâce au changement de paradigme dans la façon de concevoir les classifications après la publication de l'Origine des espèces (Darwin, 1859), les relations de parenté entre groupes de plantes commencent à être mises en évidence et que les premières phylogénies informelles des angiospermes apparaissent dès la première moitié du 20ème siècle [66,67]. Finalement, et regroupant une grande partie des données moléculaires disponibles depuis la fin des années 1990, *l'Angiosperm Phylogeny Group* a décidé de proposer une classification consensuelle et synthétique des plantes à fleur. Celle-ci est mise à jour périodiquement [68–71].

2.4. Classification actuelle des angiospermes : du grade ANA aux magnolidées, monocotylédones et eudicotylédones

Les angiospermes forment un groupe monophylétique soutenu par des données moléculaires et morphologiques [18,69]. En effet, certains caractères clés les séparent du reste des plantes à graines. Parmi ces caractères, les deux principaux sont les ovules enclos dans un carpelle et protégés par deux téguments et la présence d'une structure particulière au sein de laquelle se trouvent les organes reproducteurs de la plante, la fleur [20].

Au sein du groupe, de nombreuses relations sont encore discutées. C'est notamment le cas des groupes ayant divergé tôt au cours de l'évolution des Angiospermes et composant le grade ANA (traditionnellement connu comme ANITA [70,71]) et dans lequel se trouvent les ordres des Amborellales, Nymphéales et Austrobaileyales. Même si une majorité d'études proposent le genre *Amborella* comme étant le premier groupe à diverger parmi les plantes à fleur, le consensus n'a pas toujours été évident par le passé [72–74]. Pourtant, la résolution des relations phylogénétiques au sein de ce grade (même s'il regroupe moins de 0,1% des espèces d'angiospermes) reste indispensable pour la compréhension de l'origine et de l'évolution précoce des plantes à fleur.

Si certaines relations évolutives restent débattues, la structure générale de l'arbre phylogénétique des angiospermes est suffisamment stable pour pouvoir la considérer

comme résolue [27,68,75]. Ainsi, le groupe des plantes à fleurs est composé, chronologiquement par ordre de divergence à partir de l'ancêtre commun hypothétique : 1) du grade ANA, 2) des Magnolidae, comprenant 17 familles et plus de 10 000 espèces, 3) les Monocotylédones (ou « monocots » en anglais), avec plus de 90 familles, 62 000 espèces et présentant des caractéristiques morphologiques particulières telles qu'un seul cotylédon, une vascularisation généralement parallèle des feuilles et une homorhizie, et 4) les Eudicotylédones (ou « eudicots » en anglais) [20,58].

Le groupe des Eudicotylédones correspond au clade le plus riche en termes de nombre d'espèces parmi les plantes à fleur, représentant à lui seul entre environ 75% de la diversité des angiospermes. Les plantes appartenant à ce groupe se caractérisent par la production de pollen tricolpé et par une des fleurs souvent tétra- ou pentamères [58]. Le premier ordre à diverger parmi les Eudicotylédones est l'ordre des Ranunculales composé de sept familles et plus de 4400 espèces [20,69,76]. Le reste des Eudicotylédones est contenu dans le clade des "core" (vraies) Eudicotylédones, avec plus de 90% de la diversité du groupe. Il est composé des Superrosidae (incluant les Rosidae [39% de la diversité des Eudicots] et les Superasteridae [incluant les Asteridae [50% de la diversité des Eudicotylédones]) [76].
Il est important de noter que même si généralement les vraies Eudicotylédones présentent un périanthe composé de deux types d'organes (calice et corolle), la diversité morphologique florale du groupe est telle qu'il n'y a pas une structure florale unique et commune qui puisse le définir [17,20,58] .

2.5. Origine des angiospermes : un mystère toujours en attente d'être résolu

L'apparition des angiospermes a lieu entre la fin du Paléozoïque et le début du Crétacé, entre 140 et 250 Ma. [46,77,78]. Actuellement, même si d'importants efforts reliant des études moléculaires et le registre fossile ont essayé d'établir l'âge des plantes à fleur, les résultats restent encore vagues [19,34]. Ceci est dû, entre autres, à un biais entre le registre fossile et les données moléculaires. Ce biais peut être expliqué, en partie, par le fait que la fossilisation a pu être rendue difficile par les conditions du milieu dans lequel les premières plantes à fleur ont vu le jour [79].

Assez rapidement après leur apparition (100 – 65 Ma.), les angiospermes ont subi pendant le Cénozoïque une grande diversification qui s'est accompagnée de l'évolution d'adaptations remarquables [47–49]. La difficulté à appréhender la rapidité de cette diversification a été qualifié par Darwin d' "abominable mystère" [80].

Une part importante de la diversité morphologique florale au sein des angiospermes peut être expliquée par la combinaison de facteurs génétiques et développementaux, et cette diversité est soumise à la sélection naturelle [22,74,81,82]. La compréhension du lien existant entre ces phénomènes permet de mieux aborder l'étude de l'évolution des plantes à fleurs.

3. L'évo-dévo

3.1. Définition et historique

Les premières études visant la compréhension du lien entre le développement des organismes et l'évolution des lignées datent du 19ème avec les études en zoologie de von Baer, considéré comme le père de l'embryologie [83], et de Meckel [84] considéré comme l'un des fondateur de la tératologie et qui postula un parallèle entre le développement des animaux "supérieurs" et "inférieurs". Toutefois, ce type d'études cherchant une compréhension de l'évolution à travers, entre autres, de l'analyse morphologique des transformations d'individus isolés, sera mis de côté pendant la première moitié du 20ème siècle au profit d'études visant à comprendre l'évolution à travers des analyses à plus grande échelle, populationnelle par exemple, et avec d'autres types de données, telles que les données moléculaires [85]. C'est par exemple le cas de la théorie synthétique de l'évolution (aussi appelée néodarwinisme) qui vise à faire une synthèse entre les principes de l'hérédité biologique, la génétique des populations et la sélection naturelle [86]. Plus tard, dans les années 1970, Gould [87] rétablira les bases des études du développement dans un contexte évolutif [85,88–90].

De nos jours, et grâce aux avancées en génétique et dans les techniques d'imagerie, l'évo-dévo cherche à comprendre le contrôle génétique des processus développementaux dans un contexte évolutif et dans un cadre taxonomique comparatif [85]. Les travaux en évo-dévo ont souvent pour objectif d'étudier les gènes mis en jeu dans l'origine et l'évolution de la diversité morphologique au sein d'un clade animal ou végétal.
C'est dans ce contexte qu'a été proposé un modèle combinatoire qui permet d'expliquer l'établissement de l'identité des organes de la fleur au cours du développement.

3.2. Le modèle combinatoire ABCDE : la genèse d'une fleur

Les premières études du contrôle génétique du développement floral ont été réalisées à la fin des années 1980 et pendant les années 1990 sur des mutants d'*Arabidopsis thaliana* (L.) Heynh. (Brassicaceae) et *d'Antirrhinum majus* L. (Plantaginaceae) principalement [91–95]. C'est ainsi qu'a été proposé un modèle combinatoire expliquant, à travers l'interaction de certains gènes qui codent des facteurs de transcription et qui

s’expriment pendant le développement de la fleur (gènes homéotiques), l'établissement de l'identité des organes floraux. Ce modèle a été appelé modèle ABC [96–99]. Quelques années plus tard, d'autres régions génétiques importantes pour l'établissement de l'identité des organes floraux ont été identifiées [98,100–102]. C'est le cas du groupe des gènes de la fonction E, indispensables de l'établissement du contexte floral général et permettant l'activité des gènes des fonctions B et C [103,104]. L'identification de cette fonction a permis la proposition du modèle ABCE reflétant les interactions entre les gènes ABC et les gènes de la fonction E [105]. Un autre modèle a été proposé mettant en évidence la place prépondérante des fonctions B et C, le modèle (A)BC [98]. Enfin, la mise en évidence de la fonction D dans l'établissement de l'identité des ovule [106] a permis le développement du modèle ABCDE, modèle proposé par Thießen et Saedler [107].

Actuellement, le modèle ABC modifié inclut les fonctions D [108] et E [103], et est appelé le modèle ABCDE [109,110]. Dans ce modèle, les gènes de la fonction A interagissant avec les gènes de la fonction E déterminent l'identité sépale dans la fleur. L'interaction des gènes des fonctions A+B+E déterminent l'identité pétale, ceux des fonctions B+C+E déterminent l'identité étamines, ceux des fonctions C+E déterminent l'identité carpelle et, finalement, ceux de la fonction D participent au développement des ovules (Figure 2). Il est important de remarquer que la plupart des gènes (sauf ceux de la fonction A) et des mécanismes génétiques associés sont conservés à travers les angiospermes [97] et permettent donc d'émettre des hypothèses avec un niveau de généralité assez élevé.

Toutes les études qui ont permis la mise en évidence de ce contrôle génétique lors du développement floral ont été menées avec l'utilisation de mutants. En effet, les mutations qui produisent des organismes déviants (aussi appelés tératologiques) permettent d'interpréter l'action de chacun de ces gènes homéotiques sur le phénotype floral. Ces organismes ont développé des organes floraux ailleurs dans la fleur que la région qui leur correspondait ou n'ont pas développé certains de ces organes.

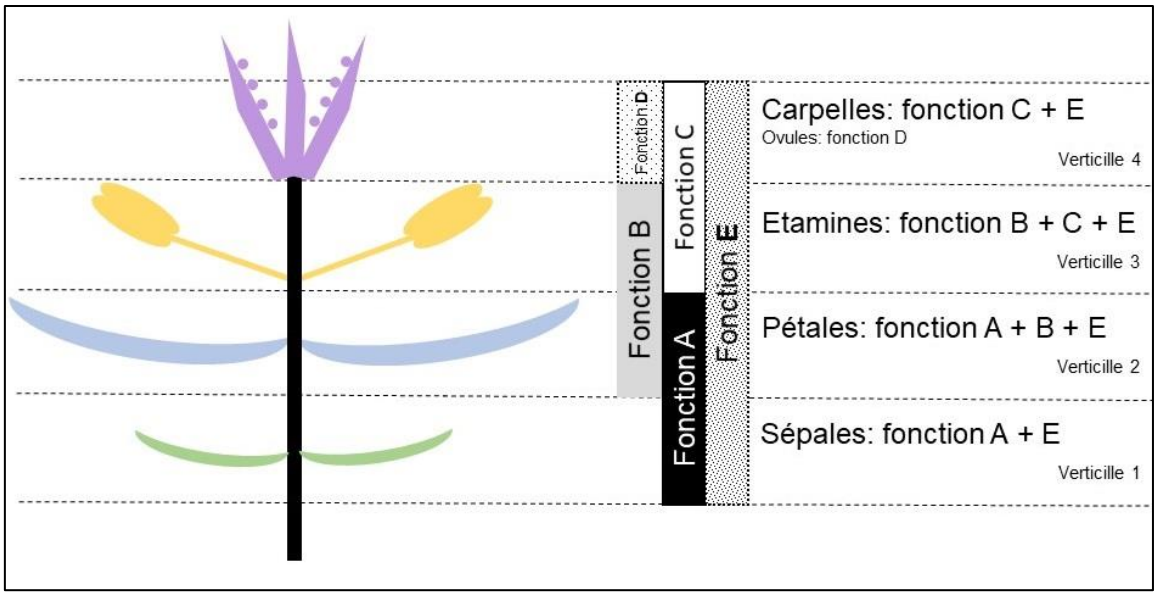

Figure 2. Schéma représentant le modèle combinatoire ABCDE. L'interaction entre les gènes des différentes fonctions permet l'établissement de l'identité des différents organes floraux. Figure adaptée de Rudall (2013).

4. La tératologie : l'anormal qui peut expliquer le normal

4.1. Définition

Le terme tératologie est définie par le centre national de ressources textuelles et lexicales (CNRTL), comme étant :

> "La science des monstres qui traite plus particulièrement des anomalies congénitales ou héréditaires les plus aberrantes, en établit les classements d'après leur aspect anatomique (*tératologie morphologique*), étudie le développement de l'embryon mal formé (*tératologie pathologique*) et tente de déceler les causes de ces malformations (*tératologie étiologique* ou *tératogenèse*)" [111].

Baillon (1892) la définit dans le *Dictionnaire de botanique* comme étant :

> "Partie de la science qui traite des monstruosités des plantes" [112],

Tandis que Moquin-Tandon (1841) définit les monstruosités comme étant :

> "des déviations du type spécifique, ordinairement congénitales, plus ou moins graves et plus ou moins complexes ; elles produisent des difformités vicieuses et gênent ou arrêtent l'exercice des fonctions" [113].

Et Calado et dos Anjos Pires la propose comme la science qui

> "Etudie les variations congénitales ou les déviations dans le développement ...[114]"

Dans ce travail, on considérera la tératologie comme étant la science qui étudie les individus déviants morphologiquement et de façon drastique par rapport à leur morphologie typique. On considérera les déviations étant issues de causes intrinsèques (par exemples, les mutations) ou des causes extrinsèques (par exemple, les conditions environnementales).

4.2. Cadre général

Depuis le 17ème siècle, l'étude des organismes tératologiques a joué un rôle fondamental dans la compréhension des mécanismes biologiques de contrôle du développement floral, de son organisation et de son évolution [113,115,124,125,116–123]. En effet, l'étude des *terata* (ou individus tératologiques) peut donner des informations importantes sur l'évolution des phénotypes sauvages. Ainsi, de nombreuses études en évo-dévo, cherchant à comprendre l'évolution florale au sein des angiospermes, ont analysé des plantes présentant des anomalies dans les organes stériles ou sexuels de la structure florale. Par exemple, le passage d'une symétrie bilatérale à une symétrie radiale (ou pélorie) chez les Orchidaceae [126,127], ou le développement d'organes surnuméraires chez les Begoniaceae [128] et les Malvaceae [129], ou la présence de

méristèmes d'inflorescences sans fleurs chez les Asteraceae [130] ont pu être étudiés grâce aux apports de la tératologie. Par ailleurs, ces études peuvent avoir des applications directes dans le développement de cultivars (ou variétés horticoles) aux fleurs plus attractives pour l'œil humain [131–133] ou dans l'évaluation de l'intérêt agricole des plantes présentant des anomalies [134].

4.3. La tératologie chez les Ranunculaceae : une science ancienne

4.3.1. Introduction

La diversité et complexité que présentent les fleurs des Ranunculaceae ont fait de cette famille une famille d'intérêt depuis plusieurs siècles. De nombreuses études ont visé la compréhension de la complexité florale au sein du groupe. L'une des disciplines ayant alimenté et inspiré ce champ de recherches est la tératologie.

De nombreuses descriptions botaniques et quelques spécimens conservés en herbiers témoignent du dynamisme de la tératologie chez les Ranunculaceae par le passé. Les premières descriptions des temps modernes de fleurs déviantes, et appartenant à cette famille, nous parviennent du 17ème siècle, avec quelques descriptions de fleurs anormales d'*Aquilegia* L., *Delphinium* L. et *Nigella* L. Plus tard, l'âge d'or des études de tératologie, le 19ème siècle, nous laissera un registre très riche des études sur des fleurs déviantes chez les Ranunculaceae. En effet, à cette époque de nombreuses études ont été réalisées visant l'analyse des anomalies affectant la couleur, la forme des organes, leur identité, la symétrie de la fleur, et le nombre des organes.

Le milieu du 20ème siècle a vu une diminution de l'intérêt pour la tératologie dans les études scientifiques. Toutefois, et récemment, quelques auteurs ont essayé de proposer un renouvellement de l'intérêt de l'étude des organismes tératologiques par le potentiel qu'ils représentent dans la compréhension de l'évolution végétale.

La préparation du cadre théorique de ma thèse m'a permis de collaborer à un travail de revue historique de l'application de la tératologie à la famille des Ranunculaceae. Ceci dans le but de 1) faire un état de l'art, 2) faire une classification des types d'anormalités florales possibles dans la famille, 3) faire une synthèse des études en évo-dévo sur les

anomalies florales, se focalisant particulièrement sur la famille des Ranunculaceae et 4) aborder la pertinence du potentiel évolutif de tels organismes.

Cette revue est parue dans Flora en 2016. Référence :

Jabbour, F., Nadot, S., Espinosa, F., & Damerval, C. (2016). Reprint of “Ranunculacean flower terata: Records, a classification, and some clues about floral developmental genetics and evolution”. *Flora*, *221*, 54-64.

Dû au fait que la publication n'a pas été proposée en open access, je présente ici la dernière version du manuscrit soumise avant la publication. Pour cette raison, elle suit les indications de présentation de la revue, particulièrement en ce qui concerne la bibliographie.

4.3.2. Histoire de la tératologie au sein des Ranunculaceae :

Ranunculacean flower terata: records, a classification, and some clues about floral developmental genetics and evolution

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Running title: Ranunculacean flower terata

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Abstract

Teratological organisms originate from developmental anomalies, and exhibit structures and a body organization that deviate from the species standard. In plants, teratological forms are often of horticultural interest. However, besides their aesthetic value, these monsters give essential clues about the formation of the wild-type groundplan. We focus on flower terata, which can be affected in their sterile and/or fertile organs, with special emphasis on the Ranunculaceae. The diversity of perianth shapes and organizations in flowers of this family is huge, and is even increased when anomalies occur during organo- and/or morphogenesis. In order to galvanise research on Ranunculacean flower terata, which has been overlooked since the middle of the 20th century, we provide the necessary material to inspire future studies in this field. We I) recount the history of the science of flower teratology, II) review records of flower terata in the buttercup family, II) propose a system to classify the changes affecting canonical development, IV) synthesize key studies on the developmental genetics of the flower terata with a focus on Ranunculaceae, and V) address the issue of their evolutionary potential. We expect Ranunculaceae species to become model organisms in flower teratology studies, focusing on morpho-anatomy as well as on evo-devo or evolutionary ecology.

Keywords: Delphinieae; evo-devo; homeosis; peloria; proliferation; virescence

I. Flower terata through the historical lens: ancient and modern observations

The study of terata had its golden age during the 19th century when reporting and exhibiting human and animal monsters was the object of scientific (and sometimes less scientific) curiosity (Geoffroy Saint-Hilaire E., 1826; Geoffroy Saint-Hilaire I., 1832). Abnormalities in developing and adult structures have also been widely documented for plants. Concerning flowers, reports of terata are known since the Greek and Roman Antiquity (Aristotle (translated version: Barthélémy-Saint Hilaire, 1887); Pliny the Elder (translated version: Ajasson de Grandsagne, 1831)). Later on, flower terata have inspired the works of many botanists, ontogeneticists and naturalists, and many of these flower phenotypes have been maintained for horticultural purposes. During the 19th century and in the first half of the 20th century, German, British, and French botanists were leaders in these studies. Some of the very influential botanists who contributed pivotal works to the science of flower teratology (Jäger, 1814; De Candolle, 1827; Moquin-Tandon, 1841; Brongniart 1844; Masters, 1869; Penzig, 1890) openly built on the theories of their predecessors (Linnaeus, 1744; Goethe, 1790). Figure 1 presents a timeline showing a chronological selection of important contributions to the study of flower terata and of Ranunculacean flower terata in particular. We arbitrarily chose the description of *flore pleno* (filled or full flower) variants of *Aquilegia*, *Consolida*, *Delphinium*, and *Nigella* made by Clusius in 1601 as the starting point. The next most important contribution to flower teratology is undoubtedly the description by Linnaeus of a peloric (after the Greek word for monster or wonder) *Linaria* collected in 1742 by one of his students, Magnus Ziöberg (Gustafsson, 1979). The molecular mechanisms responsible for this anomalous flower were elucidated more than two hundred years later (Cubas et al., 1999).

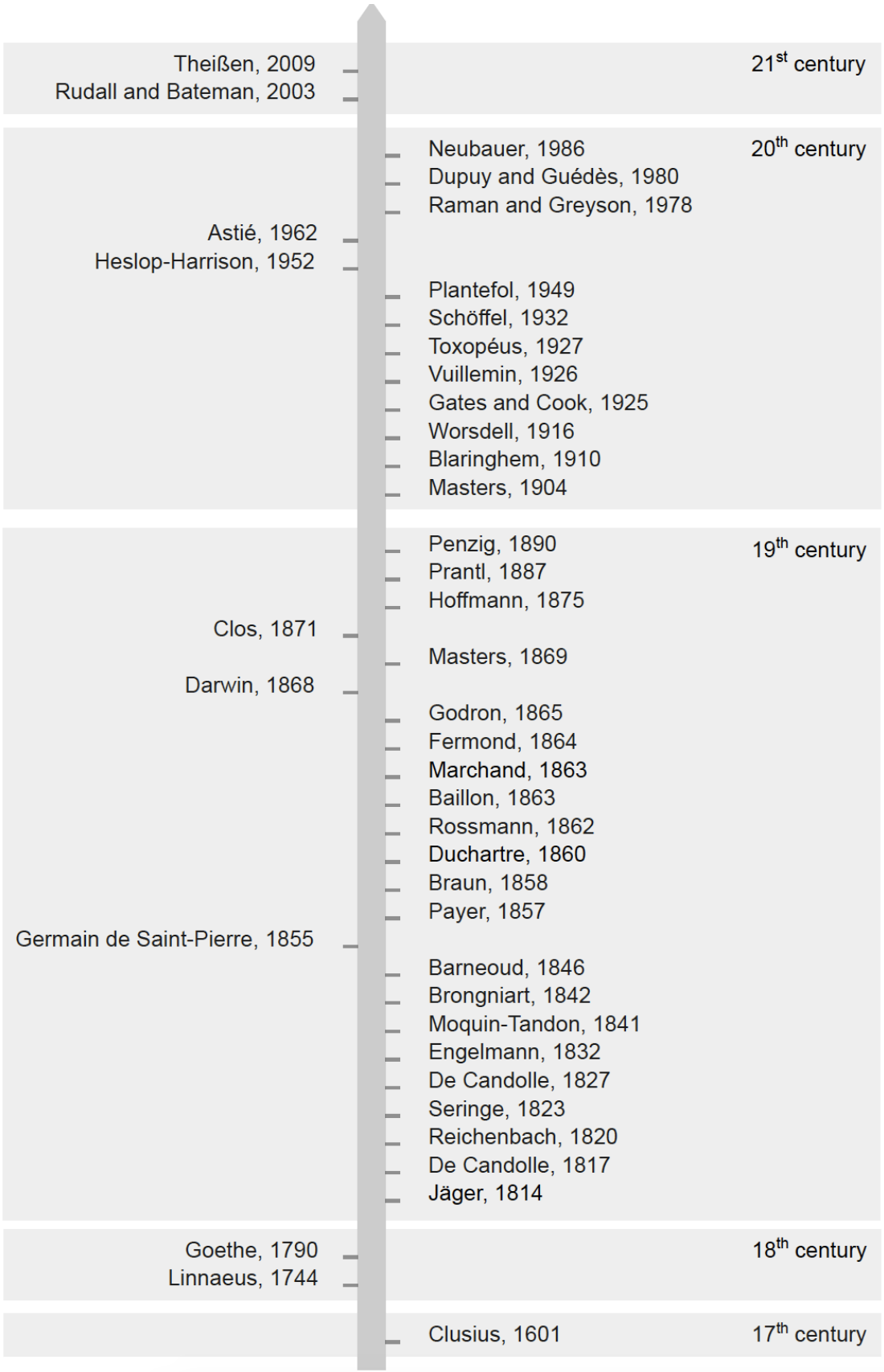

Fig. 1. Timeline presenting studies on Ranunculacean flower terata (right), and some pivotal works dealing, in whole or in part, with flower terata in angiosperms (left).

The flower is typically defined as a condensed structure gathering organs differing in their identity (sepal, petal, stamen and carpel), shape and function, organized as concentric whorls or following a tight spiral (Bateman et al., 2006). Flower traits provide criteria of primary importance for plant systematics, because their variability among angiosperms is discontinuous, enabling species typification. Therefore, referring to a species standard is crucial in detecting and describing a teratum. For instance, when a species shows flowers with natural meristic variation as is often the case in flowers with spirally inserted organs (e.g., Lehmann and Sattler, 1994; Kitazawa and Fujimoto, 2014), defining a monster based on the number of floral organs will require a more extensive study than in a species with a fixed merism. Identifying a teratum requires a keen taxonomic expertise, otherwise the temptation to propose a new species name for the specimen with unusual flowers would be strong.

Abnormal flowers deviating from the species standard do not necessarily have to be rare in nature, at least locally. Deviations can affect a single or several aspects of floral organization, such as organ identity, number, position, and functionality (resulting for instance in sex-specific sterility, or in a less conspicuous perianth). A frequent source of anomaly arises from quasi-normal organ(s) developing in a wrong place. Such misspecification may appear either within (for example a spurred petal normally observed only in dorsal position also appeared in place of all other petals) or between whorls (for example petals appear in place of stamens). In other cases, floral organs may develop as leaf-like structures. Such changes have been termed homeotic. Homeosis was first defined by Bateson in 1894, as a type of variation where “something has been changed into the likeness of something else”. Goethe (1790) had already described the phenomenon a hundred years before in “The Metamorphosis of Plants”. Additionally he had underlined the interest of what he called irregular metamorphoses to understand regular metamorphoses, an idea that was resurrected later on (Moquin-Tandon, 1841; Brongniart, 1844; Plantefol, 1949; Meyerowitz et al., 1989).

Extensive works on teratological flowers of Orchidaceae and Lamiales have been published (reviewed in Rudall and Bateman, 2003). Another group of flowering plants that has attracted the attention of botanists with respect to abnormal flowers is Ranunculaceae, in which teratological flowers are known to be common in cultivated as well as in wild plants (Baillon, 1863). In this family, flower organs are generally inserted following a tight spiral that enables differentiating pseudo-whorls of sepals,

petals, stamens and carpels. The natural variation in perianth structure and merism, and in the shape of nectariferous petals is remarkable (Schöffel, 1932; Endress, 1995; Wang et al., 2009). In addition, about one-quarter of the species, all belonging to the tribe Delphinieae, produce zygomorphic (bilaterally symmetrical) flowers.

In the next four sections of this article, we: I) review the records of flower terata in Ranunculaceae in a phylogenetic framework, II) propose a classification system of the teratological flower forms, III) synthesize studies about the developmental genetics of abnormal flowers with a focus on the rare cases investigated so far in Ranunculaceae, and IV) address the issue of the evolutionary potential of these terata.

II. Records of teratological flowers in Ranunculaceae

Our survey of the literature shows that teratological flowers belonging to members of the Ranunculaceae have been observed in at least 20 different genera, among the 62 comprised in the family (Stevens, 2001 onwards). The position of these genera (except *Ficaria* and *Staphisagria*) in the most recent phylogeny of Ranunculaceae is highlighted in Fig. 2, which shows that teratological flowers can be found in all subfamilies of Ranunculaceae, except in the two earliest diverging ones, namely Glaucidioideae and Hydrastidoideae (both monotypic). Teratological flowers can be affected in their general shape, mostly in terms of floral symmetry, and any part of the flower (perianth, androecium or gynoecium) can be modified compared to the standard groundplan (Figs. 3A–M). The heritability of these variants is generally unknown. Deviation from the standard floral organisation of the species varies according to the records: in some of them, only the perianth is affected whereas in other ones, all floral parts are affected. We give below a synthetic account of the various kinds of teratological flowers found in the genera of Ranunculaceae that were most often studied in this regard. For the genera not treated in the paragraphs below, we recommend looking at the very detailed catalogue of flower terata by Otto Penzig (1890), as well as at the contributions of the botanists whose names appear in Fig. 1. Tribes and subfamilies of Ranunculaceae are those recognized by Wang et al. (2009).

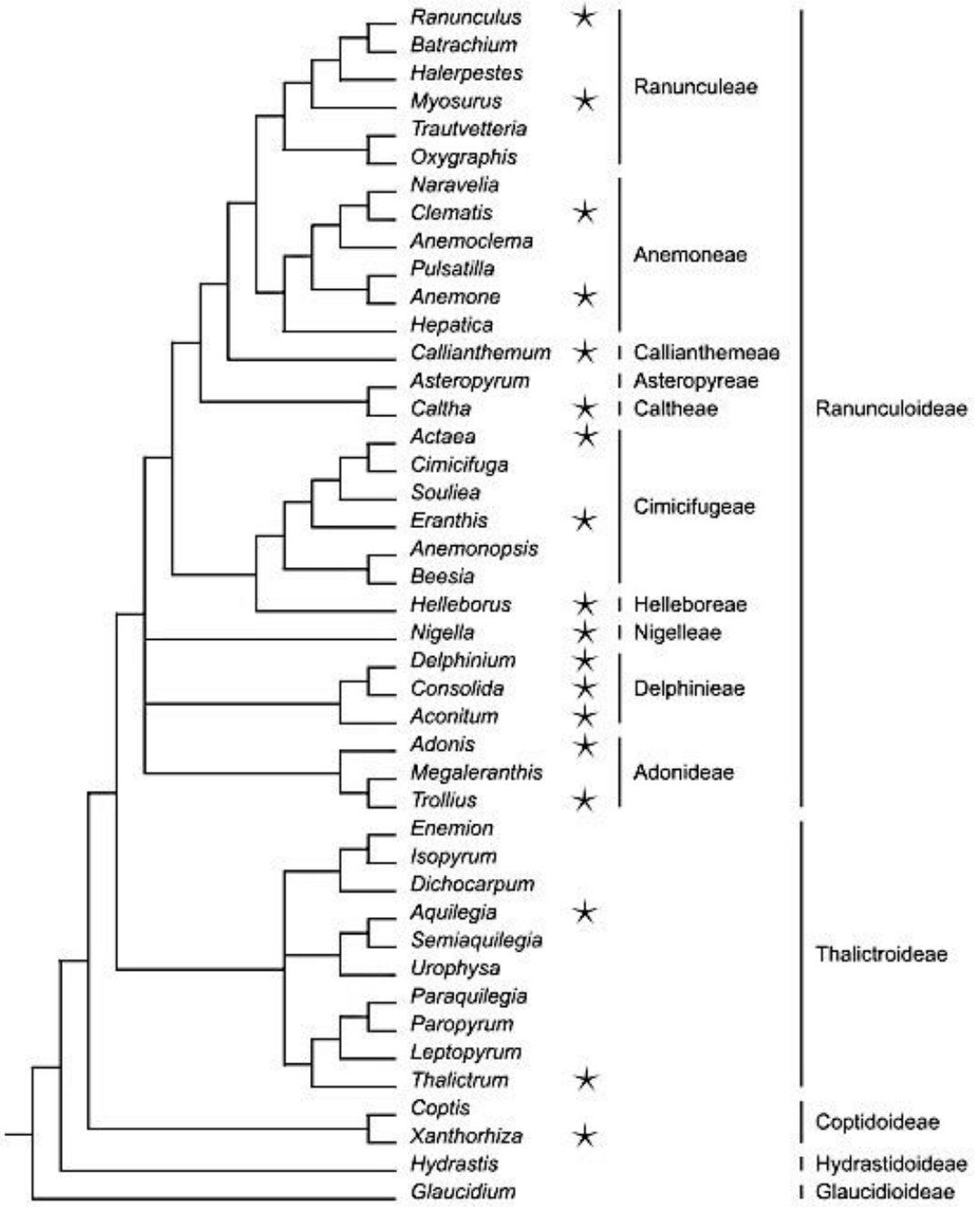

Fig. 2. Phylogenetic relationships among the genera of Ranunculaceae where flower terata have been reported. On a phylogeny adapted from Wang et al. (2009), eighteen of these genera are marked with a star. Two more should have been added (*Ficaria* and *Staphisagria*) but they were not included in the sampling of the original phylogeny. The tribes and subfamilies of Ranunculaceae are indicated.

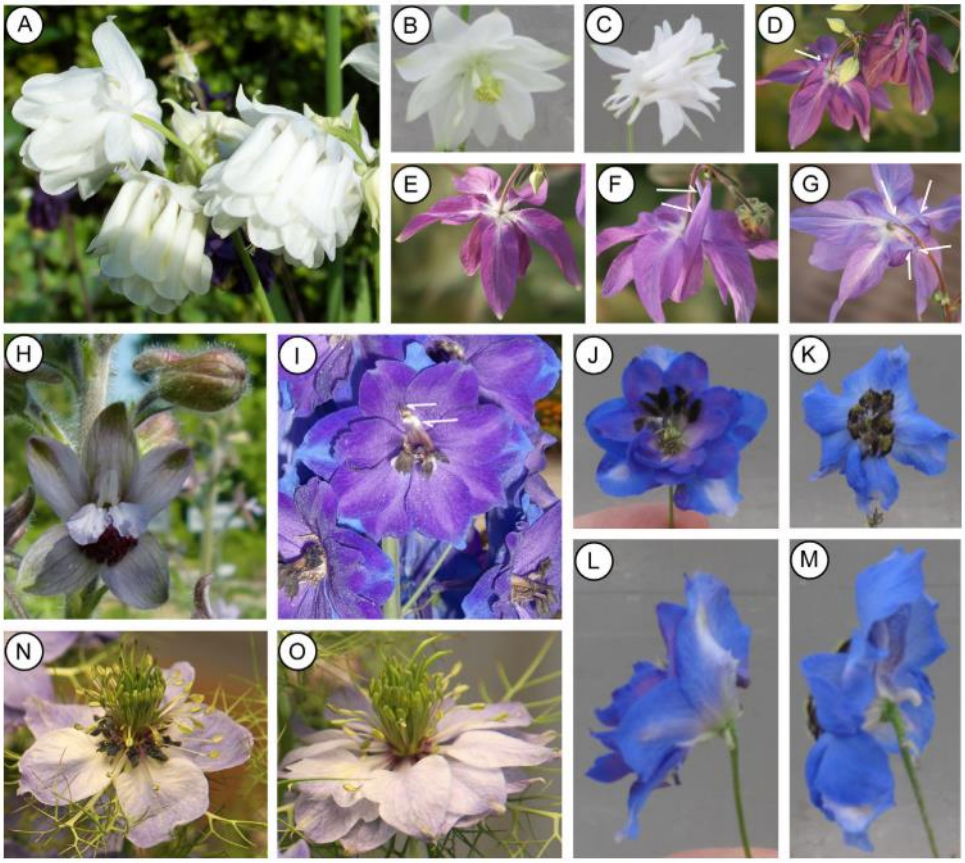

Fig. 3. Pictures of wild-type and teratological flowers of Ranunculaceae. (A) Cultivated *Aquilegia* with supernumerary spurred petals, the spurs being inserted in one another. (B, C) Two individuals of *Aquilegia x hybrida* 'Green Apples' showing a flower with supernumerary and spurless perianth organs and a flower with supernumerary spurred petals, respectively. (D–G) Five phenotypes of *Aquilegia vulgaris* flowers: wild-type with five spurred petals (right in D), spurless (E), with a single spur (left in D), with two (F) or four (G) spurs. Spurs of the teratological flower phenotypes of *A. vulgaris* are indicated by white arrows. (H) Wild-type flowers of *Delphinium exaltatum*. The perianth consists of five petaloid sepals (the dorsal one being spurred), two dorsal spurred petals, and two lateral flat petals. (I) Flowers of *Delphinium x cultorum* 'Magic Fountains' with supernumerary perianth organs. A dorsal sepal is spurred, and two of the spurs of petals (white arrows) point out, as they cannot find their way through the calyx. (J–M) Front and side views of *Delphinium x pacific* 'Blue Jay', showing supernumerary perianth organs. The dorsal sepal may (M) or may not (L) develop a spur. (N–O) Wild-type and *flore pleno* morph ([T] sensu Gonçalves et al. (2013)) of *Nigella damascena* flowers. Pictures were taken in a garden in Orsay, France (A), at the lab 'Génétique Quantitative et Evolution – Le Moulon', Gif-sur-Yvette, France (B–G, J–M), at the Botanical Garden of Munich, Germany (H), in the gardens of the National museum of Natural history, Paris, France (I), and in a garden in the suburbs of Paris (N–O).

Tribe Delphinieae (subfamily Ranunculoideae)

The vast majority of records of teratological flowers in Ranunculaceae concern the tribe *Delphinieae*, which includes approximately 650 species with highly elaborate flowers (Fig. 3H; Jabbour and Renner, 2012). Flowers in this tribe are zygomorphic; they possess five petaloid sepals, a reduced corolla with two (in the genera *Aconitum* and *Gymnaconitum*) or four (in *Delphinium* and *Staphisagria*) petals located dorsally, numerous stamens and a gynoecium composed of one to five free carpels. Only *Consolida* s.l. (including *Aconitella*), a subgroup of *Delphinium* (Jabbour and Renner, 2011), has a single petal and a single carpel. The dorsal sepal, spurred or hooded, encloses the nectariferous petals (two in all genera, except for *Consolida* s.l.).

Figure 4 (A–E) shows some illustrations of floral monsters belonging to Delphinieae, extracted from relatively old publications (Reichenbach, 1820; Seringe, 1823; Braun, 1858; Cramer, 1864; Masters, 1904). Anomalies most often result in a loss of the zygomorphic groundplan, which can take place either because of homeosis acting within a whorl (strictly speaking pseudo-whorl) or between whorls. Most monster flowers in the genus *Delphinium* are affected in the shape and number of petals: the fully developed petals become as numerous as the sepals and are more or less all identical to the dorsalmost petals, with a spur, making them look similar to columbines (genus *Aquilegia*) (Seringe, 1823; Baillon, 1863). Such peloric flowers are mostly found in a terminal position in the inflorescence (Godron, 1865; Figs. 5D, G), but a specimen with the whole inflorescence composed of peloric flowers has been recorded in *Delphinium grandiflorum* (formerly *D. chinense*) (Godron, 1865). Specimens bearing peloric flowers with petaloid stamens and sterile carpels have been found in this species, showing that all organs of the flower, and not only the perianth, can be affected. Interestingly, Baillon (1863) points out a striking morphological similarity between peloric double varieties of *Delphinium* (also described by Masters, 1869) and double varieties of *Nigella* (Fig. 3O).

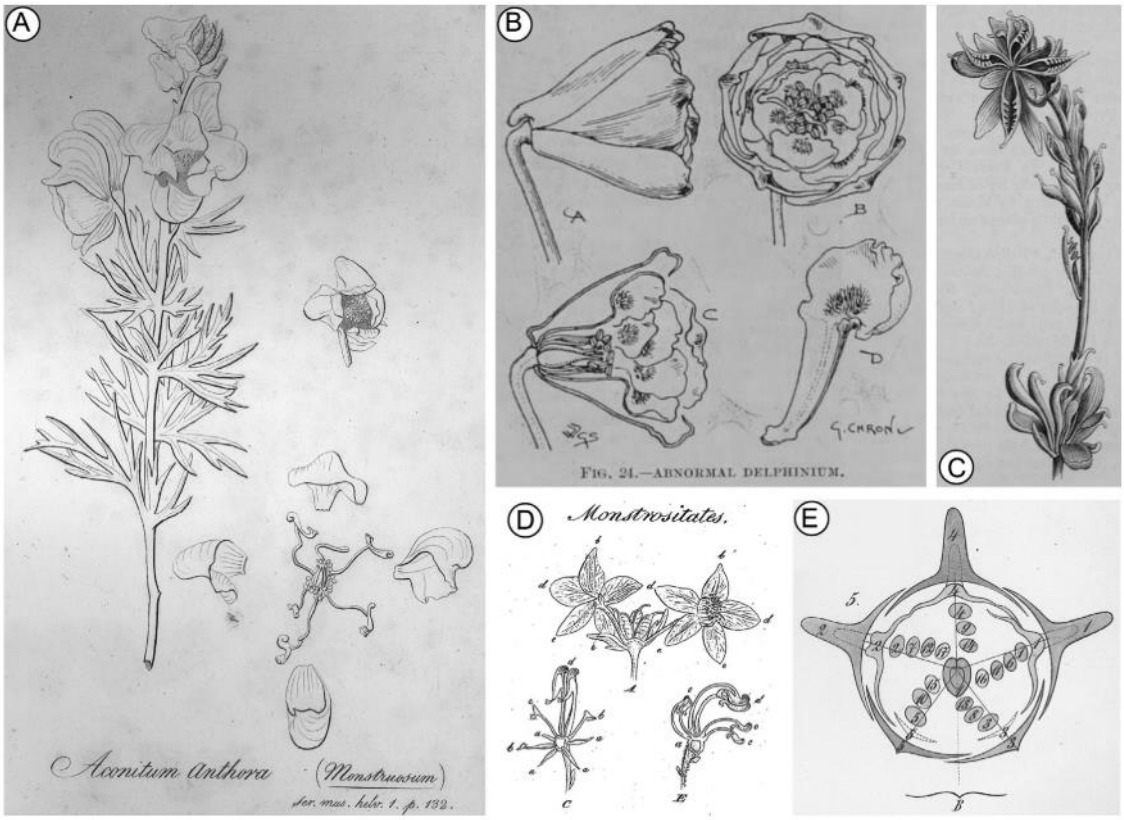

Fig. 4. Illustrations of Delphinieae flower terata, reproduced from (A) Seringe (1823), (B) Masters (1904), (C) Cramer (1864), (D) Reichenbach (1820), and (E) Braun (1858). (A) Teratological flower phenotype of *Aconitum anthora* showing a peloric flower with only four sepals (all hooded) and six petals displaying more or less accurately the typical shape of the dorsal petal of the wild type flower (a stalk, a labium, and a spur). (B) Double flower of *Delphinium* with supernumerary petals. The flower is almost peloric. Note the spur of the dorsal sepal that is very much reduced in size. (C) Teratological flower phenotype of *Delphinium*. The axis elongates from the centre of the flower (lower part of the image) and is terminated with a second flower (upper part of the image). (D) A proliferous flower of *Aconitum* and two types of corollas of *Aconitum* displaying additional fully developed and spurred petals. (E) Floral diagram of an abnormal flower of *Consolida ajacis* showing three spurred sepals and three spurred petals.

Fig. 5. Two herbarium specimens from the collection of Dominique-Alexandre Godron, kept at NCY. (A–E) Godron s.n., 1864, cultivated (NCY017202). Flowers and inflorescences of *Delphinium elatum*, the terminal flowers being peloric with five spurs. (B–E) Magnifications of the label and of the samples mounted on the specimen. (C) The terminal flower (star) is peloric with five spurs. (F, G) Godron s.n., 1864, Varangéville, near Saint-Nicolas-de-Port (NE France) (NCY017203). Inflorescence of *Consolida regalis* (= *Delphinium consolida*). Flowers have a calyx with five sepals, the dorsal one being spurred (standard), and supernumerary petals. (G) The terminal flower (star) is peloric and spurless.

The shape of sepals and petals can be affected to various degrees. For example, in *Consolida orientalis*, a specimen bearing a single terminal peloric flower has been found by Godron in 1865, with spurless sepals and numerous flat petals devoid of nectaries. This flower has numerous short carpels and no stamens. A similar phenotype has been found in a terminal flower of *C. regalis* with a regular calyx and a corolla consisting of several spurless petals (Fig. 5G). Flowers of *Staphisagria requienii* with eight spurless petals (Barneoud, 1846; Payer, 1857) and of *C. ajacis* with a spurless perianth were observed (Penzig, 1890). In *C. regalis*, monster flowers with flat petaloid sepals and devoid of petals and carpels (but with stamens) have been found (Godron, 1865). Schöffel (1932) reports peloric flowers with a perianth composed of seven flat organs with no nectaries in *Aconitum*, and there is also an old report of monster flowers with flat sepals and petals in this genus (Reichenbach, 1820). In annual larkspurs, other forms with numerous petaloid bracts completely lacking sexual organs were also recorded (Masters, 1869). Flowers of *C. ajacis* with stamens transformed into spurred petaloid organs were described (Penzig, 1890); the supernumerary spurs are inserted within the spur of the underlying petal, as in the terata of *Aquilegia* with supernumerary petals. An extreme case of flowers with leaf-like sepals, no petals, and carpels in which the ovule development was drastically affected was recorded by Marchand (1863) in *Delphinium grandiflorum*. It can be noted that greening of floral parts (a case of virescence) is often reported in *Delphinium* (Penzig, 1890; Gates and Cook, 1925).

In many *Delphinium* species (but especially in *D. formosum*) as in *Aconitum lycoctonum*, the upper sepal can have a bifid spur with each lobe enclosing one of the petal spurs (Worsdell, 1916). The flowers have otherwise normal organs. In the annual larkspurs *C. regalis* and *C. ajacis*, the single upper spurred petal can be replaced by a pair of spurred petals, which has been interpreted as a reversal toward the ancestral condition (Worsdell, 1916), consistently with the phylogenetic placement of *Consolida* within the genus *Delphinium* (Jabbour and Renner, 2011). A few flowers with a zygomorphic calyx but a peloric corolla devoid of spurs have been observed (Gates and Cook, 1925). These flowers had a normal androecium but a reduced number of carpels. An interesting teratological form of garden *Delphinium* affected only in the perianth was described by Masters (1904) (Fig. 4B). The flowers are almost peloric, the dorsal sepal bearing a very short, almost inconspicuous spur. The petals are in increased number, stalked and spurless, and the blade is fringed with orange-yellow hairs.

Aquilegia (subfamily Thalictroideae)

Normal columbine flowers are actinomorphic and composed of five petaloid flat sepals, five spurred petals (with a nectary in the tip of spur) (Fig. 3D), numerous whorled stamens and a few free carpels. A single species, *A. ecalcarata*, has spurless petals in its normal form (Leppik, 1964). In *A. alpina*, teratological flowers without spurs or with one to several petals lacking a spur (making the flower irregular) have been recorded (Fig. 3D-G). The stellate form of garden columbine has double flowers in which the petals are flat and spurless much like sepals, and often in greater number than in its wild form (*A. vulgaris*), due to petalody (transformation of stamens into petals) (Worsdell, 1916; Masters, 1869) or to the transformation of stamens and petals into sepals (Penzig, 1890 and references therein). In this species, various types of monster flowers producing foliaceous organs have been recorded, affected by frondescence (proliferation of foliaceous perianth organs) or phyllody (foliaceous carpels bearing naked ovules on their margins) (Masters, 1869).

Ranunculus (tribe Ranunculeae, subfamily Ranunculoideae)

With approximately 600 species, the genus *Ranunculus* is one of the most species rich genera of Ranunculaceae (Hörandl and Emadzade, 2012). The flowers are actinomorphic and generally possess five greenish sepals, five flat yellow petals bearing a nectary pit at their base, numerous stamens and numerous free carpels. In this genus, floral abnormalities affecting either the perianth or the sexual organs (or both) have been recorded in many species. The transformation of various floral parts (calyx, corolla and carpels) into leaves (a case of virescence different from the first one cited above) was recorded in *R. tuberosus* together with an elongation of the floral axis (Marchand, 1863). Strangely, the stamens were morphologically normal. Transformation of stamens into petals was recorded in at least three species (*R. hebecarpus*, *R. sardous* and *R. polyanthemos*), and transformation of sepals into petals was recorded in *R. repens* (Penzig, 1890, and references therein). Supernumerary petals have been observed in R. *abortivus*, *R. acris*, *R. recurvatus* and *R. septentrionalis,* the extreme situation being found in horticultural forms of *Ranunculus* with double flowers in which the sexual organs are missing and replaced by petals (Smith, 1928). Synanthia (grouping of flowers that can share part or all of the sterile whorls) were found in three different species (*R. flammula*, *R. repens* and *R. velutinus*) (Penzig, 1890).

Anemone (tribe Anemoneae, subfamily Ranunculoideae)

The flowers of *Anemone sensu* Hoot et al. (2012) possess five to numerous petaloid sepals of various colours, and numerous stamens and carpels as in *Ranunculus*. Petals are absent. The bracts, which form an involucre around the flower, are frequently affected, and transformed more or less completely into sepals (Worsdell, 1916; Penzig, 1890), or the involucre is doubled as in *Anemone hepatica* (Masters, 1869). Other teratological forms include double flowers, in which the carpels or the stamens are transformed into sepals (Penzig, 1890 and references therein). Such forms with supernumerary perianth organs were recorded for example in *Anemone japonica* (Prantl, 1887) and described as showing a 'monstrous filling'.

Helleborus (tribe Helleboreae, subfamily Ranunculoideae)

In this small genus which has flowers with five large and flat petaloid sepals, and numerous tubular petals, double flowers occur by transformation of stamens into petals (Worsdell, 1916). As in many members of the family (including *Caltha palustris*, and the cases mentioned above), greening and transformation of floral organs into leaves have also been observed (Masters, 1869; Penzig, 1890 and references therein). Surprisingly, flowers with a zygomorphic calyx have been recorded in *H. caucasicus* (Penzig, 1890). Irregular monster flowers deviating from actinomorphic normal forms can therefore occur in unrelated species (the other one being *Aquilegia alpina*, mentioned above).

Other occurrences of teratological flowers

Occurrences of teratological forms are recorded in a few other genera of Ranunculaceae, to a lesser extent compared to the above listed genera. Petalody due to transformation of stamens was observed in *Clematis* (tribe Anemoneae, subfamily Ranunculoideae), resulting in double flowers (Penzig, 1890; Worsdell, 1916). Petaloid stamens were also observed in *Actaea spicata* (tribe Cimicifugeae, subfamily Ranunculoideae), in *Anemonella thalictroides* (formerly *Thalictrum anemonoides*) (Penzig, 1890), in *Caltha palustris* (tribe Caltheae, subfamily Ranunculoideae) in which sexual organs can be completely replaced by petals in horticultural forms named double marsh marigolds (Smith, 1928; Wijnands, 1993). In *Thalictrum thalictroides*, all organs can be replaced by petaloid sepals, and such variants are used in horticulture (Galimba

et al., 2012). Flowers in which the involucral bracts become proliferous to the exclusion of all the other parts of the flower have been found in some of the cultivated double varieties of *Nigella damascena* (Masters, 1869).In *Xanthorhiza simplicissima* (formerly *X. apiifolia*, subfamily Coptidoideae), flowers can abort or become unisexual flowers, with reduction in stamen and carpel number (Penzig, 1890).

III. A classification of the flower terata in Ranunculaceae

Different systems for classifying flower terata were elaborated in the last two centuries. They focused on taxonomy (Clos, 1871; Penzig, 1890), on the category of organs affected (De Candolle, 1817; Masters, 1869; Worsdell, 1916), or on the type of anomaly (De Candolle, 1827; Moquin-Tandon, 1841; Kirschleger, 1845, Masters, 1869; Vuillemin, 1926). Some authors coined specific terms for the floral abnormalities they observed (e.g., Vuillemin, 1926), and/or used a jargon that may confuse or discourage the reader not willing to continuously go back to a glossary. In the following, we clarify the different conceptions of peloria, doubling, proliferation, and virescence, before introducing our own classification.

The term peloria has a more or less identical meaning for most botanists, and is used to describe a radialized flower compared to its bilaterally symmetric standard form (but see Vuillemin (1926) for whom a peloric flower is a flower radialized in a single whorl only, in contrast with actinomorphosis, leading to a fully radialized flower). Traditionally, two types of peloria have been recognized (Godron, 1865; Masters, 1869; Worsdell, 1916; Gates and Cook, 1925): *peloria anectaria* (radialized flower devoid of nectariferous spurred organs) and *peloria nectaria* (the number of nectariferous spurred organs equals flower merism; this is the type of peloria described by Linnaeus in 1744). The terms pseudo-peloria or semi-peloria were used to describe orchids with supernumerary spurs that were not fully radialized (Rudall and Bateman, 2003; and see Braun (1858) for semi-peloric *Consolida*, Fig. 4E). The definition of peloria we adopt in our classification is the following: radialization of one or several whorls rendering the whole flower actinomorphic whereas the species standard flower is zygomorphic.

In contrast, the terms doubling and proliferation have been defined in many different ways. In 1790, Goethe defined flowers in which the stamens and the carpels are transformed into petals as ‘double’. De Candolle (1827) and Moquin-Tandon (1841) distinguished the doubling by lateral multiplication of organs within a single whorl,

from the doubling by multiplication of whorls of organs from the same category (doubling *sensu* Germain de Saint Pierre (1855)). Moquin-Tandon (1841) further introduced the following categories: semi-doubling (part of the androecium is converted into supernumerary corollas), doubling (all the stamens are converted into petals), and full or filled flowers (androecium and gynoecium converted into corollas, elsewhere designated as *flore pleno*). It is to be noted that the *flore pleno* morph of *Nigella damascena* described by Clusius in 1601 was rather a double flower, with an androecium and a gynoecium. De Candolle (1827) proposes slightly different categories: to him, double flowers included the *flores petalodei* (all floral organs or only some of them are converted into petals), the *flores multiplicati* (*flores petalodei* with an increase in the total number of organs), and the *flores permutati* (the abortion of the male or female organs leads to a change in the shape of the calyx or the corolla). In our classification, a double flower has more perianth organs (sepals and/or petals) organized in an equal number of whorls or more whorls compared with the species standard, and a filled flower (or full, or *flore pleno*) has all of its stamens and carpels transformed into perianth organs.

Germain de Saint-Pierre (1855) qualified as 'proliferous' flower specimens that showed an indefinite spiral of petaloid organs, as well as flowers developing additional floral buds initiating at the axil of organs. For Moquin-Tandon (1841), 'proliferous' defined a flower with an extended axis with or without additional flowers (see Goethe, 1790). Penzig (1890) defined 'prolification' as the emergence of flowers or even inflorescences at the axil of organs or at the centre of a primary flower. Our definition of proliferation embraces all of these definitions, and we consider a flower proliferous when a vegetative or flowering axis emerges from the centre of the primary flower or from the axil of one or several of its organs (as in Fig. 4D).

Similarly, the term virescence has been subject to several interpretations. In their work on *Delphinium*, Gates and Cook (1925) explained that virescence could refer to 1) the greening of floral organs belonging to the same category (sepal, petal, stamen, or carpel) or to different categories, or 2) the greening of flower parts and additional acquisition of leaf-like characters (frondescence). In our classification we make a distinction between the simple greening of parts of a flower (virescence), and the homeotic change of some or all floral organs to leaf-like organs (frondescence).

The classification of flower terata that we propose in Table 1 focuses on the objects affected by the anomaly and the type of change. In addition to the terms defined

above, we chose to use a simple vocabulary in order to avoid any misinterpretation of terms, and to make our classification readily understandable. First, we consider the flower in its context (the inflorescence, where the anomaly can be systemic, scattered, or affect only the terminal flower). Second, we list possible changes in organ identity, number, and arrangement. All of these changes can happen independently or in combination, leading to a wide diversity of teratological phenotypes. The potential influence of the deviation from the standard phenotype on the relationship with pollinators and on fertility can be deduced from the way in which the flower is affected (in floral symmetry, nectariferous organs, and sexual organs). Minor anomalies affecting colour, size, and pubescence are not considered.

IV. From flower terata to developmental genetics and vice versa

Studies describing teratological flowers often provide hypotheses to explain the emergence of abnormal shapes, by investigating their developmental causes and their genetic bases. While the "Mutationstheorie" of De Vries (1901) largely influenced the study of flower terata, the opinion that environment can favour the emergence of such forms was however shared among many botanists. Effectively, flower terata may be non-heritable, induced by environmental stress such as epidemic-like transmission of the monstrous phenotype caused by insect parasitism or attacks (Molliard, 1900; Gates and Cook, 1925), or other unknown external or internal causes. Phytoplasmas are insect-transmitted pathogenic bacterias that induce many developmental defects in plants, including phyllody and virescence; the molecular mechanisms underlying such transformation of flowers into leaf-like vegetative tissue have been recently unravelled (Du Toit, 2014; MacLean et al., 2014; Maejima et al., 2014). Moquin-Tandon (1841) and Masters (1869) believed that soil nutrients had a major influence on the formation of flower terata. Alternatively, Raman and Greyson (1977, 1978) showed that a modification in the plant hormonal metabolism could possibly trigger terata formation.

Some scientists mostly interested in plant physiology and morphology developed protocols to generate flower monsters. This field of botany, called experimental teratology, flourished in the second half of the 20th century (Astié, 1962; Dupuy and Guédès, 1980). Nowadays, chemical mutagenesis (using EMS: Ethyl methanesulfonate, or T-DNA: Transfer-DNA; the plant is transformed with *Agrobacterium*; e.g., Latado et al., 2004; Dinh et al., 2014) and physical mutagenesis (using X-rays; e.g., Cremer et al.,

2001) are used to generate floral mutants for developmental genetics and horticultural purposes.

Heritable floral mutants have been largely used to decipher flower developmental genetics in the model species *Arabidopsis thaliana* and *Antirrhinum majus*. Studies of simple and double mutants with homeotic phenotypes led to an interpretative framework of the flower groundplan, the so-called ABC model (Haughn and Somerville, 1988; Bowman et al., 1989; Carpenter and Coen, 1990; Coen and Meyerowitz, 1991). According to this model, three functions, alone or in combination, specify the four types of floral organs: sepals are directed by the A function, petals by A+B, stamens by B+C and carpels by the C function. The genes responsible for the homeotic mutants have then been cloned, first the *DEFICIENS* (*DEF*, B-class) gene in *Antirrhinum* and the *AGAMOUS* (*AG*, C-class) gene in *Arabidopsis* (Sommer et al., 1990; Yanofsky et al., 1990). All of them were found to encode transcription factors, most of which belong to the MADS-box gene family (Theißen et al., 2000). Later on, the model was made more complex adding the E-function, which is a required partner of the ABC functions to specify floral organ identity in *Arabidopsis* (Pelaz et al., 2000, 2001). This model has constituted the initial framework for floral evo-devo studies in angiosperms (for review e.g. Causier, 2010).

The ABC model is also of heuristic value for testing hypotheses about the genetic and molecular origin of abnormal flowers in distantly related species. For example, in filled flowers, stamens and carpels are replaced by sepals and petals; according to the ABC model, C-class gene expression is expected to be affected in the innermost whorls. Such a hypothesis has been confirmed in filled flowers of *Camellia japonica* (Sun et al., 2014) and *Ipomoea nil* (Nitasaka, 2003). Similarly, double flowers with an increased number of petals and/or petaloid stamens and a decrease in stamen number, may correspond to mutation(s) shifting the expression domain of C-class genes. Such a shift in the expression domain of *AG* homologues has been described in species with double flowers belonging to *Rosa* sp, *Prunus lannesiana*, *Camellia japonica* and *Lilium* (Dubois et al., 2010; Liu et al., 2013; Sun et al., 2014; Akita et al., 2008, 2011). In the Orchidaceae family, the characterization of genes from the extended ABCE model, with a focus on the B-class genes has given clues about the genetic origin of peloric flowers in some species. Up to four *DEF* paralogs have been found that correspond to three ancient duplications specific to the family (Mondragón-Palomino

and Theißen, 2008, 2009). Specific combinations of *DEF* paralogous gene expression appear to be linked to the different identities of the perianth organs, in a so-called 'orchid code' that accounts for the different types of peloria defined on morphological grounds (Rudall and Bateman, 2002, 2006; Mondragón-Palomino and Theißen, 2011; Mondragón-Palomino, 2013, for review). Beyond that, modularity in the control of individual organ types achieved through the combined expression of these paralogous genes may have offered the opportunity for separate morphological diversification, accounting for the astonishing diversity of orchid flowers (Theißen, 2010).

Indeed, peloria seems to rely on different types of genetic and molecular mechanisms. In *Antirrhinum majus*, *radialis (rad)*, *dichotoma (dich)* and *cycloidea (cyc)* single mutants are semi-peloric whereas double *cyc dich* mutants are fully peloric, with all petals displaying the ventral wild-type identity; all these mutants are impacted on genes encoding transcription factors (Luo et al., 1996, 1999; Galego and Almeida, 2002; Corley et al., 2005). It has been shown that the CYC transcription factor, acting in combination with the three others, directs the unidirectional development of the flower in this species; specific combinations of the four transcription factors define the identity of each of the five petals (Corley et al., 2005). In the peloric form of *Linaria* described by Linnaeus, it has been demonstrated that the gene homologous to *CYC* is extensively methylated and transcriptionally silent, leading strictly speaking to an epimutant (Cubas et al., 1999). Another case of peloria affecting only the terminal flower in a short raceme in *A. majus* has been shown to be controlled by the *CENTRORADIALIS* gene (Coen and Nugent, 1994).

Few genetic studies have been conducted in teratological flowers of Ranunculaceae. All of them pertain to double-flowered and *flore pleno* phenotypes and rely on a candidate gene approach based on the extended ABCE model. In *Thalictrum thalictroides*, the cultivar "Double White" is sterile, all floral organs being replaced with white petaloid sepals. In agreement with the ABC model, the expression of the C function *ThtAG1* gene is reduced, associated with structural defects in the genomic region, which entail the production of abnormal mRNAs and truncated proteins unable to interact with proteins encoded by the putative E-function gene *ThtSEP3* (Galimba et al., 2012). In *Nigella damascena*, a widespread floral mutant coexists with the normal type in gardens and flowery meadows. In this mutant the small nectariferous petals are replaced by numerous petaloid sepals (Fig. 3O), suggesting a disruption in the B

function. This hypothesis has been confirmed by the identification of the locus responsible for the mutation, which has been found to be a B-class gene, *NdAP3-3*, whose expression is abolished in the floral mutant (Gonçalves et al., 2013). Similar candidate gene approaches could be designed to uncover the origin of other homeotic mutants described above: for instance in *Ranunculus repens* with a shift from sepal to petal identity or in *R. hebecarpus* with a shift from stamen to petal identity, modifications in the expression patterns of A, B and/or C-class genes could be investigated. Similarly, peloria in the terminal flowers of *Delphinium* species are reminiscent of the *cen* mutant phenotype in *A. majus*. Such approaches should be coupled with functional ones in order to ascertain the genetic origin of the concerned terata.

V. Evolutionary potential of flower terata: myth or reality?

Homeosis has been suggested to be a strong driver of flower evolution (e.g., Ronse De Craene, 2003; Theißen, 2010). This idea also underlies the refinements of the ABC model proposed to account for the diversity of floral architectures in angiosperms (shifting/sliding boundary, fading border, for review Soltis et al., 2006). Many spontaneous floral mutants are due to homeotic changes. An interesting question is whether such mutants could survive in the wild, and whether they have the potential to ultimately give rise to novel species. This question relates to the debate about gradual *vs* saltational mode of evolution. Darwin, and later the proponents of the Modern evolutionary Synthesis, argued that evolution takes place through the gradual accumulation of small changes over time, which should be sufficient to account for the present biodiversity. Gradualism as the sole evolutionary mechanism has been questioned early after the publication of Darwin's Origin of species, and ever since (e.g., Gould, 1977). One of the most famous scientists disputing the primacy of gradualism in explaining abrupt evolutionary transitions was Richard Goldschmidt, who coined the popular expression 'hopeful monsters' (1940). Although he did not deny that gradualism can account for microevolution, and that most macromutations resulting in discontinuous phenotypic changes ('monsters') are disadvantageous, he also thought that in rare cases, macromutations can be successful, producing by chance phenotypes adapted to particular environmental conditions. Only such saltational events could account for species differences that he saw as 'bridge-less gaps' (see also Bateman and DiMichele, 2002; Theißen, 2006 and 2009 for a discussion of these ideas).

Survival and evolutionary success depend on the fitness of the mutant. Changes in flower design may result in changes in signals for pollinators, affecting in turn gene flow and reproductive success. Few data are available on the fate of such floral mutants. In the widespread shepherd's purse (*Capsella bursa-pastoris*), a homeotic mutant in which petals are replaced with stamens (variety *Stamenoid petal*, *Spe*) has been observed for a long time, and in a number of wild habitats. Wild-type plants are visited by insects about twice as much as the mutant plants. While the seed set per fruit is similar in both types of plants, wild-type plants produce more flowers and fruits, but *Spe* seeds have a better germination rate than wild-type seeds. Thus, the overall fitness is similar in both types of plants but with compensatory performances in plant architecture and germination. Insect visitation and floral architecture seem to have a low incidence on global fitness, possibly because the species is mainly self-pollinating (Ziermann et al., 2009).

A recent study showed the persistence in the wild for more than 160 years of a double flower mutant of *Vinca minor* where the five stamens are changed into petals (Wang et al., 2011). This mutant also displays modifications in the number and colour of petals of the second whorl (purple instead of blue) compared with the standard, and occasionally 'flowers within flowers' develop on elongated pedicels originating from the axil of floral organs of the primary flower. Some flowers of the *flore pleno* morph still develop stamens that are morphologically normal, explaining the survival in the wild of such a phenotype. Moreover, sexual reproduction is likely to play a minor role in the persistence of a local population, since it was reported that both wild-type and the teratological variety are able to propagate vegetatively and form clonal colonies (Wang et al., 2011).

Traits pertaining to plant architecture, organ shape or structure are often governed by a limited number of genes (Gottlieb, 1984) most probably acting at key points during development. Therefore major phenotypic changes may in fact be underpinned by limited genetic changes affecting the level and/or pattern of key developmental gene expression. This seems to be the case for at least some of the teratological mutants, as described above. However, it is worth mentioning that in several cases the exact number of genes and molecular events (sequence mutation, epigenetic change) are not fully elucidated.

Thus even though the gradual hypothesis can hold at the genetic level, adaption and evolution play with phenotypes, and the issue is whether major ruptures in the phenotype are the source of innovations and lineage diversification. Investigating the evolutionary potential of flower terata may provide insights into this question. A wide range of Ranunculacean flower terata are well documented in the literature. We believe that the science of eco-evo-devo, integrating genetics and development with pollination ecology studies, would benefit a lot from such model system.

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5. Groupe d'étude : les dauphins du monde végétal

Mon travail de thèse a consisté en l'étude de l'origine et de l'évolution de la diversité florale avec pour genre focal *Delphinium* L. Celui-ci appartient à la tribu des Delphinieae qui est le seul clade des Ranunculaceae à présenter des fleurs à symétrie bilatérale (ou fleurs zygomorphes) [26,135]. Cette tribu se situe au sein de la sous-famille des Ranunculoideae, dans la famille des Ranunculaceae et dans l'ordre des Ranunculales (Figure 3) [69].

L'ordre des Ranunculales est l'un des premiers ordre à diverger parmi les Eudicotylédones, et le groupe frère des Eudicotylédones vraies [72,136,137]. Les espèces au sein de cet ordre se caractérisent par une grande diversité morphologique, notamment florale, et de mode de vie [18,137–139]. C'est pourquoi l'ordre entier a été proposé en tant que groupe modèle pour des études en évo-dévo [101,137,140–143].

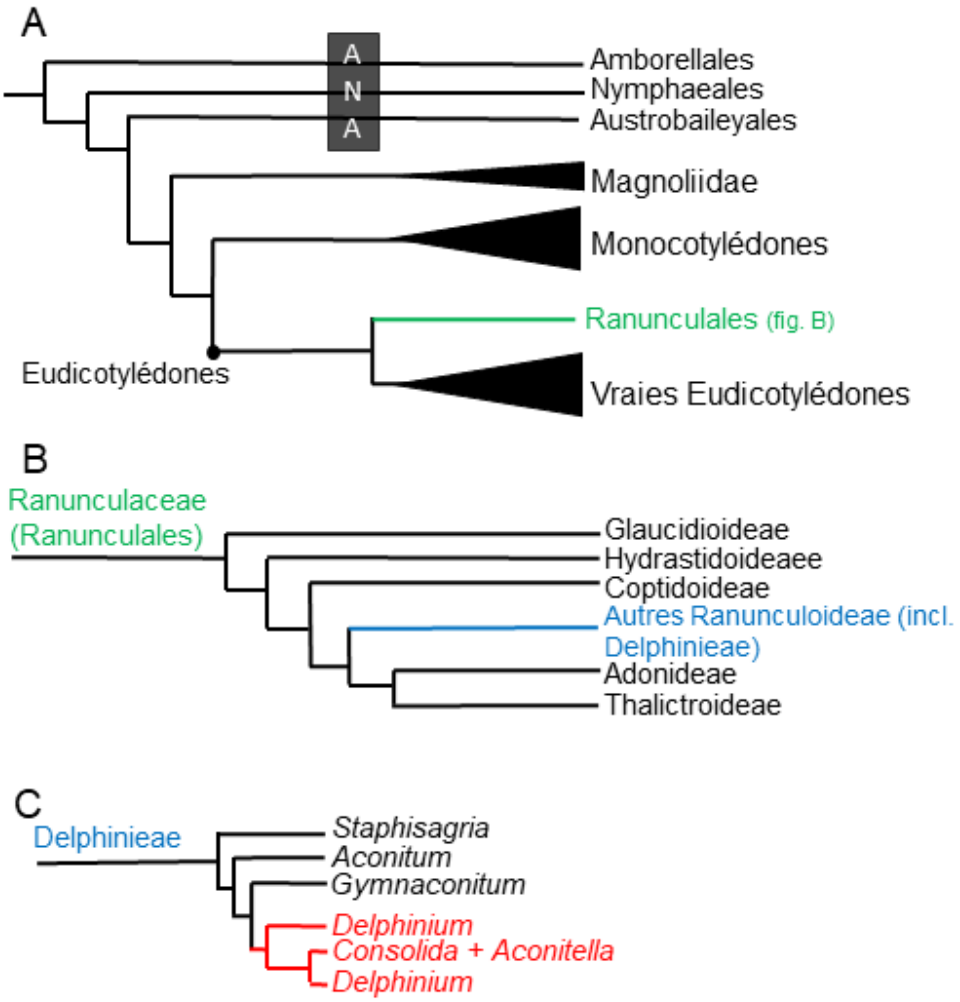

Figure 3. Phylogénie simplifiée : A- Clade des Angiospermes, en vert est signalé l'ordre auquel appartient le genre *Delphinium*; B- Clade de la famille des Ranunculaceae, en

bleu est signalée la tribu à laquelle appartient le genre *Delphinium*; C- Clade de la tribu des Delphinieae, en rouge sont marqués les clades composant le genre *Delphinium.*

5.1. La famille des Ranunculaceae : une famille à la morphologie florale très diverse

La famille des Ranunculaceae est la plus grande en termes de nombre d'espèces au sein de l'ordre des Ranunculales avec environ 2300 espèces réparties en 62 genres [140,144]. La famille présente une grande diversité florale incluant une partie des variations florales observées chez les Eudicotylédones. Ainsi, au sein du groupe il est possible de trouver des genres avec des fleurs qui ne présentent pas de pétales à l'état adulte (genre *Thalictrum* L. ou *Caltha* L.), avec des fleurs à symétrie radiale (par ex. *Paraquilegia* Drumm. & Hutch), ou à symétrie radiale avec des structures nectarifères et des staminodes (par ex. *Aquilegia* L.), ou à symétrie bilatérale avec des structures nectarifères (par ex. *Delphinium* L.) avec des variations dans le nombre et le type d'organes du périanthe au sein de la même espèce (par ex. *Nigella damascena* L.), avec une réduction dans le nombre d'organes du périanthe (par ex. *Cimicifugeae* L. ex Wernisch.), entre autres.

Preuve de l'énorme diversité florale et de la complexité du périanthe chez les Ranunculaceae, et en lien avec le développement de structures telles que les éperons nectarifères, et à l'ambigüité de l'origine évolutive des organes du périanthe [145], de nombreuses dénominations de ces mêmes organes ont été proposées. Certains auteurs ont considéré les organes nectarifères comme issus des étamines et ont proposé les dénominations suivantes : "staminodia"[146], "nectary organs"[147] ou "petals" [138,145,148–151]. Dans d'autres cas ce sont les organes latéraux de la corolle qui ont été considérés comme des "staminoides" [152]. Ainsi, les organes composant le calice ont été appelés "tepals"[147] ou "sepals"[138,145,148–151] (pour plus d'informations, voir [26]). Dans ce travail on suivra la terminologie proposée par Jabbour et Renner (2012) : on utilisera "organes W1" pour faire référence aux cinq organes du périanthe les plus externes (qui s'initient en premier au cours du développement) et "organes W2" pour faire référence aux organes internes du périanthe.

Depuis plus de deux siècles la famille a été sujette à de nombreuses études morphologiques [142,145,153–157], phylogénétiques [139,140,158] et, plus récemment, des études en évo-dévo [21,101,159,160], ce qui a permis d'établir un cadre solide pour les études des mécanismes évolutifs responsables de la diversification morphologique chez les plantes à fleur.

5.2. Le genre *Delphinium* : des Ranunculaceae à symétrie bilatérale

La fleur typique du genre *Delphinium* a une symétrie bilatérale et est composée de quatre type d'organes : deux types d'organes formant le périanthe, W1 et W2, et deux types d'organes sexuels, les étamines et les carpelles. Les organes externes (W1) sont pétaloïdes, libres et disposés en quinconce : deux ventraux, deux latéraux et un dorsal éperonné. On rencontre ensuite quatre organes internes pétaloïdes libres (W2) disposés uniquement dans la partie dorsale de la fleur ; les deux organes dorsaux présentent des éperons nectarifères insérés dans l'éperon de l'organe W1 dorsal, et les deux organes dorso-latéraux sont sans structure nectarifère. Les organes W2 ventraux sont initiés mais leur développement s'arrête précocément. Enfin, la fleur présente de nombreuses étamines disposées en huit spirales, et entre trois et cinq carpelles libres (Figure 4) [26,153,161,162]. Tous les organes de la fleur sont disposés en spirale.

La symétrie bilatérale de la fleur des *Delphinium* résulte de deux phénomènes indépendants : 1) le développement d'éperons sur les organes dorsaux W1 et W2, et 2) le développement non homogène des organes W2, restreignant les organes adultes à la partie dorsale de la fleur [135].

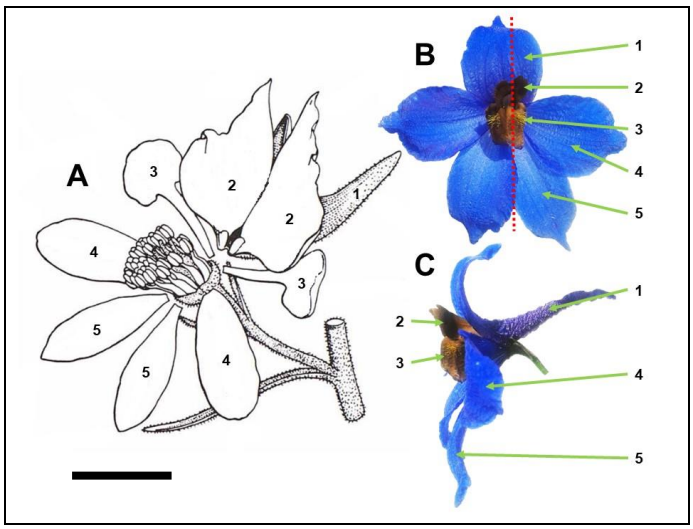

Figure 4. Structure de la fleur typique du genre *Delphinium* L. : A- Dessin représentant la structure de la fleur de *Delphinium verdunense* Balb. adapté de Blanché; B-Vue frontale de la fleur de *D. elatum* L., la ligne en pointillé rouge représente le plan de symétrie de la fleur; C- Vue latérale de la fleur de *D. elatum* L. 1- Organe W1 dorsal; 2-Organes W2 dorsaux; 3- Organes W2 latéraux; 4- Organes W1 latéraux, 5-Organes W1 ventraux. Echelle : 1 cm.

Problématique et objectifs de la thèse

La diversité morphologique au sein des Angiospermes s'exprime notamment au travers des variations morphologiques des organes floraux. Ces variations peuvent être des variations de forme, taille, nombre d'organes ou positions de ceux-ci au sein de la structure florale.

La compréhension de l'origine et de l'évolution de cette diversité morphologique florale peut nous aider dans la compréhension de l'évolution des plantes à fleurs. Pour cela, on se demandera :

De quelle façon la diversité florale prend-elle place au cours du développement et quels processus de développement contribuent à son évolution ?

Une approche possible pour répondre à cette question consiste en l'étude des phénotypes déviants, d'un point de vue développemental et génétique. Pour répondre à cette question, j'ai choisi de prendre le genre *Delphinium* L. (Ranunculaceae) comme modèle. Dans une première partie, j'analyserai les bases développementales conduisant à une fleur de *Delphinium* à symétrie radiale. Dans une deuxième partie, j'étudierai les possibles bases moléculaires responsables de la diversité florale dans des variétés horticoles de *Delphinium*. Enfin, dans une troisième partie je discuterai des implications taxonomiques qu'entraîne l'apparition de nouvelles morphologies florales au sein du genre *Delphinium*.

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Matériels et méthodes

Dans cette partie je présenterai de façon générale les manipulations réalisées pendant ce travail de thèse, volontairement sans précision sur la qualité et le nombre de spécimens. A part quelques exceptions indiquées dans chacun des chapitres, trois types principaux de manipulations m'ont permis de répondre aux différentes questions posées : 1) l'observation de la morphologie des fleurs adultes, 2) l'observation de la morphologie de boutons floraux à différentes étapes du développement et 3) l'analyse anatomique des organes de la fleur.

Ces manipulations m'ont permis de répondre aux questions suivantes : 1) quelles sont les variations phénotypiques présentes chez les plantes analysées ? 2) A quels moments du développement floral apparaissent ces variations ? et 3) Comment sont vascularisés les organes des fleurs présentant des écarts par rapport à la morphologie typique de l'espèce ? Les éléments de réponse à ces questions ont alimenté ma réflexion sur les bases moléculaires possibles de ces phénotypes déviants, et sur le potentiel évolutif de ces lignées présentant des écarts à la morphologie standard.

1. Origine du matériel analysé

Pendant cette thèse j'ai travaillé avec deux types de matériel : du matériel frais provenant de plantes cultivées sous serre, en chambre de culture, ou dans les parterres du Jardin des Plantes, et du matériel d'herbier.

1.1. Matériel frais

Le matériel frais a été prélevé puis fixé dans de l'alcool acétique formolé, dont l'abbreviation en anglais est FAA : 90% éthanol, 5% formaldéhyde en solution aqueuse, 5% acide acétique). L'utilisation du FAA permet de préserver la forme des cellules végétales en empêchant leur déshydratation afin de pouvoir réaliser des observations fines en morphologie et anatomie. Postérieurement, le matériel a été transféré dans une solution d'éthanol à 70% pour la suite des manipulations.

1.2. Matériel sec

Le matériel sec provient de spécimens conservés en herbier. Les spécimens analysés ont été récoltés à partir de la première moitié du 19ème siècle et jusqu'en 2015 (plus de

détails sur ces spécimens sont présentés dans le chapitre 2). La réhydratation d'une partie du matériel a été faite en faisant baigner le matériel (boutons floraux) dans une solution aqueuse d'ammoniac à 10% pendant 24 h et à une température de 60°C. Une fois réhydraté, le matériel a été passé dans du FAA pendant 24h puis conservé dans une solution d'éthanol à 70% afin de le préparer pour les manipulations à suivre.

2. Observation sur les fleurs adultes

Afin de pouvoir identifier les variations morphologiques observées sur le matériel choisi, j'ai observé à l'œil nu puis à la loupe binoculaire des fleurs adultes de chacun des spécimens étudiés. Ensuite, j'ai disséqué une fleur adulte des spécimens sélectionnés. Les fleurs adultes "éclatées" ont été montées sur une feuille d'herbier suivant la disposition des organes sur le réceptacle floral et sont conservées à l'Herbier national de Paris (P) (Figure 1).

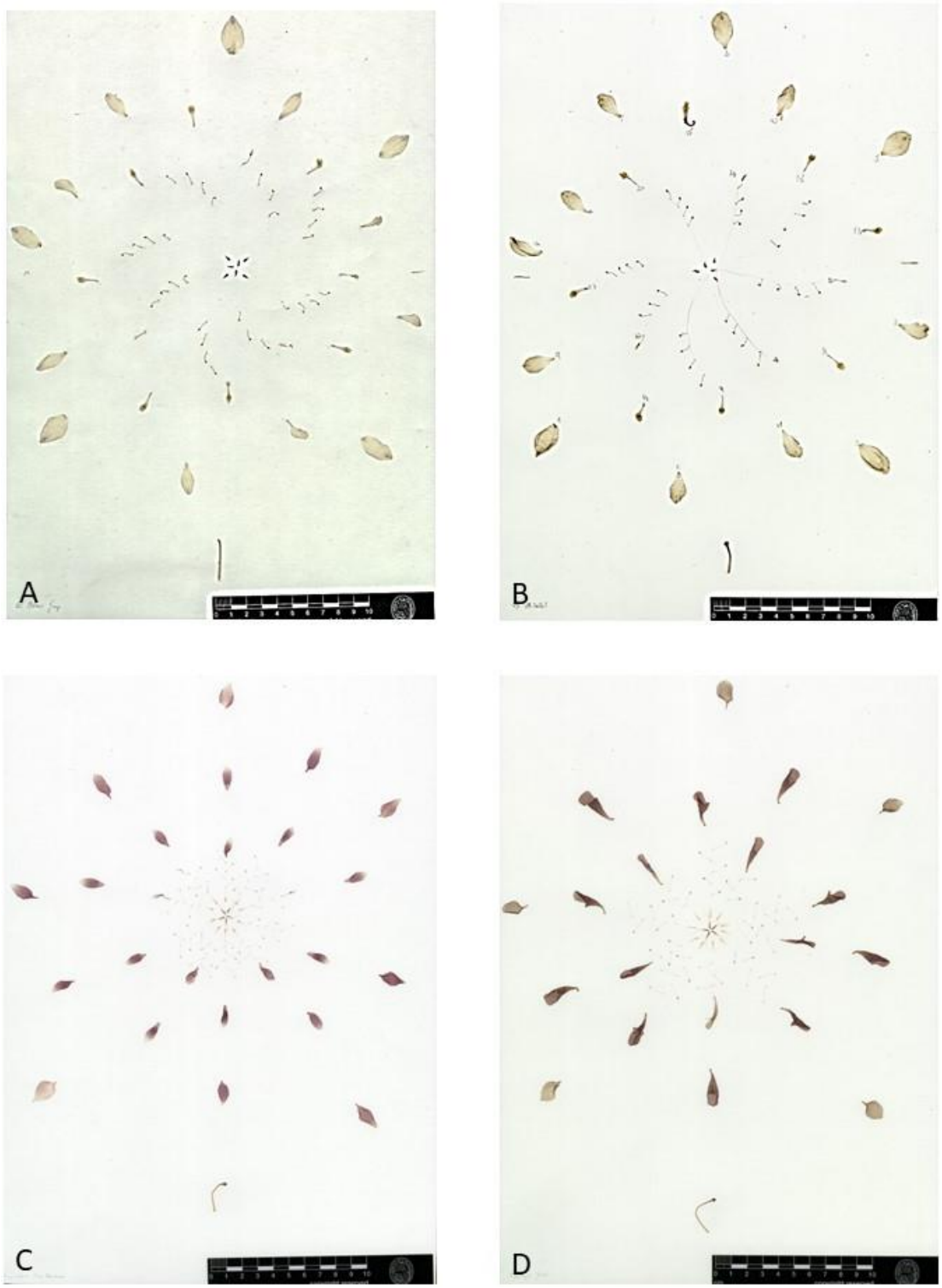

Figure 1. Exemples de fleurs éclatées des cultivars de *Delphinium* (A, B), et *Aquilegia* (C, D) conservées à P : A- *D.* × *pacific* 'Blue Jay'; B- *D.* × *pacific* 'Astolat'; C- *A. vulgaris* 'Nora Barlow'; D- *A. vulgaris* 'Leprechaun Gold'.

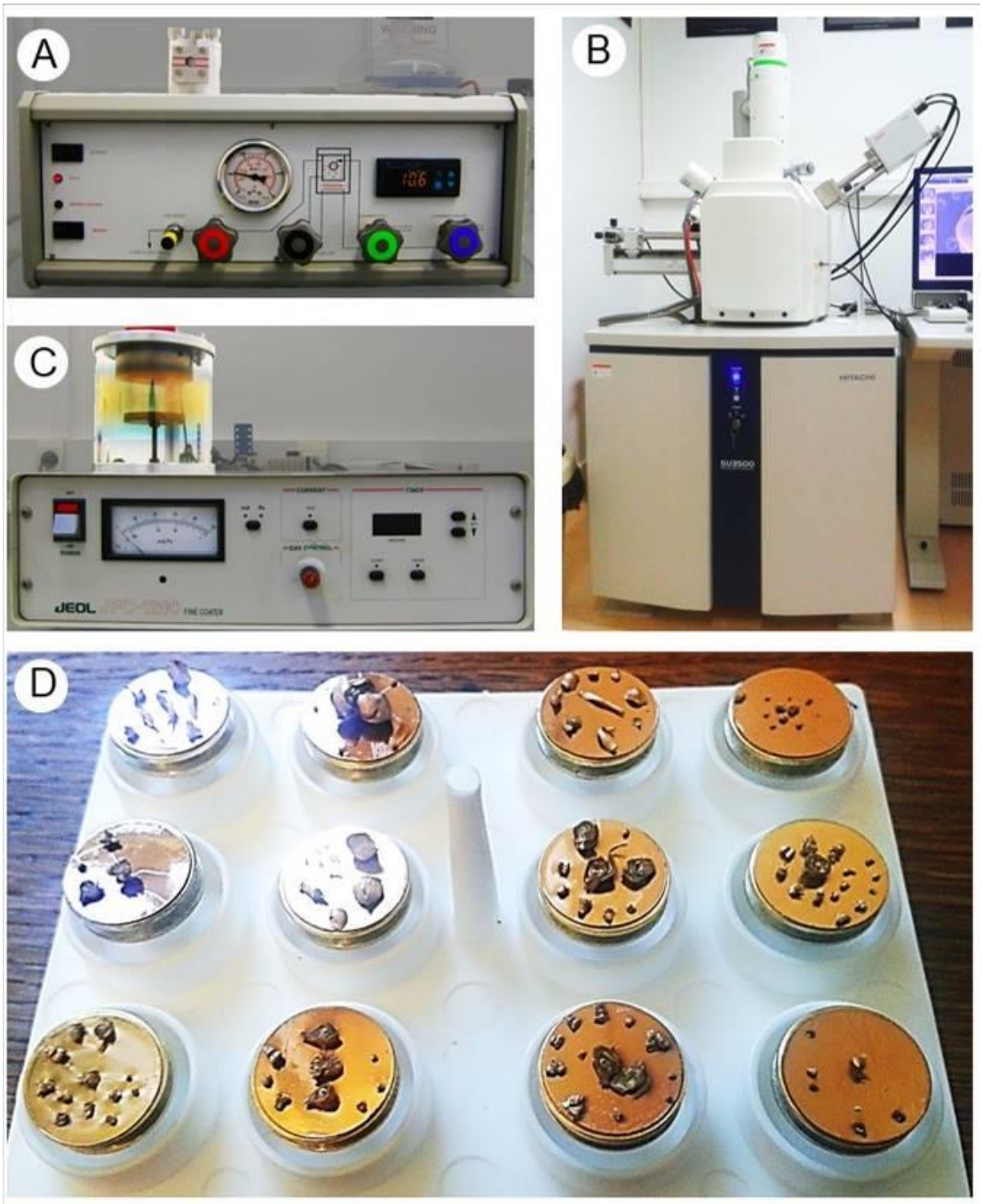

Figure 2. Appareils utilisés pour la préparation du matériel pour les observation au MEB : A- Sécheur au point critique Emitech K850; B- Microscope électronique à balayage (MEB) SU 3500 (Hitachi); C- Métalliseur Jeol LFC 1200; D- Matériel disposé sur les plateaux d'aluminium et métallisé, prêt pour être observé au MEB.

3. Observation du matériel au Microscope Electronique à Balayage (MEB)

Afin de pouvoir observer les différentes étapes du développement floral, ainsi que d'autres caractères invisibles à l'œil nu ou à la loupe binoculaire, j'ai observé au MEB des dissections de boutons floraux, des grains de pollen et des organes d'intérêt provenant de fleurs adultes. Sauf pour le pollen (plus de détails dans le chapitre 1), le reste du matériel a subi la préparation suivante :

1) Une déshydratation du matériel à travers une série de bain successifs d'éthanol (une moyenne de 10 minutes par bain) à 70%, 80% et finalement dans de l'éthanol absolu.

2) Un séchage au point critique au CO_2 (figure 2.A). Le principe de ce séchage est que, sous une pression et température adéquate, l'éthanol liquide dans lequel est immergé le matériel est remplacé par du CO_2 liquide qui, suite à une augmentation continue et régulière de la température et de la pression, passera par son point critique et se transformera quasi instantanément en CO_2 gazeux. Le matériel est ainsi complétement déshydraté sans subir de modifications morphologiques.

3) Le matériel déshydraté est ensuite monté sur des plateaux d'aluminium surmontés d'un film adhésif.

4) Le matériel déposé sur ce film adhésif est recouvert d'une fine couche d'or (90 secondes de métallisation) pour qu'il soit rendu conducteur d'électrons (figure 2 B-D).

5) Le matériel a été observé au MEB du Plateau Technique de Microscopie Electronique et de Microanalyses du MNHN (figure 2 C).

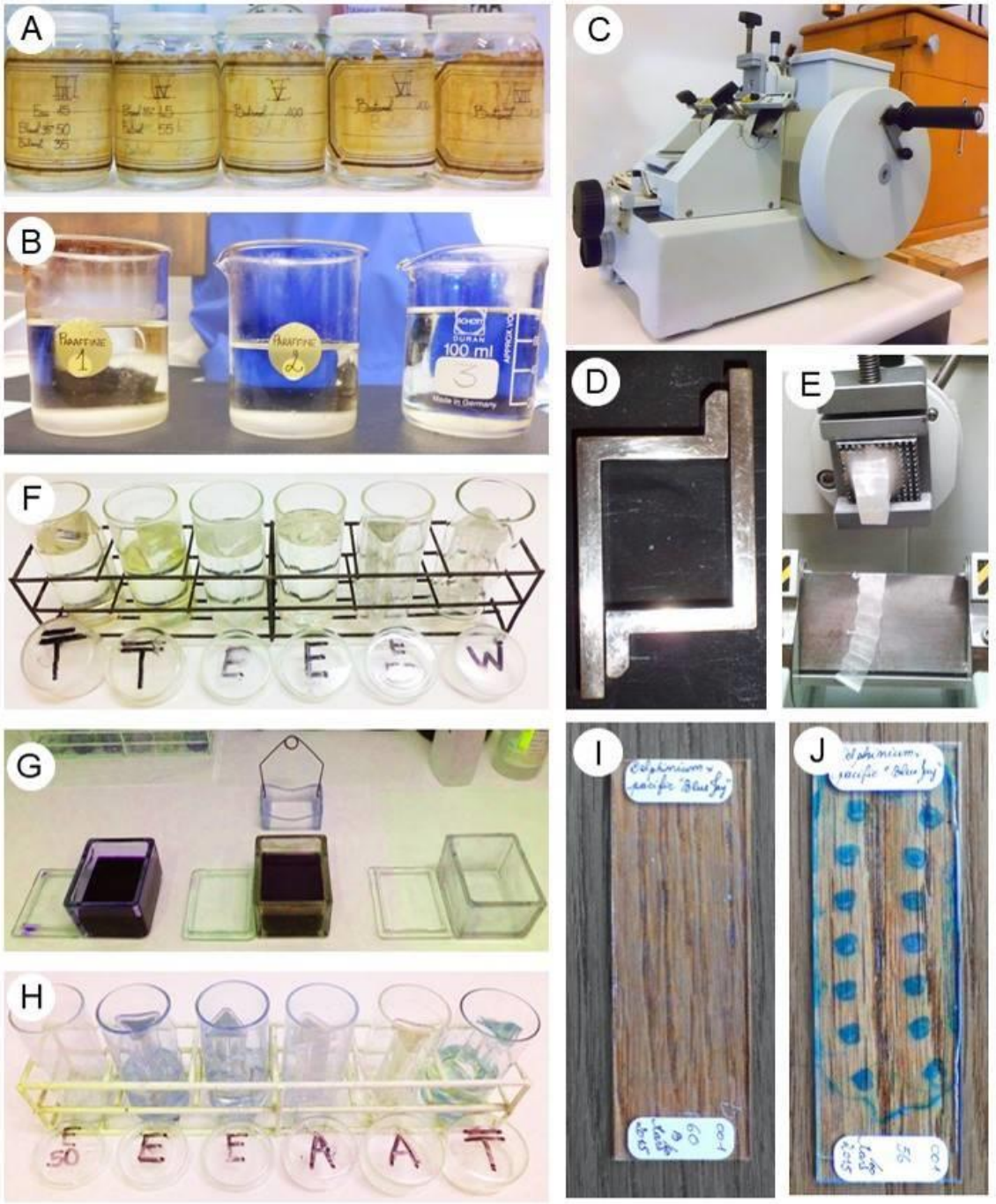

Figure 3. Etapes de préparation du matériel pour l'analyse anatomique : A- Bains de butanol pour la déshydratation du matériel ; B- Bain de paraffine pour inclusion du matériel ; C- Microtome rotatoire Leitz 1512 ; D- Barres de Leuckart ; E- Débit du cube de paraffine contenant le matériel ; F- Bains de toluène et éthanol pour la réhydratation du matériel ; G- Bains de coloration (Bleu Astra à gauche et Fuchsine de Zhiel à droite ; H- Bains de déshydratation à l'éthanol et l'acétone ; I- Lame avec le matériel fixé avant coloration; J- Lame avec le matériel fixé après coloration.

4. Etude anatomique

L'étude anatomique des boutons floraux m'a permis de reconstruire la vascularisation de la fleur. Cette étude a aussi été indispensable dans la compréhension de l'organisation et la disposition des organes floraux, ainsi que pour suggérer l'identité de chacun des organes, par comparaison avec la vascularisation des organes des fleurs à la morphologie typique. Dans cette étude j'ai analysé des boutons floraux frais et réhydratés. Pour cela le matériel a subi la préparation suivante :

1) Une déshydratation dans une série de bains de butanol à cinq concentrations différentes (eau/éthanol 95%/t-butanol : I) 150/50/35; II) 0/45/55; III), IV et V) 0/0/100). Les bains I), II), III) et VI) ont duré trois heures, le dernier a duré six heures.

2) Une inclusion dans trois bains successifs de paraffine à 60°C (chaque bain avec une durée de 6h sauf le dernier avec une durée de 12h), puis l'inclusion du matériel dans un cube de paraffine réalisé à l'aide de barres de Leuckart (un bouton par cube).

3) Les cubes ont été débités en coupes successives de 12 μm d'épaisseur. Les coupes ont été collées sur des lames à l'aide d'un mélange eau-gélatine.

4) La paraffine a été enlevée des lames avec du toluène, puis le matériel a subi une réhydratation avec successivement un bain d'éthanol à 95%, un autre d'éthanol à 50%, puis de l'eau.

5) Une coloration faite avec un bain dans du Bleu Astra 5%, suivi d'un bain de Fuchsine de Ziehl 10% (chaque bain a duré 5 minutes) afin de pouvoir différencier les tissus lignifiés en rouge et les parois pectocellulosiques en bleu.

6) Une déshydratation du matériel avec une succession de bains d'éthanol puis d'acétone.

7) Une fixation des lamelles.

La reconstruction de la vascularisation a été réalisée grâce à une sélection des dessins des coupes calquées à l'aide d'une chambre claire.

5. Utilisation des spécimens d'herbier dans les études en morphologie et anatomie

5.1. Introduction

Les études menées pendant ce travail de thèse m'ont amené à utiliser des spécimens d'herbier vieux de plus d'un siècle, conservés à l'Herbier national de Paris (P), et d'un herbier envoyé en prêt de l'herbier de Nancy (NCY), ainsi qu'à examiner les collections de *Delphinium* du bassin méditerranéen. Leur analyse m'a montré le potentiel de conservation morphologique et anatomique que peuvent représenter les herbiers. En effet, c'est grâce à la collection de l'Herbier national (P) que j'ai pu avoir accès très rapidement, facilement (deux étages au-dessus de mon bureau) et économiquement (gratuit) à des fleurs et des boutons floraux d'espèces provenant de Turquie. J'ai eu la chance d'être invité à écrire une note technique sur l'utilisation des spécimens d'herbier dans les études en morphologie et anatomie. Dans celle-ci, j'ai présenté, en collaboration avec Myreya Pinedo Castro (Faculté des sciences de l'Université Javeriana de Bogota, Colombie), les différentes méthodes actuelles de restauration et d'exploitation du matériel d'herbier utilisées dans les études en morphologie et anatomie et visant entre autres à répondre à des problématiques en alpha taxonomie.

Cette note technique a été publiée dans un numéro spécial de *Botany Letters* paru en 2018 et intitulé « Herbarium-based science in the 21st century ». Référence :

Espinosa, F., & Pinedo Castro, M. (2018). On the use of herbarium specimens for morphological and anatomical research. *Botany Letters*, *165*(3-4), 361-367.

Dû au fait que la publication n'a pas été publiée en open access, je présente ici la dernière version du manuscrit à être soumise avant publication. Pour cette raison, elle suit les indications de présentation de la revue, particulièrement en ce qui concerne la bibliographie.

On the use of herbarium specimens for morphological and anatomical research

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On the use of herbarium specimens for morphological and anatomical research

Herbarium material is crucial to answer questions in plant systematics and evolution. However, the exploitation of pressed and dried biological material from herbarium specimen can be challenging. Although some protocols have been designed to analyze fresh and dried material indistinctly, dried material may require additional treatments before being further exploited. In this technical note, we present general or more particular methods of restoration and exploitation of herbarium material used for morphological and anatomical studies in order to solve alpha systematic problems and address theoretical plant morphology and anatomy.

Keywords: Dyes, Herbarium specimen; Plant Anatomy; Plant morphology; Restoration

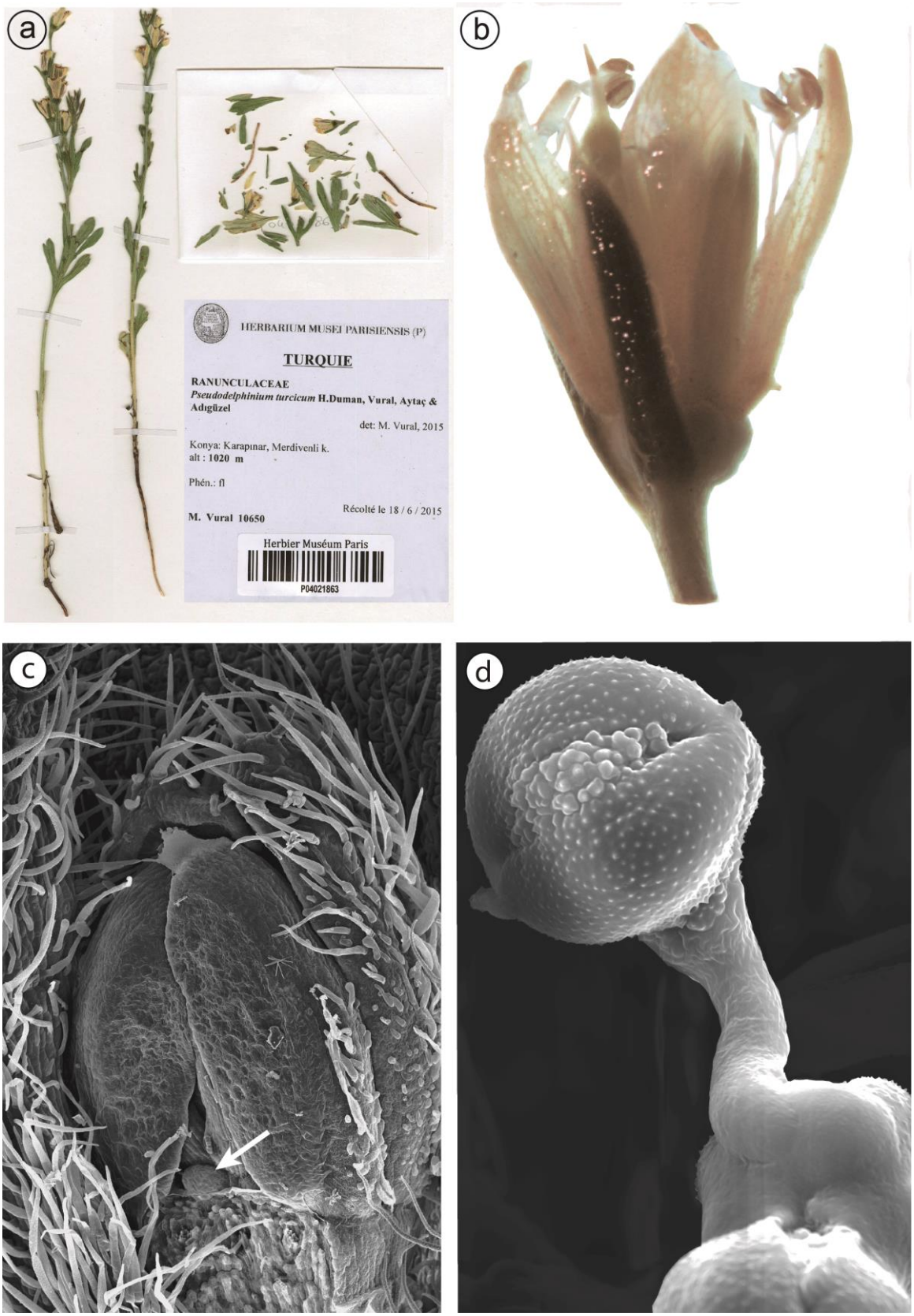
Introduction

Herbaria have been repositories of biodiversity and centres of taxonomic expertise since the 18th century (Fosberg 1946, Alberch 1993, Suarez and Tsutsui 2004). The materials conserved correspond to whole plants or representative parts that are dried, associated with a label gathering information about the collection, and stored for future researches (Figure 1a, Forman and Bridson 1989). Nowadays, efforts to make herbarium informations more accessible are undertaken by some governments or institutions mainly through online databases that facilitate data mining and taxonomic research (Beaman and Cellinese 2012, Campostrini et al. 2015, Page et al. 2015).

From the beginning of anatomical studies in plants, emphasis was put on some structural features such as cell walls (e.g. pectocellulosic, lignified, suberized, cutinized or silicified structures) and rather stable cell contents (e.g. starch grains, crystals, gums, resins or latex) (Malpighi 1675; Grew 1682). All of these are well preserved during the drying process of herbarium specimen preparation and recognizable after a suitable restoration protocol, aiming to 1) prevent these relevant characters from alteration, 2) regain the original volume and outline, and 3) prepare the tissues for further section and staining.

Dry herbarium material is not always as easily observable as fresh material could be. Indeed, pressed herbarium material is often deformed, making some morphological characters unrecognizable. It should be noted, however, that spirit collections (specimens preserved in fluid) better conserve the morphology of particular samples that would lose all or part of their scientific interest if pressed in two dimensions (e.g. fleshy fruits, densely packed inflorescences). Techniques and protocols have been designed to study the morphology and anatomy of plant structures from herbarium material that is typically dried, and more or less deformed. When selecting the most appropriate dye to stain a structure, one should take into consideration both the type of organ targeted (e.g. reproductive, vegetative) and the taxon (e.g. characterized by vegetative structures with a secondary growth, or lacking these structures). However, the techniques and protocols are not always standard and have sometimes to be adapted or adjusted for each sample (Langeron 1942, Keating 2014), depending on the research goal. Although most of the time morphological observations will not require the material to be treated, other studies, such as anatomical investigations or observations using scanning electron microscopy (SEM), require a deep transformation of some parts of the specimens.

This paper is a technical note, based on authors' experience and for the most part on a literature review on some aspects of the use of herbarium specimens for morphological and anatomical studies. First, we briefly introduce general principles in anatomy researches. We then present studies for which there was no destructive handling (i.e. direct observation without treatment or restoration allowing subsequent drying) of the herbarium material and studies for which an invasive analysis of the herbarium material was required. Finally, we discuss some other cases where the herbarium material should be a pertinent tool for theoretical studies. We focus in this note on the study of vegetative and floral morphology using optical tools, and on the study of anatomy using dyes.


a
HERBARIUM MUSEI PARISIENSIS (P)
TURQUIE
RANUNCULACEAE
Pseudodelphinium turcicum H.Duman, Vural, Aytaç & Adıgüzel
det: M. Vural, 2015
Konya: Karapınar, Merdivenli k.
alt : 1020 m
Phén.: fl
Récolté le 18 / 6 / 2015
M. Vural 10650
Herbier Muséum Paris
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b
c
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Figure 1. Microscopic observations of herbarium specimens of *Delphinium turcicum*. (a) Herbarium specimen, *M. Vural 10650a* (right sample) and *10650b* (left sample, scale = 2 cm). (b) Detail of a flower restored with an aqueous solution of 10% ammonia (scale = 0,5cm). (c) SEM micrograph of floral bud (scale = 1 mm. The arrow points to a young organ with an arrested development considered as a petal primordium. (d) SEM micrograph of germinating pollen grain on a stigma (scale = 10 µm). Adapted from Espinosa et al. (2017).

General methods of restoration and exploitation of herbarium material

Boiling or tepid water, or, better, solutions of washing powder or liquid detergent have been used for restoring plant tissues. Caustic soda and potash solutions have also been used, but too often caused a decomposition of lignin, which is no more stainable, and even a disintegration of tissues. Restoration by hot lactophenol after Amann or chlorallactophenol was recommended (Dop and Gautié 1928) but samples, suitable for morphological studies, are not suitable for paraffin embedding.

Aqueous solutions of 5-10% ammonia (NH_4OH) at 60°C can solve this issue: samples are regaining their original size, without wall alteration and with a gradual removal of tannins, so that stainable tissues are obtained. Some artefacts cannot be avoided: e.g. accurate corolla morphology cannot be retrieved in Convolvulaceae, and some kinds of trichome may be lost. A post-fixation in FAA is needed, so that ammonia is neutralized and walls become wholly stainable. Samples may be then kept in the classical preservative mixture (Glycerol/Water/95% Ethanol, equal volumes) allowing them to be easily examined and dissected. When tissues are highly heterogeneous in a sample (e.g. fibers or sclereids in spongy parenchyma, as in many magnoliaceous flowers or palm stipes), it is recommended to use additional 4% ethylenediamine baths (Carlquist 1982), in order to prevent any tearing. After embedding in hard paraffin (melting point 60-70°C) it is useful to put the block in water for 48 hours or more, so that walls rehydrate and recover some elasticity (Gerlach 1984). Entire and seriate paraffin sections may be thus easily done at thicknesses of 10-20 µm, allowing usual staining procedures (e.g. Toluidine blue after Sakai, or combination Astrablue/Ziehl's Fuchsine).

Non-destructive studies of herbarium material

Morphological studies without treatment of herbarium specimens

Morphological descriptions mostly rely on external characters that can be observed by eye or with a stereomicroscope. No specific treatment is needed to make the structures observable and interpretable.

Gymnosperm and angiosperm general morphology

In the classification of the genus *Gnetum* L. (Gnetaceae) proposed by Biye, Balwill and Cron (2014), the authors examined 300 herbarium specimens using a stereomicroscope. They statistically analyzed the measurements, revised the description of the genus and made a comparison with the original description. The systematics of the group was revised and the authors proposed two new species for the genus. Similarly, in the south Andean genus *Laretia* Gillies & Hook (Apiaceae), Fernández, Ezcurra, and Calviño (2016) made measurements of the lamina of the leaves on the scanned pictures from the JSTOR Plant Science online database (http://plants.jstor.org/) using a computer process, which is by definition non-invasive.

Corney et al. (2012) evaluated the efficiency of morphometric analyses using scanned pictures from 1895 specimens of *Tilia* L. (Malvaceae) using a software that automatically extracted leaf characters such as length and width. The distances they measured were smaller than the values measured from cultivated specimens from this genus.

Angiosperm reproductive organ morphology

Chartier et al. (2016) used sample trans-illumination and traditional morphometrics to study flower morphology in afro-montane specimens of *Delphinium* L. (Ranunculaceae). Trans-illuminated herbarium flowers were analyzed with geometric morphometrics by Chen et al. (in this Special Issue) to study the shape variation at the level of the tribe Delphinieae (Ranunculaceae).

Espinosa et al. (2017) observed pollen grain morphology of three species of *Delphinium* (Ranunculaceae) aiming to clarify the taxonomical position of one of them. The authors fixed the non-restored material onto an adhesive tape and coated it with gold. In this case, the particular pattern of aperture of the pollen grain and the variations of grain shape allowed the authors to hypothesize that one of the species might correspond to a hybrid population.

Looking for the interpretation of morphological and anatomical divergences of some populations of the genus *Ixora* L. (Rubiaceae) in New Caledonia, Mouly et al. (2016) studied fruit morphology and texture combining information from alcohol preserved specimens and from field notes. They noticed that fruit texture varied in response to an ecological gradient. In the study of the south Andean genus *Laretia*. (Apiaceae), Fernández, Ezcurra, and Calviño (2016) used morphological traits from hydrated fruits of 80 specimens for their taxonomic revision of the genus.

Fern morphology

Dubuisson et al. (2013) conducted an exhaustive morphological study of numerous herbarium specimens, analyzing stipe, frond and sorus morphology to clarify the systematics of the fern genus *Crepidomanes* C.Presl (Hymenophyllaceae). This analysis allowed them to circumscribe morphological groups and provide names for unidentified and problematic specimens.

Restoration of herbarium material

Restoration of material is needed to recover the original form of the structure that was pressed during the drying of the specimen, facilitating morphological descriptions and/or preparing material for subsequent studies. Different protocols are available, depending on the taxon and on the structure investigated.

Angiosperm leaves restoration

To describe leaf teeth types from specimens of the genus *Casearia* Jacq. (Salicaceae), Fernandes et al. (2016) rehydrated herbarium material using the protocol proposed by Smith and Smith (1942) based on boiling water and a solution of 2% potassium hydroxide. In order to observe and describe venation patterns in *Didymaea* Hook.f. (Rubiaceae), authors cleared the material in a sodium hydroxide solution, transferred it in sodium hypochlorite and finally washed it with tap water (Pacheco-Trejo, Terranzas, and Ochoterena 2009).

Leaf adaptations to xeric conditions in two species of the genus *Satanocrater* Schweinf. (Acanthaceae) were observed on softened material (Tripp and Fatimah 2012). Leaves were removed from herbarium specimens and softened in 1.5% Contrad 70 solution as proposed by Schmid and Turner (1997), rinsed with deionized water then soaked in FPA (a 1:1:18 ratio of 37% formaldehyde-propionic acid-70% ethanol).

Angiosperm flowers and pollinaria restoration

To study floral morphology in *Delphinium* (Ranunculaceae), Espinosa et al. (2017) restored the material using a 10% NH_4OH aqueous solution then fixed it in FAA (90% ethanol 70%, 5% formalin, 5% acetic acid, Figure 1b). Mouly et al. (2016) examined flowers of *Ixora* (Rubiaceae) clearing their material using a 50% NH_4OH aqueous solution before fixing the material with FAA.

A study of the pollinaria was conducted in *Capanemia* Barb.Rodr. (Orchidaceae) (Buzzatto et al. 2012) based on herbarium material restored with concentrated ammonium hydroxide and described using a calibrated microscope. In this study, the authors described fresh flowers and rehydrated pollen to clarify the systematics of the genus.

Fern root restoration

Schneider (2000) used a 10% solution of Tinoventin to soften roots from herbarium specimens to conduct an anatomical study of the fern tribe Trichomaneae H.Schneider (Hymenophyllaceae).

Invasive treatments

Many structures conserved in herbarium specimens cannot be directly observed and studied but become exploitable only when using specific techniques and tools. Here, we will focus on SEM observations and leaf and floral anatomical studies by staining and sectioning material. Other techniques such as transmission electron microscopy will not be treated here.

Leaf morphology analysis of angiosperms using SEM

Leaf surface can be analyzed using SEM to identify particular structures in the framework of systematic studies. Chen, Tan, and Wong (2015) analyzed the silvery lower leaf lamina surface of the leaves in *Timonius* DC. (Rubiaceae) excising a portion of leaf from the herbarium specimens. The material was attached onto an adhesive tape and coated with gold. Hairs producing the silver color were observed and characterized with SEM. Differences in hair morphology and density were identified among the species groups, establishing the taxonomic utility of leaf micromorphology in this genus.

Fernandes et al. (2016) used SEM to observe the presence and characterize the theoid teeth on the leaf margin in *Casearia* (Salicaceae). In their work, rehydrated material was then dehydrated in an ethanol series, critical point dried using CO_2, fixed and coated with gold.

Sosnovsky et al. (2017) softened leaves of *Rhododendron* L. (Ericaceae) using an equal solution of distilled water, glycerin, and alcohol to observe the anatomy in light microscopy. Samples of herbarium leaves were coated with gold and observed using SEM.

Floral morphology analysis using SEM

Developing structures such as floral buds can be studied from herbarium material, which makes it possible to propose hypotheses on the organ development and floral evolution of the studied plant trough detection of time-frozen events (Figure 1c, and d, Espinosa et al. 2017). The identification of young peloric flowers with an arrested development in *Delphinium turcicum* (H.Duman, Vural, Aytaç & Andigüzel) Espinosa (Ranunculaceae) allowed the authors to make hypotheses about the developmental origin of these flowers.

Leaf anatomy in angiosperms

In order to describe the structure of glands on the leaf margins in *Casearia* (Salicaceae), Fernandes et al. (2016) used a solution 50% alcoholic fuchsine according to Vasco et al. (2014) and toluidine blue at pH 4.7. Authors used methacrylate resin to embed and cut the material using an automatic rotary microtome before mounting it on slides with resin.

To study leaf anatomy in *Didymaea* (Rubiaceae), Pacheco-Trejo, Terrazas, and Ochoterena (2009) revealed the venation against the background by staining cleared herbarium material with safranin O in 96% ethanol, then de-staining it partly with 100% ethanol. Leaves were then mounted with synthetic resin.

Anatomy of reproductive structures in Angiosperms

Mouly et al. (2016) analyzed the anatomy of *Ixora* (Rubiaceae) stigmas after dehydrating the rehydrated herbarium material using t-butylic alcohol, and embedding it in paraffin. Then, the material was sectioned and stained with an aqueous Astrablue 0.5% and Ziehl's Fuchsine 10% combination and mounted in Eukitt.

To study floral anatomy of Convolvulaceae, several and prolonged 20% ammonia baths to clear the tissues were required (Deroin 1992; e.g. in *Humbertia*, Figure 2a, b).

To complete species descriptions of *Laretia* (Apiaceae), Fernández, Ezcurra, and Calviño (2016) rehydrated fruits in hot water with few drops of detergent, then sectioned and observed them using a stereomicroscope.

Fern stem anatomy

In Cyatheaceae, White and Turner (2017) stained stems with safranin and fast green according to Johansen (1940).

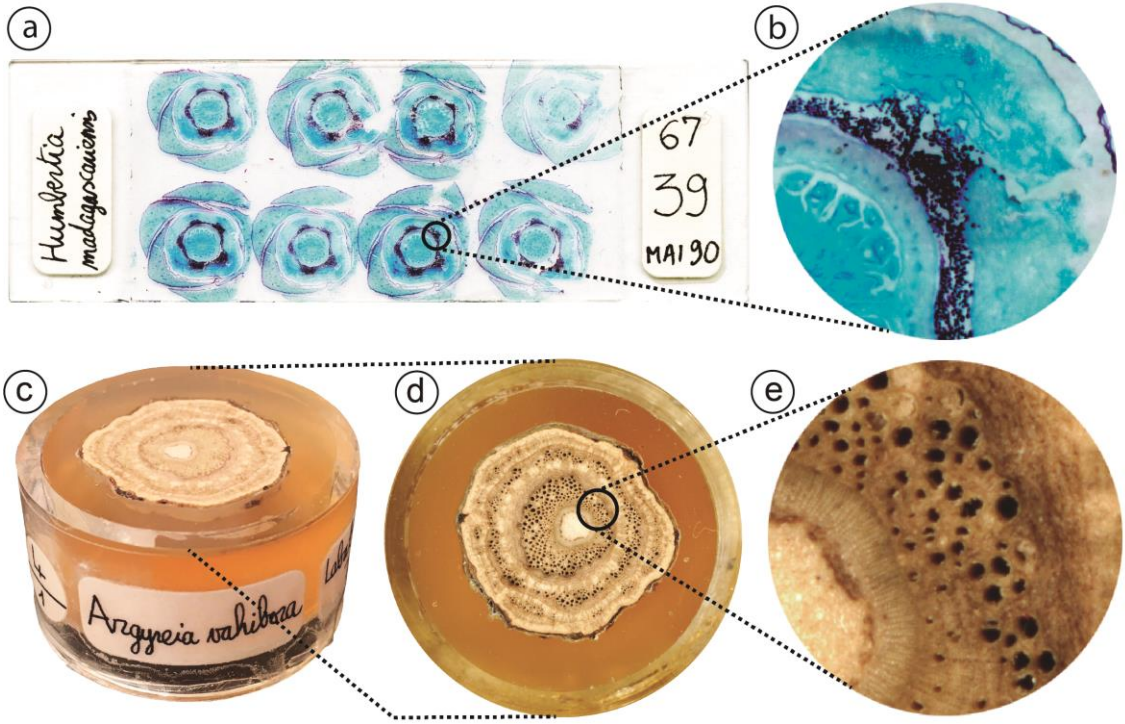

Figure 2. Stem and floral bud anatomy in two Malagasy species of Convolvulaceae. (a and b) Section of *Humbertia madagascariensis* Lam. floral bud (voucher: *Service Forestier 1955*). (a) Serial cross-sections colored and mounted in Eukitt (scale= 1 cm). (b) Detail of a cross-section showing different kinds of stained tissues (scale= 1 mm). Material coming from Deroin (1992). (c, d and e) *Argyreia vahibora* Deroin stem section (from the specimen *Labat & Deroin 2325;* P00427134) impregnated in Eukitt resin and quick-hardening araldite. (c) General view (scale= 1 cm). (d) Section view (scale= 1 cm). (e) Detail of the section view showing different tissues of the woody twining stem (scale= 1 mm). Material coming from Deroin and Falaise (1995).

Uses in theoretical plant morphology and anatomy

In some cases, restored samples are prepared by quick methods (e.g. hand sections, no staining and mounting in glycerol or glycerol/gelatine) before examination under a stereoscopic microscope. Precious (e.g. types), rare and remarkable samples can be dehydrated and permanently mounted (e.g. in Euparal, after ethanol 95%). Such mountings are useful for nodal anatomy, and wide views of wood and bark. Double-inclusion turned out to be interesting for research, teaching and even museographical purposes (Deroin and Deroin 1997): the sample is first impregnated in Eukitt resin, then dried in the oven, embedded in a quick-hardening araldite, and polished to reveal the histological arrangement. That should be studied by light reflection only as in metallography, all hollow structures emphasized by the slight retraction of Eukitt. Conclusive results were obtained with the stems of some woody lianas in Convolvulaceae (Deroin and Falaise 1995; Figure 2b, c, d). These blocks are easy to manipulate and to store. Tissue colours and textures are often contrasting, and nicely underline the complex anatomy due to secondary and tertiary xylem and phloem.

Using a reliable protocol allows to describe accurately the floral anatomy, especially the vasculature, even for rare taxa, and even for those known by the holotype only. Placentary supply was analysed in the endemic Malagasy genus *Takhtajania* Baranova & J.-F.Leroy (Winteraceae), based on a herbarium flower collected by Perrier de la Bâthie 90 years before (Deroin and Leroy 1993), and then compared to that in fixed fruits from living individuals found again six years later. In the same way, a short study of the floral vasculature of another endemic Malagasy genus *Fenerivia* Diels (Annonaceae), considered extinct at that time (Deroin 2007), stimulated further a new interpretation of morphogenetic evolution of flowers in Annonaceae (Saunders 2010) and allowed to recognize several other species until then considered as belonging to *Polyalthia* Blume (Annonaceae). All data provided by restored materials are fragmentary and need to be compared to better-known structures (i.e. from living and well-fixed samples), and interpreted in a developmental frame when available.

Conclusions

The exploitation of herbarium specimens remains an essential tool in anatomy, systematics and in the understanding of plant evolution. Although some techniques can

be destructive, the transformed material (e.g. stained sections on slides, clearings, inclusions) is to be safely kept in dedicated repositories, linked to the collections, for potential future studies. All these processes will be made much easier by a parallel computerization of herbarium sheets, samples and slides in tight link with librarian resources (books, articles, illustrations), which appears significant and feasible only by several specialists collaborating in a well-defined project, both in scope and time.

Disclosure statement

No potential conflict of interests was reported by the authors.

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Myreya Pinedo Castro is a junior researcher at the Biology Department at the Pontifical University Xaverian. She investigates the population genetics of neotropical mammals and flower evolution in Orchidaceae and Ranunculaceae.

FE wrote the first draft of this manuscript and both co-authors prepared the last version of it.

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Chapitre 1 : Histoire d'un *Delphinium* avec des fleurs à symétrie radiale

1. Introduction

Au sein d'une espèce, les déviations du plan général d'organisation florale sont rarement maintenues du fait qu'elles touchent des organes participant à la reproduction de la plante. En effet, les variations du plan de base des fleurs, généralement bien adaptées à un type particulier de pollinisation, peuvent s'avérer désavantageuses pour la reproduction et donc sans issue sur le plan évolutif. Les organismes tératologiques naturels sont donc rares mais leur étude peut nous donner des informations importantes sur l'origine des nouvelles formes dans la nature. Le registre d'individus tératologiques naturels est une opportunité rare d'analyse, d'autant plus quand les populations concernées se maintiennent à l'état sauvage pendant quelques années ou dizaines d'années.

En 2012, un groupe de chercheurs a décrit une population de plantes présente dans le bassin du lac salé de Tuz Gölu en Turquie. Les individus de cette population présentent des caractéristiques morphologiques de l'appareil végétatif et reproducteur qui se retrouvent chez les Ranunculaceae. Ils ont considéré les plantes de cette population comme étant morphologiquement proches du genre *Delphinium* L. Toutefois, et à cause d'une particularité importante du périanthe (unisérié et à symétrie radiale) (figure 1), ils ont décidé de l'inclure dans un nouveau genre, *Pseudodelphinium* H.Duman, Vural, Aytaç & Adigüzel, et de la décrire *a fortiori* comme une nouvelle espèce, *Pseudodelphinium turcicum* H.Duman, Vural, Aytaç & Adigüzel.

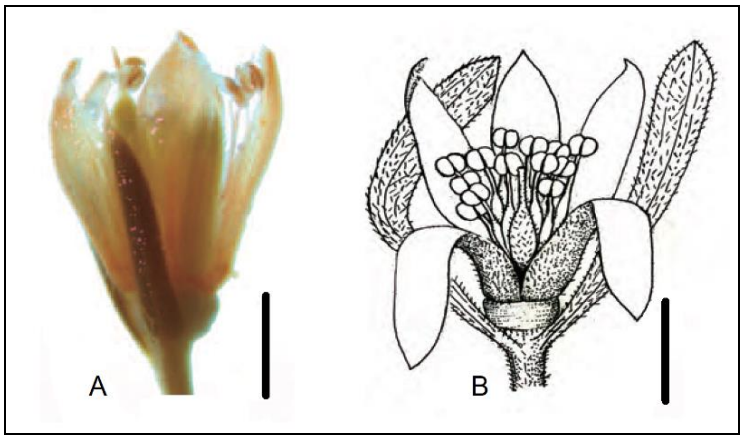

Figure 1. A- Fleur restaurée de *Pseudodelphinium turcicum*, image modifiée de Espinosa et al. 2017; B- Dessin de la fleur de *P. turcicum* modifié de Vural et al. (2012). Echelle = 0,25 cm.

Suite à l'analyse de la description de cette espèce et des images disponibles, nous avons décidé de l'étudier plus en détail en nous posant les questions suivantes : quelle est la position phylogénétique de la nouvelle espèce *Pseudodelphninium turcicum* par rapport au genre *Delphinium* ? Et quelles sont les bases développementales gouvernant une telle morphologie ?

Pour répondre à ces questions, j'ai 1) réalisé une étude morphologique des fleurs adultes (afin de mieux comprendre la morphologie du groupe), des observations de boutons floraux (afin d'avoir une vision plus détaillée des organes présents sur la fleur) et des grains de pollen (le pollen étant une source de caractères pour la détermination taxonomique ainsi que pour la compréhension de certains traits biologiques), 2) reconstruit une phylogénie moléculaire (pour trouver la position de la nouvelle espèce au sein de la famille des Ranunculaceae) et 3) réalisé une étude anatomique (afin de reconstruire la vascularisation de la fleur et inférer l'identité probable des organes floraux).

Cette étude a été publiée dans *International Journal of Plant Sciences* parue en 2017. Référence :

Espinosa, F., Deroin, T., Xiang, K. L., Wang, W., Pinedo Castro, M., Byng, J. W., Aytaç, Z., Nadot, S. & Jabbour, F. (2017). The Turkish Endemic *Pseudodelphinium turcicum* (Ranunculaceae): An unusual population of *Delphinium* with peloric flowers that has persisted in the wild for 20 years. *International Journal of Plant Sciences*, *178*(7), 546-555.

Dû au fait que l'article n'a pas été publié en open access, je présente la dernière version du manuscrit à être soumise avant publication. Pour cette raison, elle suit les indications de présentation de la revue, particulièrement en ce qui concerne la bibliographie.

2. Article : L'endémique turque *Pseudodelphinium turcicum* (Ranunculaceae) : une population déviante de *Delphinium* présentant des fleurs péloriques et qui se maintient à l'état sauvage depuis plus de 20 ans

The Turkish endemic *Pseudodelphinium turcicum* (Ranunculaceae): an unusual population of *Delphinium* with peloric flowers that has persisted in the wild for 20 years

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Shortened title: *Pseudodelphinium* is a *Delphinium* with peloric flowers

Abstract

Premise of research. The Turkish endemic *Pseudodelphinium turcicum* (Ranunculaceae) was described by Vural et al. in 2012 based on a single population found in the salt lake basin of Tuz Gölü, Konya Province. Although, the authors noticed morphological similarities between the plants and *Delphinium* or *Garidella*, they highlighted unusual features, which led them to describe a new genus consisting of a single species, *P. turcicum*. The aim of this study was to investigate the morphological particularities and evolution of *Pseudodelphinium*.

Methodology. We carried out morphological, anatomical, and palynological studies on individuals of *P. turcicum*. We also conducted a molecular phylogenetic analysis to identify the closest relatives of this species.

Pivotal results. This combination of approaches shows that the species belongs in the group of Mediterranean species in *Delphinium* subg. *Delphinium*. Based on our sampling *P. turcicum* is more precisely sister to the Turkish endemic *D. venulosum*. The perianth of *P. turcicum* flowers consists of tepals that are anatomically speaking similar to the sepals of *Delphinium*. A second type of perianth organs was evidenced. They are located between the tepals and the stamens, and their development is arrested at an early stage, therefore being comparable to the ventral petals of *Delphinium* flowers. The pollen of *P. turcicum* is heteromorphic.

Conclusions. We conclude that the population of *P. turcicum* is an unusual population of *Delphinium* presenting peloric flowers (through ventralization) that has been maintained in the wild for at least two decades. We provide hypotheses concerning the identity of the floral organs, as well as an explanation for the origin of the floral particular morphology.

Keywords: anatomy, *Delphinium*, morphology, palynology, peloria, *Pseudodelphinium*

Introduction

The buttercup family (Ranunculaceae), belonging to the order Ranunculales (Wang et al. 2009; APG 2016), arose in the early mid-Cretaceous (c. 108 Ma) and went through a rapid diversification during the Campanian between 83 and 74 Ma (Wang et al. 2016a). Currently, the family is divided into five subfamilies and consists of about 43 genera and 2346 species (Christenhusz & Byng 2016), mostly herbaceous (Stevens 2001 onwards, Wang et al. 2009). Even though the family has a worldwide distribution, most species are restricted to the north temperate regions (Tamura 1993, Stevens 2001 onwards). The tribe Delphinieae (subfamily Ranunculoideae) comprises c. 700 species, approximately a quarter of all species diversity within the family and is mainly distributed from the Mediterranean Basin to Korea and Japan, Siberia and North America (Jabbour & Renner 2012a, but for the tropical African species of *Delphinium* see Chartier et al. 2016). In Turkey, the family is represented by c. 230 species distributed among more than 19 genera (Güner et al. 2012), *Ranunculus* being the largest genus with 100 taxa 20 of them endemic for Turkey (Davis et al. 1965). The genus *Delphinium* has 33 taxa and 17 of them are endemic (Günter et al. 2012). *Delphinium sensu lato* (*i.e.* including *Consolida* and *Aconitella* [*sensu* Jabbour & Renner 2011]) comprises 58 species (Ilarslan 1996, Özçelik & Korkmaztürk 2012), which makes it one of the most species rich genera of angiosperms in Turkey. Davis et al. (1965) noted that *Delphinium* is a taxonomically 'difficult' genus in Turkey due to the fact that many species are connected by hybrid intermediates.

Recently, Vural et al. (2012) reported the occurrence of a population in central Turkey, in the basin of the large Salt Lake Tuz Gölü in Konya Province (fig. 1). The lake is one of the largest salt lakes in the world and the surrounding ecosystems are known for their high level of plant endemism. Öztürk & Çetin (2013) reported 54 species endemic to the region. According to Vural et al. (2012), the single population of *P. turcicum* known to date was collected for the first time in 1997 (*M. Vural 7793*, kept in GAZI and KNYA [herbarium acronyms follow Thiers 2017]) and is composed of short and virgate herbaceous plants with, from the base to the top of the plant, leaves divided in three linear segments and simple linear leaves. The inflorescences are racemose with pubescent bracts and bracteoles similar to the leaves. Flowers are actinomorphic with five free petaloid tepals, numerous free stamens, and three free carpels longitudinally striate (Vural et al. 2012). These plants show features of annual species of *Delphinium*

L. but due to significant differences in the floral structure, the authors suggested that plants from this population could also relate morphologically to *Garidella* L. However, without any additional argument (e.g. molecular phylogeny) that could have helped them interpret those morphological particularities, Vural et al. (2012) considered that these plants should be attributed to a new genus, *Pseudodelphinium* H.Duman, Vural, Aytaç & Adigüzel (Ranunculaceae) comprising a single species, *Pseudodelphinium turcicum* H.Duman, Vural, Aytaç & Adigüzel.

The aim of this paper was to improve our knowledge on the monotypic genus *Pseudodelphinium* and to better understand its morphological peculiarities and evolution. To do this we carried out a molecular study to place the genus in the phylogenetic tree of Ranunculaceae. Then we undertook a morphological-anatomical study to describe the particular floral organization in the genus. This led us to provide an updated taxonomic treatment for the taxon. In this paper, the vocabulary relative to the perianth organs of *Delphinium* follows Rasmussen et al. (2009).

Fig. 1 Map of Turkey. The zoomed section (Imagery ©2017 DigitalGlobe, Map Data ©2017 Google, scale = 2 km) corresponds to the locality of the single population of *Pseudodelphinium turcicum* currently known: "Turkey. C4 Konya : Karapınar to Konya road, 30 km, Merdivenli village, 1020 m" (Vural et al. 2012). The inferred GPS coordinates are 37.722779, 33.201391. Yellow lines indicate the road.

Materials and Methods

Taxon sampling, DNA sequencing, alignment, and phylogenetic analyses

Plastid and nuclear markers were sequenced from four specimens from the known population of *P. turcicum*, namely *M. Vural 10643 & O. Tugay* (kept at P; [barcode P04021862], available following the link https://science.mnhn.fr/institution/mnhn/collection/p/item/p04021862), *M. Vural 10650a*, and *M. Vural 10650b* (kept at P; [P04021863], https://science.mnhn.fr/institution/mnhn/collection/p/item/p04021863; these two individuals are mounted on a single sheet), and *M. Vural & O. Tugay 10643.1* (kept at PE; sequenced by Xiang et al. (2017) for a study focusing on *D.* subg. *Delphinium*). DNA isolation and sequencing relied on the DNeasy 96 Plant Kit (Qiagen) and the DNeasy Mini Plant Kits (Tiangen Biotech, Beijing, China), and the universal primers of Taberlet et al. (1991) for amplifying and sequencing the *trn*L intron and adjacent *trn*L-*trn*F intergenic spacer. The nuclear ribosomal internal transcribed spacer ITS-1, the 5.8S gene, and ITS-2 were amplified with the external primers of White et al. (1990). PCR products with the expected size were detected by agarose gel electrophoresis before sequencing (Eurofins Genomics, MWG/Operon, Les Ulis, France). Sequencing reactions for the *M. Vural & O. Tugay 10643.1* accession were conducted using BigDye Terminator kits (Applied Biosystems, Foster City, CA, USA) with an ABI 3730 DNA automated sequencer (Applied Biosystems).

Sequence assembly of forward and reverse strands was carried out in MEGA v.7 (Kumar et al. 2016), and sequences were subjected to a BLAST-search in GenBank. All newly obtained *trn*L-F and ITS sequences of *P. turcicum* were very similar (more than 98% identity) to sequences from species belonging to *D.* subg. *Delphinium*. In order to clarify the systematic placement of *Pseudodelphinium*, we thus restricted our sampling to the tribe Delphinieae, to which *Delphinium* belongs. Three accessions of *P. turcicum* (*Vural 10650a* was discarded due to an unsuccessful sequencing of the ITS marker) were added to the *trn*L-F + ITS alignment of Wang et al. (2013) which provides the most complete sampling of Delphinieae accessions to date and includes two outgroup accessions from the species *Nigella damascena* and *Megaleranthis saniculifolia*. Sequences were aligned by eye and the final alignment was 2193 position long and comprises 290 accessions. The phylogenetic reconstruction relied on maximum likelihood (ML) as implemented in raxmlGUI (Silvestro & Michalak 2012), using the GTR + G + I model (the most general evolutionary model proposed). Statistical support

for nodes was assessed by bootstrap resampling of the data under the same model (500 replicates).

Plant material for the morphological-anatomical study

The morphological-anatomical study was carried out on *P. turcicum* as well as on two species of *Delphinium*. We selected *D. venulosum* and *D. virgatum* because *P. turcicum* is sister to *D. venulosum* and is nested within the group of Mediterranean *Delphinium* in *D.* subgenus *Delphinium*, of which *D. virgatum* is a representative. In addition, a phylogenetic analysis focusing on *D.* subgenus *Delphinium* using four markers (*trnK-matK*, *trnS-trnG*, *trnL-F*, and ITS) showed that the accessions of *P. turcicum* (*M. Vural & O. Tugay 10643.1* [PE], also included in this study, and *M. Vural & O. Tugay 10643.2* [PE]) formed a strongly supported clade sister to *D. virgatum* and *D. venulosum* (bootstrap percentage [BP] = 95, posterior probability = 1.0; Xiang et al. 2017). An additional (and geographic) argument is that *D. venulosum* (Turkish endemic) and *D. virgatum* grow in the vicinity of the area where *P. turcicum* was collected (Davis et al. 1965).

Flowers, buds, and pollen grains of *D. venulosum* and *D. virgatum* were taken from dried specimens at P herbarium, National Museum of Natural History Paris. The material for *D. venulosum* was obtained from the Turkish specimen *P.E.E. Sintenis 4627* [P00201219]. Material of *D. virgatum* was taken from the Croatian specimen [P02681093] (Unio itineraria, 1829). Material of *P. turcicum* was sent by M. Vural (*M. Vural 10643 & O. Tugay* and *M. Vural 10650a* and *10650b*) and is now kept at P. Floral buds for the anatomical study were sampled from the specimen *M. Vural & 10643 O. Tugay*, and young floral buds and pollen grains for the morphological analysis were sampled from the specimens *Vural 10650a* and *10650b* (fig. 2*A*).

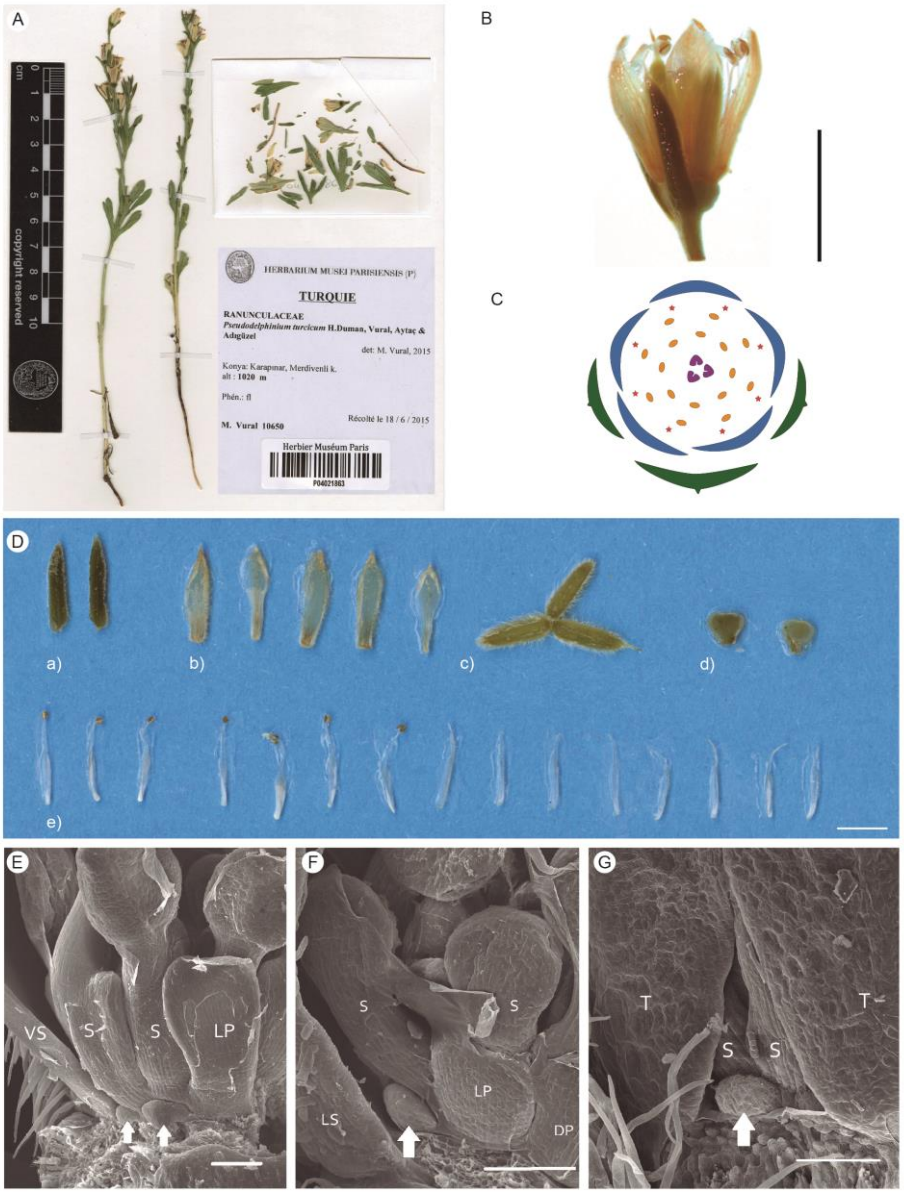

Fig. 2 A-D- *Pseudodelphinium turcicum* flower morphology. A- Sheet *Vural 10650a* and *10650b* [P04021863] housed at P. B- Detail of a restored flower, scale = 0.5 cm. C- Floral diagram: bract and bracteoles are in green, perianth in blue, stamens in orange, carpels in magenta and red stars indicates the hypothetical position of young perianth organs with an arrested development. D- Floral dissection of the restored flower, scale = 1 cm: a) bracteoles b) tepals c) carpels d) receptacle e) stamens (stamens at the right lost their anthers during dissection). E-G- SEM micrographs of floral buds. E- *Delphinium venulosum*, scale = 1 mm. Arrows show young ventral petal organs (one lateral and one ventral sepal were removed). F- *Delphinium virgatum*, scale = 1 mm. The arrow points to a young ventral petal (the dorsal sepal was removed). G- *P. turcicum*, scale = 1 mm. The arrow points to a young dorsal organ with an arrested development (the dorsal tepal was removed). In all the figures: T = tepal, VS = ventral sepal, LS = lateral sepal, LP = lateral petal, DP = dorsal petal, S = stamen.

Restoration of the material

The dried flowers sampled from herbarium specimens were restored in an ammoniacal 10% aq. solution at 60°C during 24 hours, then fixed with FAA (90% ethanol 70%, 5% formalin, 5% acetic acid) for 24 hours, and finally stored in a mixture of water, ethanol and glycerol in equal volumes.

Dissection and observation of adult flowers

Three adult flowers of *P. turcicum* were dissected using a stereomicroscope (Nikon SMZ 745T). Two of the flowers were dissected without being restored, and one was restored as detailed above. The dried flowers were mounted and put in an envelope pasted on the sheet [P04021863]. The restored flower was mounted on a glass slide, which is kept at P.

Scanning electron microscopy observations

Fourteen restored buds (four for *D. venulosum*, four for *D. virgatum*, and six for *P. turcicum*) were sampled. Buds 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 gold (60 seconds of metallization) using a JFC–1200 fine coater (JEOL), and observed using an SU3500 scanning electron microscope (Hitachi). Pollen grains were fixed directly on aluminum stubs with colloidal graphite and observed using the same scanning electron microscope. Samples are kept at P slide library.

Anatomical sections

Three pre-anthetic floral buds were sampled for each of the species *D. venulosum*, *D. virgatum*, and *P. turcicum*, and were restored. 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 Nikon Eclipse Ci microscope. Floral vascularization was reconstructed by drawing selected serial cross

sections of the anthetic flowers using a *camera lucida* and superimposing tracing papers. Because restoration of the bud of *D. venulosum* was problematic due to the poor tissue preservation, a second bud from the same specimen (*P.E.E. Sintenis 4627*) was dissected in order to check organ arrangement. Samples are kept at P slide library.

Results

Pseudodelphinium turcicum belongs in Delphinium subg. Delphinium

The GenBank accession numbers for the *trn*L-F and ITS markers for *M. Vural 10643 & O. Tugay* and *M. Vural 10650a* and *10650b* are KY596027, KY596028, KY596029 (for *trn*L-F), KY596030, KY596031 (for ITS, the sequencing of this marker for *Vural 10650a* was unsuccessful), respectively. The reconstructed ML tree is shown in figure 3. The topology and phylogenetic relationships among accessions of Delphinieae are identical to those shown by Wang et al. (2013). For this reason, each of the *Staphisagria* lineage, the clades composing the genus *Aconitum*, and the sister group of *D.* subg. *Delphinium* were collapsed in figure 3. All three accessions of *P. turcicum* form a clade (BP = 85%), sister to *D. venulosum* (BP = 70%). *Pseudodelphinium turcicum* belongs to the clade of Mediterranean species of *Delphinium* within subgenus *Delphinium* (BP = 100%).

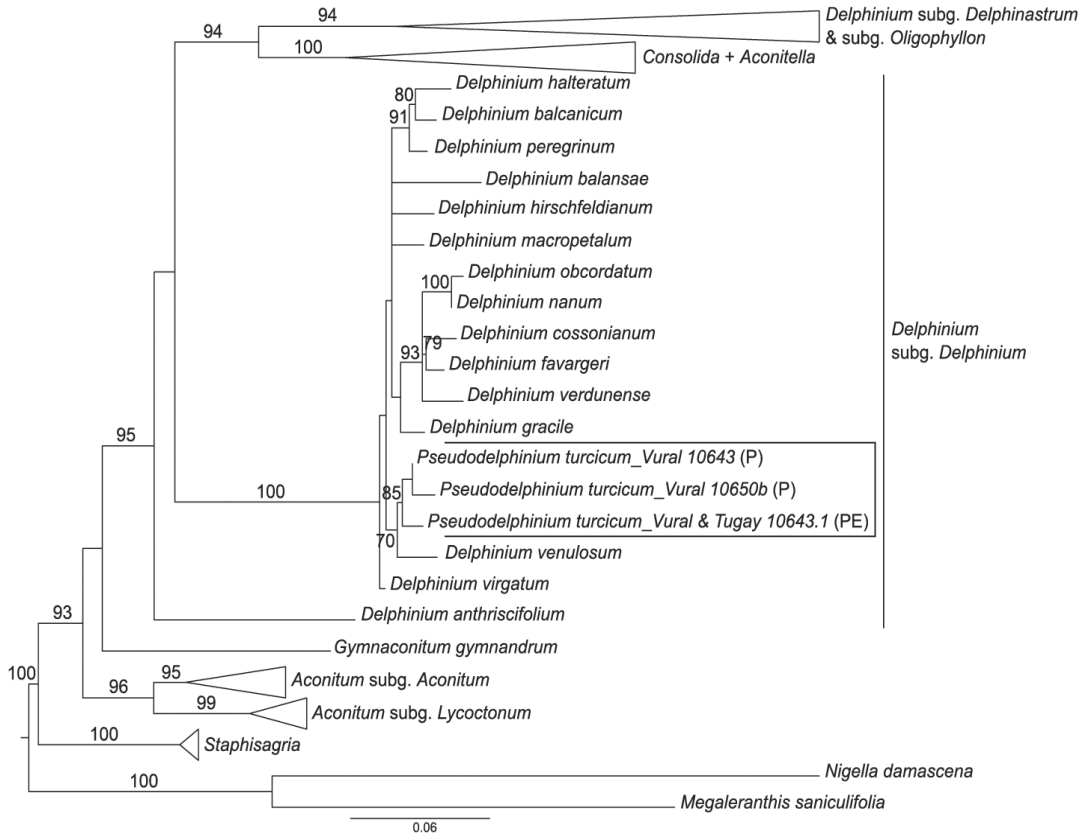

Fig. 3 ML tree of Delphinieae based on the combined *trn*L-F and ITS dataset. The focus is put on the clade of Mediterranean *Delphinium* species belonging to the subgenus *Delphinium* that includes the three *Pseudodelphinium turcicum* accessions. Bootstrap percentages ≥ 70 are indicated.

Morphology

Pseudodelphinium turcicum exhibits a short virgate stem (20 cm high) with a simple taproot, glaucous vegetative parts and alternate leaves (2–2.5 cm long x 1.2–1.8 cm wide) with palmate venation at the base of the plant and simple leaves at the top (1.3–1.7 cm long x 0.3 cm wide). The inflorescence is a raceme (sometimes panicle) with actinomorphic bisexual flowers (fig. 2*A*). The flower is enclosed by one bract and two lateral bracteoles. It consists of three whorls: perianth, androecium, and gynoecium (fig. 2*B*, 2*C*). The tepals of the uniseriate and pentamerous perianth are quincuncially overlapping (fig. 2*B*–2*D*). In the three analyzed flowers, tepals are 6–7 mm long x 1–2 mm wide, oblong to oblanceolate, with acuminate apex, truncate base (fig. 2*D*), cream to primrose *sensu* Beentje (2012; fig. 2*B*), pubescent especially on the abaxial side. The androecium is composed of 11–16 stamens with basifixed anthers and glabrous filament winged on the lower half. The wing is 0.5–0.7 mm wide. The gynoecium consists of three hypogynous and pubescent carpels (fig. 2*D*) with greenish styles (0.1–0.2 mm long) and bilobed greenish stigmas (0.29–0.35 mm long). In the floral bud of *P. turcicum*, young perianth organs with an arrested development were found between the developing tepals and the filaments (fig. 2*G*) and were not visible in the anthetic flower.

Delphinium venulosum and *D. virgatum* flowers have a similar general organization with two differentiated and zygomorphic whorls. The calyx consists of five quincuncially overlapping petaloid sepals: two ventral, two lateral and one dorsal spurred sepal. The corolla consists of four dorsal organs (the two dorsalmost being spurred). Each petal is located at the base of each stamen parastichy. They vary in size, depending on whether they are located in the dorsal or ventral half of the flower (fig. 2*E*, 2*F*).

Delphinium venulosum and *D. virgatum* have spheroidal tricolpate pollen grains, with a length of 22–31 μm, a microechinate exine and an ornamented aperture membrane (fig. 4*A*, 4*B*, shape differences are due to the desiccation of the material). The pollen grains of *P. turcicum* are heteromorphic; they vary in the number and distribution of apertures,

and in length (22–31 µm). Both di- and tricolpate pollen grains are observed. Their exine is microechinate as well, with an ornamented aperture membrane (fig. 4*C*, 4*D*).

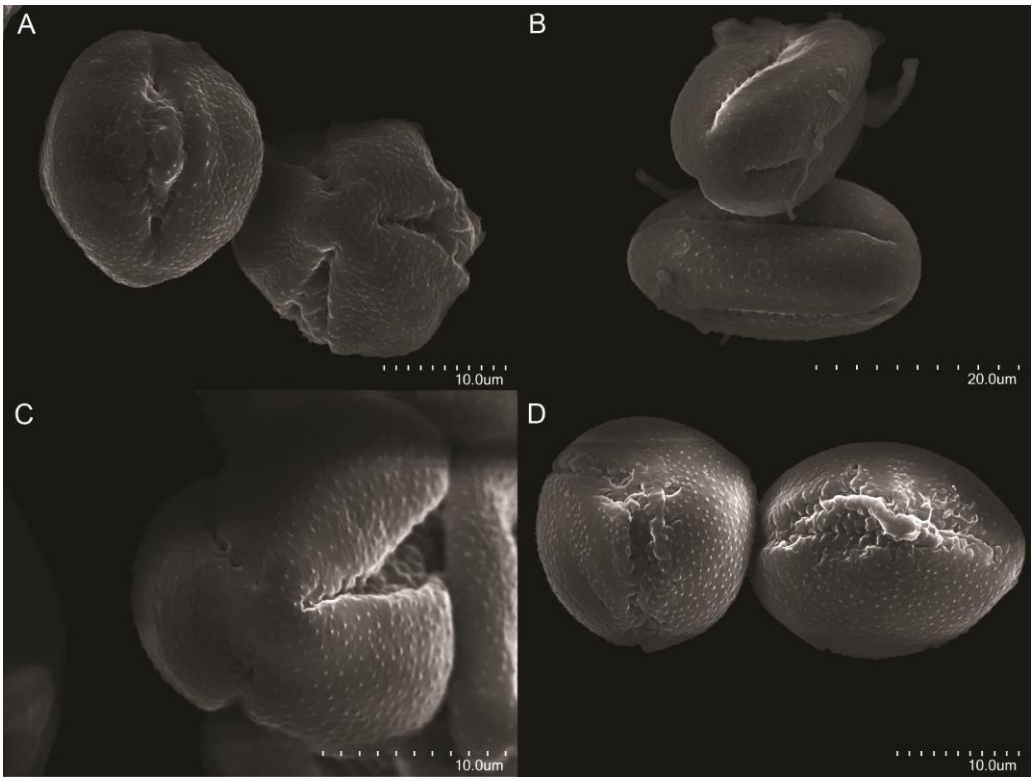

Fig.4 SEM micrographs of pollen grains of A) *Delphinium venulosum* (left: equatorial view, right: polar view), B) *Delphinium virgatum*, C) *Pseudodelphinium turcicum* (polar view), D) *P. turcicum* (left: polar view, right: equatorial view).

Anatomy

Bracts and bracteoles of the three species are supplied by five vascular bundles. In bracts, these bundles originate from three independent traces: one median non-divided and two lateral ones. Lateral bundles bifurcate after entering the organ. Bracteole bundles originate from a single trace, which gets divided at first into three (one median and two lateral), then the two lateral traces bifurcate again (fig. 5).

The sepals of *D. venulosum* and *D. virgatum*, and the perianth organs of *P. turcicum* are composed of three independent and non-ramified traces, one median and two lateral (fig. 5). Petals of *D. venulosum* and *D. virgatum* are vascularized by a single trace. In the two spurred petals, this trace bifurcates before entering the organ. Stamen vascularization in the three species is established by a single trace and carpel

vascularization is composed of three independent and non-ramified traces, one median and two lateral ones (fig. 5).

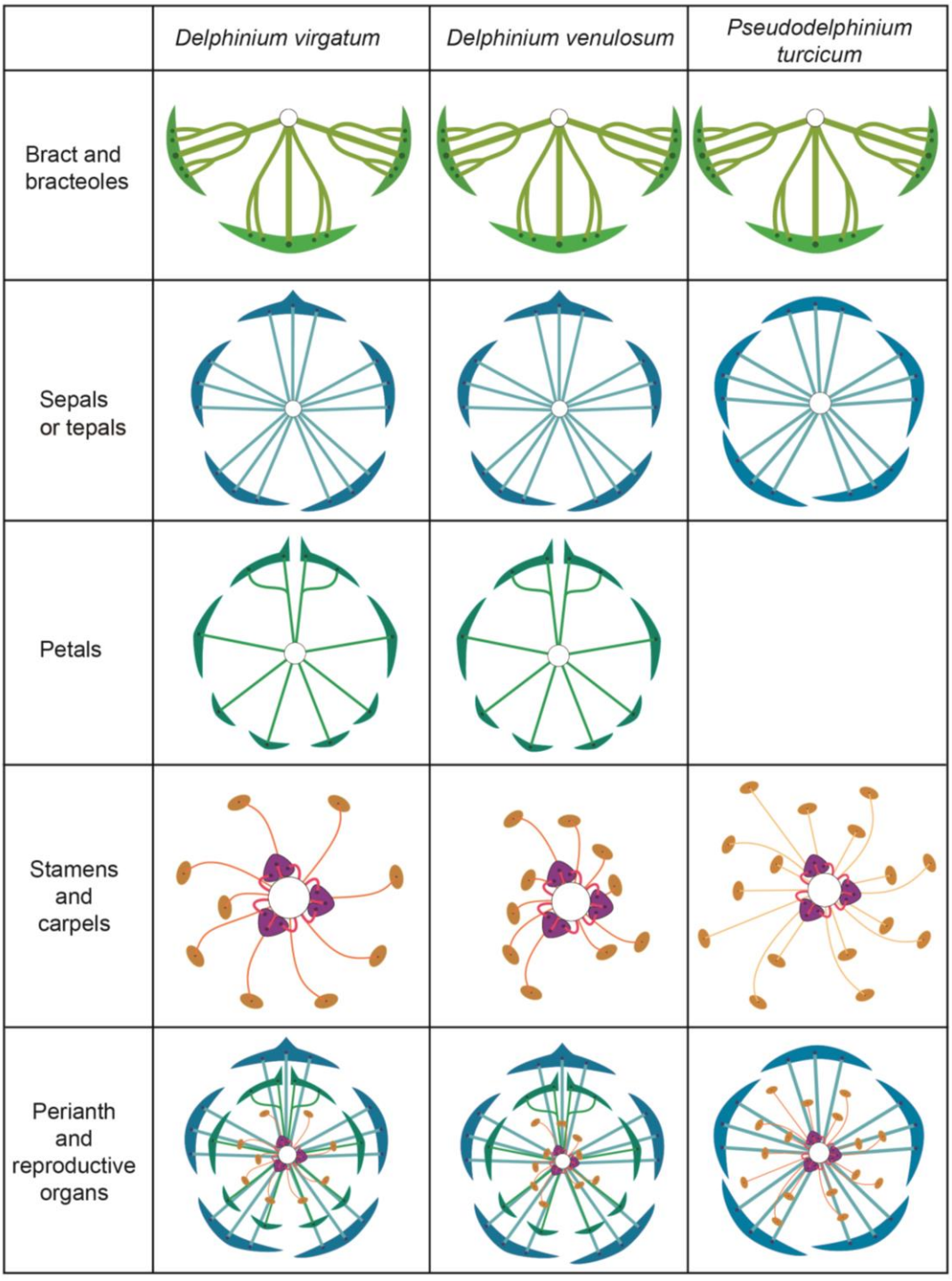

Fig. 5 Floral vascularization of each floral whorl (bract and bracteoles, sepals, petals or tepals, stamens and carpels) of *Delphinium virgatum*, *Delphinium venulosum*, and *Pseudodelphinium turcicum*. Same color code as in fig. 2*C*.

Discussion

Phylogenetic placement of Pseudodelphinium in the tribe Delphinieae

The topology and the well-supported nodes (BP >70%) of the inferred ML tree are consistent with the ML trees presented by Jabbour & Renner (2012a) and Wang et al. (2013) (fig. 3).

The three accessions of *P. turcicum* formed a well-supported clade sister to *D. venulosum* and nested within *D.* subg. *Delphinium*. A study focusing on the molecular phylogeny and biogeography of the subgenus *Delphinium* found that the two accessions of *P. turcicum* (*M. Vural & O. Tugay 10643.1*, included in this study, and *M. Vural & O. Tugay 10643.2*) formed a clade sister to [*D. venulosum* + *D. virgatum*] (BP = 95%, PP = 1.0; Xiang et al. 2017). The results of Xiang et al. (2017), based on more phylogenetic markers and a different set of accessions, are in accordance with ours (fig. 3). Following this molecular evidence *Pseudodelphinium turcicum* should be considered as a species of *Delphinium*.

Morphological similarities of Pseudodelphinium with Delphinium

The specimens of *P. turcicum* we investigated showed typical ranunculaceous features, namely herbaceous habit, palmate venation, free and numerous stamens, numerous ovules, and beaked fruits (fig. 2*A*–2*D*, Vural et al. 2012, Wang et al. 2016b).

Similarly to the annual species of *Delphinium*, *P. turcicum* exhibits glaucous vegetative parts and a simple taproot, alternate palmately-lobed leaves, and a terminal raceme or panicle (fig. 2*A*, Pawlowski 1964, Davis et al. 1965, Zohary 1966, Townsend 1980, Blanché 1990, Iranshahr 1992, Vural et al. 2012). Although *Pseudodelphinium* seems to differ from *Delphinium* by its spurless actinomorphic flowers and uniseriated perianth (fig. 2*A*–2*D*), some floral characteristics of *Delphinium* could be found in *Pseudodelphinium*: i) bisexual flowers whose perianth is composed of five free, pilose, and quincuncially overlapping petaloid organs, ii) numerous stamens with basally winged filaments, iii) superior ovaries, iv) three dehiscent follicles containing numerous ovules (fig. 2*B*–2*D*, Pawlowski 1964, Davis et al. 1965, Zohary 1966, Townsend 1980, Blanché 1990, Iranshahr 1992, Vural et al. 2012), v) black spheroidal seeds with 5–8 transversal rings of concrescent scales and an umbilical orifice occupying 1/2–1/3 of the total seed volume (Blanché 1990, Vural et al. 2012), and vi) a proportion of the pollen grains being tricolpate with microechinate exine and an ornamented aperture membrane (fig. 4*C*, 4*D*, Saidabadi 1968, Bursali & Dogan 2005, Sharifnia et al. 2013).

Pollen heteromorphism was already reported in the Ranunculaceae (Humphrey 2016, Wang et al. 2017). In order to understand the origin of pollen heteromorphism in *Pseudodelphinium* and the implications for its fitness, it would be necessary to investigate microsporogenesis and test the viability of the pollen, as well as conduct an ecological study about the fertilization success of individual pollen grains. To our knowledge, pollen heteromorphism has never been reported in *Delphinium* so far.

The perianth of *P. turcicum* is composed of a single pentamerous whorl of petaloid organs considered here as tepals (fig. 2*B*–2*D*, Vural et al. 2012). Vural et al. (2012) interpreted this whorl as the corolla. However, comparing the arrangement and the morphological characteristics of the tepals in *P. turcicum* with the sepals in *Delphinium*, we consider instead that *P. turcicum* tepals are equivalent to *Delphinium* sepals and that adult flowers lack a fully developed corolla. Additionally, *P. turcicum*, organs with an arrested development are found between tepals and stamens (fig 2*E*-*G*). These young organs are similar to the ventral petals with an arrested development in *Delphinium* flowers (Payer 1857, Kosuge 1994, Jabbour et al. 2009, Jabbour & Renner 2012b). Based on the organization of the androecium in *P. turcicum* (fig. 5) and appendix A1, we hypothesize that there are eight petals with an arrested development in this species, each one being initiated at the base of each stamen parastichy. They develop until they reach a size and shape similar to the young ventral petals of *Delphinium* flowers at the stage of the establishment of zygomorphy (see Fig. 2 in Jabbour et al. 2009).

Anatomical similarities of Pseudodelphinium with Delphinium

The perianth organs (tepals) of *P. turcicum* have a trilacunar three-traced vascularization, which is a feature of the outermost perianth organs of Ranunculales (fig. 5, Hiepko 1965, Stevens 2001, Wang et al. 2009). As in the other Delphinieae investigated so far (Novikoff & Jabbour 2014), *P. turcicum* stamens have a unilacunar one-traced vascularization and the vascularization of the ovary is equivalent as the vascularization in other *Delphinium* species (fig. 5). Based on the floral anatomy of *D. virgatum* and *D. venulosum* and on the findings of Novikoff & Jabbour (2014), the vascularization of the tepals in *P. turcicum* is identical to the vascularization of the sepals in *Delphinium*. However, none of the perianth organs of *P. turcicum* shows a typical *Delphinium* petal-like venation (fig. 5). That is an additional argument to hypothesize that *P. turcicum* adult flowers lack a fully developed corolla. A way to

determine the identity of the perianth organs in *P. turcicum* would be to investigate the expression of A- and B-function genes (Coen & Meyerowitz 1991) in dissected flowers.
During the last 200 years, numerous plants showing abnormal flowers in Ranunculaceae have been described (e.g. De Candolle 1827, Payer 1857 reviewed in Jabbour et al. 2015) some of them explaining changes in floral symmetry from zygomorphy to actinomorphy (e.g. Clusius 1601, Linnaeus 1744, Masters 1904). In most cases, changes in floral symmetry correspond to peloria (e.g. Masters 1904), i.e. a radialization of a usually zygomorphic floral groundplan. In addition, in the case of *P. turcicum*, flowers did not show any nectariferous organs, indicating a particular case of peloria, namely *peloria anectaria* (Linnaeus 1744, Rudall & Bateman 2003). The dorsal perianth organs of the flower show ventral characteristics. The floral phenotype in *P. turcicum* would then be the result of a ventralization of the flower (see Luo et al. 1996). Numerous studies demonstrated that zygomorphy depends on the expression of the TCP family genes *CYCLOIDEA* (*CYC*) and *CYC*-like genes. Some *CYC* copies act as repressors of development of the primordia in the floral meristem, or repress the development of dorsal floral organs in zygomorphic flowers (Luo et al. 1996, Citerne et al. 2000, 2006). In the flowers of *P. turcicum*, the expression of *CYC*-like genes could affect all the organs of the perianth by repressing the development of the perianth organs located between the tepals and the stamens, hence resulting in actinomorphic flowers without fully developed petals.
Nevertheless, even if genetic causes could underlie this unusual floral phenotype in a taxon which is molecularly speaking a *Delphinium* representative, and knowing that the single population of *P. turcicum* is found in a very particular ecosystem (basin of a salt lake), floral morphological-anatomical abnormalities could also be due to extrinsic causes. More studies about the distribution of the population enriched by more material and ecological data are needed at this stage to test these alternative hypotheses. We highlight that the current study relied on three specimens of *P. turcicum* only. More material is needed for future morphological and evo-devo studies to understand the bases of this unusual floral phenotype.

Based on the array evidence of molecular and morphological-anatomical data presented, the generic name *Pseudodelphinium* H.Duman, Vural, Aytaç & Adigüzel should be sunk in *Delphinium*.

The taxonomic treatment follows.

Delphinium L.

= *Pseudodelphinium* H.Duman, Vural, Aytaç & Adigüzel in Turk. J. Bot. 36(5): 428. 2012 – Type: *Pseudodelphinium turcicum* H.Duman, Vural, Aytaç & Adigüzel.

Delphinium turcicum (H.Duman, Vural, Aytaç & Adigüzel) Espinosa, *comb. nov.*

≡ *Pseudodelphinium turcicum* H.Duman, Vural, Aytaç & Adigüzel in Turk. J. Bot. 36(5): 428. 2012 – Type: Turkey. C4 Konya: Karapınar to Konya road, 30 km, Merdivenli village, 1020 m, dry plain steppes, 28 July 1998, *H.Duman* 6824 & *Z.Aytaç* (holotype: GAZI, isotypes: ANK, HUB).

Conclusions

The molecular phylogenetic evidence shows that the Turkish endemic *Pseudodelphinium turcicum* belongs in *Delphinium* and should not be considered as a distinct genus. The morphology and anatomy of the vegetative and reproductive organs of *P. turcicum* flowers lead us to conclude that the single population of the monospecific genus *Pseudodelphinium* corresponds to a population of *Delphinium* with peloric flowers and a corolla with an arrested development. The formal perianth organs in *P. turcicum* are interpreted as equivalents to sepals in *Delphinium* typical flower. Interestingly, specimens from this population of annual plants were collected already 20 years ago. The conditions of the maintenance in the wild of these plants with an unusual floral phenotype have to be investigated. Future research may address the molecular and/or environmental causes of this unusual phenotype, investigate the role of pollen dimorphism in the ecology of these plants, their mating system(s) and study the genetic relationships of this population with neighboring populations of *Delphinium*.

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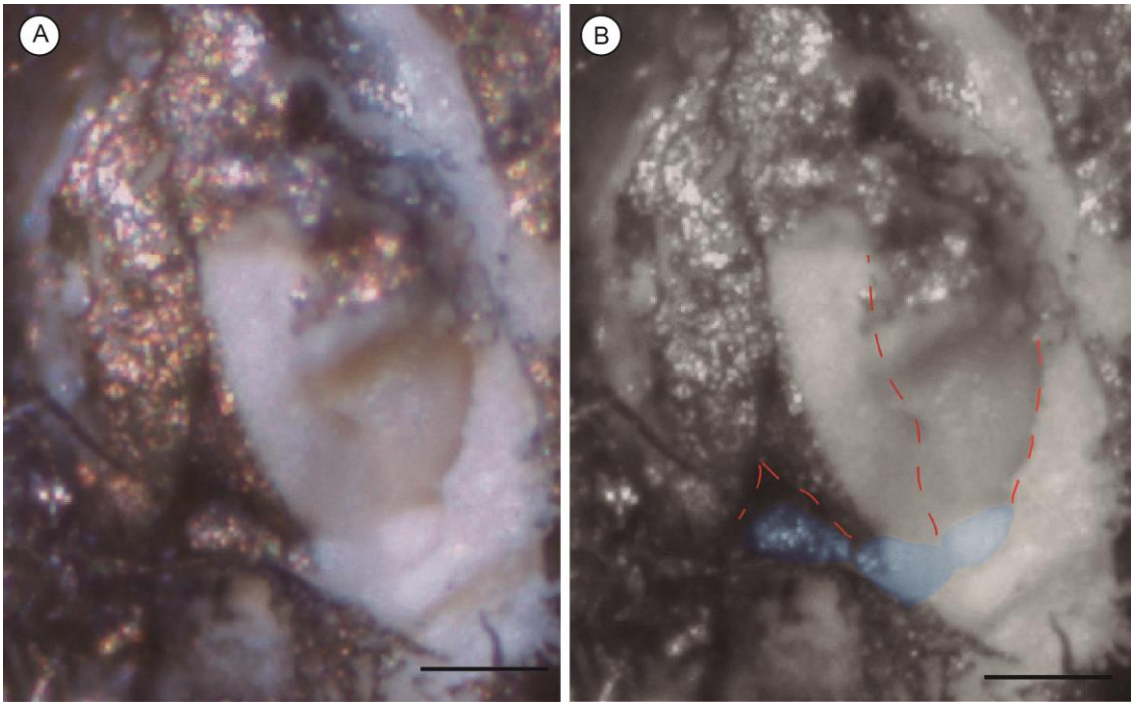

Fig. A1 Pictures of *Pseudodelphinium turcicum* floral bud used in fig. 2*G*, scale = 1µm. An additional tepal was dissected. A) Original image, B) Same image, young perianth organs with an arrested development are highlighted in blue and the borders of the filaments are shown with red dashed lines.

3. Conclusions

Les résultats obtenus ont apporté les réponses suivantes aux questions posées :

1) À la base des tépales, et autour de l'androcée, se trouve un verticille composé d'organes qui ne sont pas développés, seuls des primordia sont présents à l'état adulte. De plus, la reconstruction de la vascularisation florale a montré que les tépales formant le périanthe de la fleur présentaient la vascularisation typique des organes W1 chez *Delphinium*. Nous avons émis l'hypothèse que le périanthe des fleurs étudiées était formé uniquement par des organes W1 et que les organes W2 arrêtaient très tôt leur développement. Le retard de développement des organes W2 par rapport au reste des organes floraux, ou l'arrêt (en général en position ventrale dans la fleur lorsque la symétrie est bilatérale) de ce même développement, est un phénomène général chez les Ranunculaceae [1–3].

2) D'un point de vue phylogénétique, *P. turcicum* se place au sein du clade des *Delphinium* méditerranéens. L'espèce présente de l'hétéromorphisme pollinique (plusieurs types polliniques produits par la même fleur) qui peut indiquer une possible origine hybride ou polyploïde de cette espèce [4,5].

Nous avons conclu que cette nouvelle espèce pouvait correspondre à une population de *Delphinium* déviante présentant des fleurs péloriques, dont la corolle n'est pas développée et présente un phénomène de ventralisation du périanthe (c'est à dire que l'ensemble du périanthe présente les caractères des organes ventraux du périanthe typique). Nous avons fait l'hypothèse que des modifications dans l'expression des gènes participant à l'établissement de la symétrie bilatérale (les gènes *CYC*-like [6,7]) pourraient être à l'origine de la pélorie chez les fleurs de cette espèce. Finalement, la nouvelle combinaison *Delphinium turcicum* a été établie.

De plus, cette population présente un grand intérêt du fait de son maintien à l'état sauvage depuis de plus de 20 ans (cf. Vural et al. 2012). Les principales questions qui se posent sont les suivantes : comment expliquer le maintien de ces anormalités touchant aux organes participant à l'attraction des pollinisateurs et quel est l'impact de ces

modifications sur la valeur sélective des individus ? Pour répondre à ces questions, il serait nécessaire de travailler avec du matériel vivant pour 1) élucider l'origine génétique des anormalités (par des analyses ciblant les gènes potentiellement mutés), 2) mieux comprendre la biologie de l'espèce (par des études de caryologie et d'écologie de la pollinisation) et 3) tester la survie et le maintien de ces variations dans un environnement différent de celui du lac salé (se caractérisant par un taux élevé d'endémisme affectant différentes familles botaniques [8]).

Même si au début de l'étude nous avions prévu de réaliser des études sur des individus vivants, notamment en caryologie, nos collègues turcs ont réagi défavorablement à l'envoi de graines. Nous n'avons pas non plus réussi à avoir accès aux spécimens types de l'espèce. Nous nous sommes contentés de travailler avec les trois spécimens séchés (en fleurs et en boutons) qui nous ont été envoyés. Toutefois, cette étude m'a permis d'établir une collaboration avec l'équipe du professeur Wei Wang, en Chine, chercheur incontournable dans les recherches actuelles de la tribu des Delphinieae. Grâce à cette collaboration j'ai pu contribuer à une étude de phylogénie et de biogéographie du sous-genre *Delphinium* et cosigner un article publié dans la revue *TAXON* :

Xiang, K. L., Aytaç, Z., Liu, Y., Espinosa, F., Jabbour, F., Byng, J. W., Zhang, C.-F., Erst, A. S. & Wang, W. (2017). Recircumscription of *Delphinium* subg. *Delphinium* (Ranunculaceae) and implications for its biogeography. *Taxon*, *66*(3), 554-566.
(voir Annexe 1)

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Chapitre 2 : Analyse de la diversité morphologique florale des cultivars de *Delphinium* L. et *Aquilegia* L. (Ranunculaceae) : organogenèse et bases moléculaires potentielles

1. Introduction

Pour comprendre la diversité morphologique florale au sein des angiospermes, il est indispensable d'analyser son origine et son évolution. Ceci implique, entre autres, la compréhension des mécanismes moléculaires et écologiques sous-jacents. Les phénotypes floraux déviants apparaissent comme des outils essentiels d'analyse.
Grâce à une sélection artificielle, l'horticulture a permis l'établissement de variétés de plantes (ou cultivars) aux phénotypes floraux tératologiques. En effet, la grande majorité de ces variétés présente des modifications des caractéristiques morphologiques typiques des fleurs (par exemple une augmentation du nombre d'organes floraux, des modifications de leur forme, des modifications dans l'organisation générale de la fleur). De plus, et alors que les individus mutants sont rares dans la nature, l'accès à la plupart de ces cultivars est facile sur le marché et ils sont aisément cultivables, faisant de ces plantes des spécimens de choix pour l'étude des phénomènes à l'origine des nouveautés morphologiques.

Pour cette partie de ma thèse, je me suis posé la question suivante : quelles sont les bases développementales et moléculaires possibles responsables de la diversité florale chez les *Delphinium* L. horticoles ?

Pour répondre à cette question j'ai choisi de travailler avec cinq cultivars de *Delphinium* présentant des déviations florales. J'ai aussi utilisé quatre cultivars du genre modèle en évo-dévo des Ranunculaceae, *Aquilegia* L. afin de faciliter l'interprétation génétique de ces déviations. Pour l'analyse de ces variétés j'ai utilisé deux approches différentes :1) la description morphologique de fleurs adultes et de boutons floraux, et 2) la reconstruction de la vascularisation florale.

Cette étude a fait l'objet d'un manuscrit actuellement en révision pour la revue *PLoS ONE*. (Les avis des reviewers sont placés en Annexe 2).

Référence :

Espinosa, F., Damerval, C., le Guilloux, M., Deroin, T., Wang, W., Pinedo Castro, M., Nadot, S. & Jabbour, F. (In review). Insights into the floral morphological diversity of cultivars of *Delphinium* L. and *Aquilegia* L. (Ranunculaceae): organogenesis and potential molecular clues. *PLoS ONE.*

Dû au fait que l'article n'est pas encore accepté, je présente ici la version du manuscrit tel qu'il a été soumis. Pour cette raison, elle suit les instructions aux auteurs de la revue, particulièrement en ce qui concerne la bibliographie.

2. Article : Quelles sont les bases développementales et moléculaires possibles responsables de la diversité florale chez les *Delphinium* L. et *Aquilegia* L. horticoles ?

Insights into the floral morphological diversity of cultivars of *Delphinium* L. and *Aquilegia* L. (Ranunculaceae): organogenesis and potential molecular clues

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Abstract

Horticultural plant varieties often display deviant floral phenotypes and as such, they are an appropriate material to address the origin and the building of morphological variations. To identify the pivotal developmental stages at which floral variation may originate, and infer the putative associated genetic causes, we studied abnormal flowers in cultivars of *Aquilegia* and *Delphinium* from the Ranunculaceae, a family displaying a high range of floral diversity. Wild species of these genera are characterized by spurred flowers, but markedly differ in the floral symmetry (zygomorphic vs actinomorphic). Flower morphology was studied at different developmental stages up to anthesis in nine cultivars, and in order to infer the putative identity of the perianth organs, floral vascularization was reconstructed. We showed that floral symmetry and phyllotaxis were conserved while flower organization and vascularization were modified in cultivars in both genera compared to wild relatives. Most of the morphological and anatomical deviations impacted the perianth, including organ number and identity. We hypothesized that the floral phenotypes of *Aquilegia* and *Delphinium* cultivars result from genetic alterations affecting meristem size, frontiers between sets of organs of different identity, and organogenesis and development of nectariferous structures. This work on the morphology and anatomy of human-selected teratological phenotypes contributes to the knowledge about floral diversity in Ranunculaceae, and provides hypotheses about the molecular control of the developmental changes that could be tested in future evo-devo research.

Introduction

The flower is considered as one of the major distinctive features of angiosperms [1–3], the most diversified and successful plant group on earth with more than 90% of living land plant species (ca. 300 000) [4–6]. It is usually defined as an association of sexual organs (carpels and stamens) surrounded by sterile organs forming the perianth (petals and sepals) on a short axis. While its ground plan is highly conserved, there is a wide diversity of floral traits [1,7]. The number and form of floral organs, and their relative organization within the flower, have been shown to be linked to the pollination process and to the evolutionary success of the group [6,8–10].

The order Ranunculales is the sister group to all other eudicots, the most species rich clade of angiosperms [11–13], and is characterized by a large diversity in life history and morphology [4,13–16]. For these reasons, the entire order was recently proposed as a model order for studying flower evolution [16]. Among the seven families recognized within Ranunculales, the buttercup family (Ranunculaceae) is the largest in terms of species number (ca. 2300 species in 62 genera [17,18]) and hosts a high range of floral diversity. Since the 18th century, the family has been subject to numerous morphological studies [14,19–25], several phylogenetic hypotheses have been produced [15,17,19], and more recently evolutionary-developmental genetic (evo-devo) studies have been conducted [10,16,26,27] providing a solid background to study the evolutionary mechanisms responsible for its diversification.

Evo-devo aims at establishing a link between development, genetics and evolution [28], looking at the genetic control of developmental processes in an evolutionary context [29] to understand for instance the origin of morphological diversity in a clade. Based on the studies of floral mutants in several model species, the identity of typical floral organs has been explained by a model composed by a combination of five functions (ABCDE) involving gene families encoding transcription factors [30–36]. Modifications of the expression patterns of these genes can result in drastic changes in floral development and in adult floral morphology [37–40]. It has been shown that this model is broadly conserved at the scale of angiosperms [41,42].

Ever since the 17th century, the study of deviant organisms has played a key role for understanding the biological mechanisms controlling floral development, organization and evolution [43,44,53,45–52]; for a review in Ranunculaceae see [54] (S1 Text).

Several evo-devo studies aiming at understanding floral evolution among angiosperms have already investigated deviant organisms presenting peloric or double flowers, or naked inflorescence stems (without flowers): bilateral symmetry was studied in Orchidaceae [55,56] or in Fabaceae [57] using peloric flowers, the establishment of floral organ identity in Malvaceae [58] and Begoniaceae [59] was studied using double flowers, and interactions within proteins during the development of Asteraceae flowers [60] were analyzed using mutant organisms exhibiting inflorescence meristems without flowers. Such studies have direct applications for the development of new and more attractive cultivars [61–63] and for the assessment of the agronomical value of plant abnormalities [64].

The complexity of perianth types in Ranunculaceae linked to the petal origin and nectar production and store, has led to diverse ways of naming perianth organs (reviewed in Jabbour and Renner [65]). In this article, we examine in detail floral development in cultivars of the genera *Delphinium* L. (wild-type [WT] perianth: zygomorphic with a single outer spur, a pair of inner nectar spurs, and spirally inserted organs) and *Aquilegia* L. (WT perianth: actinomorphic with five nectar spurs, whorled and spirally inserted organs). *Delphinium* belongs to the tribe Delphinieae, the only clade of Ranunculaceae presenting zygomorphic flowers [66], and *Aquilegia* belongs to the subfamily Thalictroideae and was proposed as a model genus for evo-devo studies [24]. Following the terminology proposed by Jabbour and Renner [65], we will use "W1 (whorl 1) organs" to refer to the first five perianth organs that develop on the ontogenic spiral and "W2 (whorl 2) organs" to refer to the inner perianth organs.

The WT flower of *Delphinium* consists of four categories of organ: two categories of perianth organs (W1 and W2) and two categories of sexual organs, male and female. W1 is composed of petaloid organs arranged on a spiral and quincuncially overlapping: two ventral and two lateral organs, and a spurred dorsal one. W2 comprises four sessile petaloid organs located in the dorsal half of the flower: two in dorsal position and two in dorso-lateral position. Four primordia in the ventral half of the flower stop developing shortly after initiation [19,67]. The two dorsal W2 organs are nectariferous structures forming spurs enclosed in the dorsal W1 spurred organ. The other two dorso-lateral W2 organs are generally exsert, flat and epeltate [14,25,67,68]. The androecium is composed of numerous stamens, and the gynoecium of three to five free carpels [14].

The WT flower of *Aquilegia* is actinomorphic and composed of five pentamerous categories of organs, consisting of: two types of petaloid organs forming the perianth, the androecium, a whorl of staminodes, and the gynoecium. W1 organs are arranged on a spiral and are quincuncially overlapping. Five W2 organs are arranged on a whorl, they are sessile, spurred, nectariferous, and alternate with W1 organs. The androecium is composed of stamens arranged in 10 orthostichies of four to nine whorls and is topped with a whorl of staminodes [24]. Close affinity between stamens and staminodes in *Aquielgia* has been proposed in developmental studies [69–71]. The gynoecium usually includes five free carpels.

Based on the comparison between the WT floral morphologies of *Delphinium* and *Aquilegia* and the deviant floral characteristics obtained by artificial selection, we propose hypotheses for the putative genetic bases underlying changes in the developmental sequence of mutant flowers, and using available knowledge in other more or less distantly related species and assuming functional conservation. These hypotheses provide guidelines for future evo-devo investigations, targeting crucial groups of genes and developmental stages possibly at the origin of floral diversity in Ranunculaceae.

Materials and methods

Plant material

Young individuals from, respectively, five and four cultivars of *Delphinium* and *Aquilegia* were provided by the Jardin du Pic Vert (www.jardindupicvert.com) (Fig 1). It is likely that all *Delphinium* cultivars result from crosses involving *D. elatum* [72–74]. Those of *Aquilegia* result from crosses involving *A. vulgaris* [75]. The horticultural varieties were chosen according to 1) their availability in the catalogue of the horticulturist in November 2014 and 2) their diversity in floral morphology.

Five plants per cultivar presenting 3–4 leaves were installed in a growth chamber under long day period (18-h day at 25°C / 6-h night at 16 °C) until they reached the flowering phase. They were then transfered to a cold greenhouse. Inflorescences at various stages of development (from inflorescence initiation to anthesis) were sampled and fixed in FAA (90% ethanol 70%, 5% formalin, 5% acetic acid), and transferred to ethanol 70%.

Floral morphology

One adult flower of each cultivar was selected, dissected under a stereomicroscope (Nikon SMZ 745T) and its organs were mounted on a sheet of paper following phyllotaxis. Additionally, a herbarium specimen was made for each cultivar and deposited at the Paris Herbarium (P; Table 1).

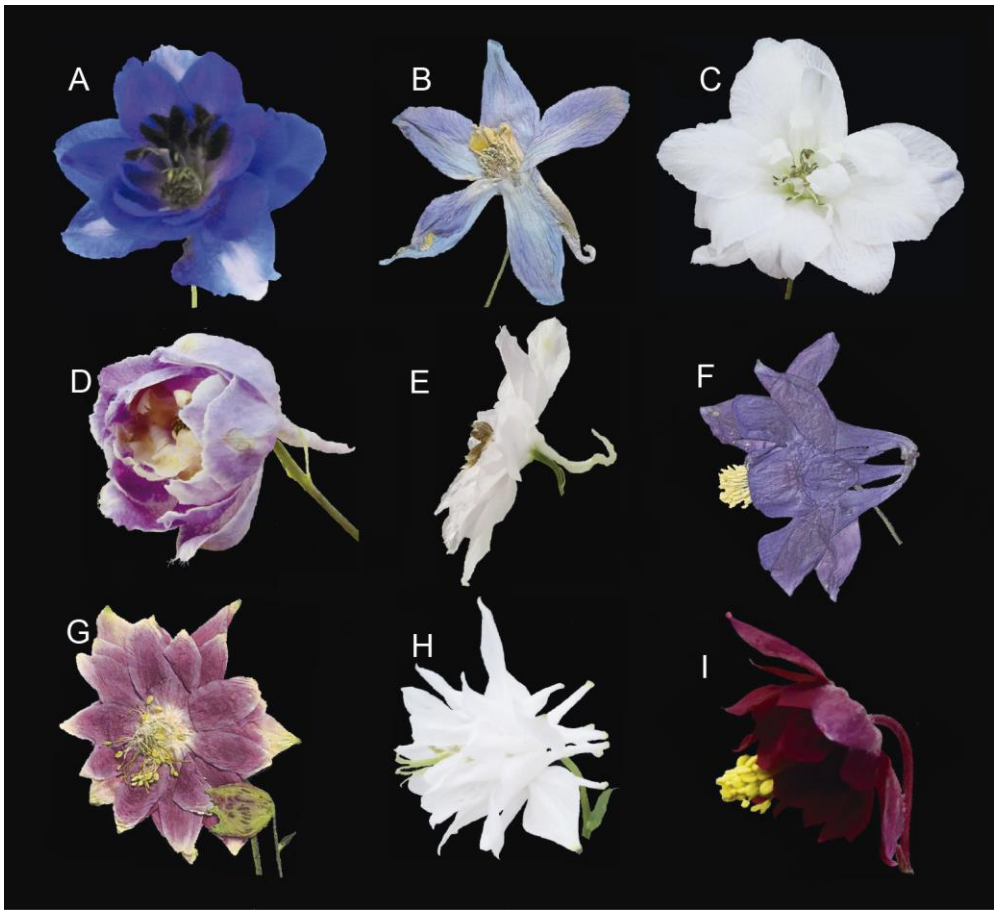


Fig 1. *Delphinium* and *Aquilegia* studied cultivars.

(A) *Delphinium* × *pacific* ‘Blue Jay’. (B) *D.* × *pacific* ‘Guinevere’. (C) *D.* × *pacific* ‘Galahad’. (D) *D.* × *pacific* ‘Astolat’. (E) *D.* × *elatum* ’Sky sensation’. (F) *A. vulgaris* ‘Leprechaun Gold’. (G) *A. vulgaris* ‘Nora Barlow’. (H) *A.* × *hybrida* ‘Green Apples’. (I) *Aquilegia hybrida* ‘Ruby Port’. Flowers from the latter variety present a very short spur on each of the *A*mW2 and *A*mW2' organs; spurs are not visible on the picture. All plants were provided by the Jardin du Pic Vert (www.jardindupicvert.com).

Fixed buds were dissected using the same stereomicroscope. They were dehydrated in an ethanol series (70%, 80%, absolute alcohol) and dried using an Emitech K850 critical-point dryer (Quorum Technologies), mounted on aluminum stubs with colloidal graphite, sputter-coated with gold during 60 s using a JFC-1200 fine coater (JEOL), and observed using an SU3500 Scanning Electron Microscope (Hitachi).

Floral anatomy

A preanthetic floral bud from a single cultivar of each genus was sampled (*D.* × *pacific* 'Blue Jay' and *A. vulgaris* 'Leprechaun Gold'), and floral organ vascularization was studied using a classic protocol to investigate plant anatomy [76]. Buds were dehydrated through a t-butyl series and embedded in paraffin (melting point: 58–60°C; [77]). Serial transverse sections were cut at a thickness of 12 µm using a rotary microtome (Leitz 1512) and then stained with Astra Blue 0.5% aqueous (Chroma 1B163) and Ziehl's fuchsine 10%. The material was mounted and observed using a Nikon Eclipse Ci microscope. Floral vascularization was reconstructed by drawing selected serial cross sections using a camera lucida and superimposing tracing papers. Samples are kept at the Paris Herbarium slide library.

Conventions for naming floral organs

In comparison with the WT flower, the mutant flowers from the analyzed *Delphinium* and *Aquilegia* cultivars include additional perianth organs. In *Delphinium*, we classified these organs in four categories from the outside in: 1) five organs quincuncially arranged, morphologically similar to the W1 organs and forming what we called *D*m W1 (*Delphinium* mutant W1); 2) supernumerary organs located between *D*mW1 and *D*mW2, arranged on a spiral, and morphologically similar to *D*mW1, therefore called *D*mW1'; 3) organs morphologically similar to the wild-type W2 organs, called *D*mW2; and 4) supernumerary perianth organs located between the perianth and the androecium, called *D*mW2', presenting abnormal features of reproductive organs.

Table 1. General symmetry and number of perianth organs in selected flowers of *Delphinium* and *Aquilegia* cultivar used in this study.

Delphinium L.		Herbarium specimen barcode	General symmetry	mW1		mW1'	mW2		mW2'	Number of perianth organs
				Flat	Spurred		Flat	Spurred		
WT			Bilateral	4	1	0	2	2	0	9
D. × pacific	'Blue Jay'	P04021845	Bilateral	5	0	7	8	0	4	24
	'Guinevere'	P04021840; P04021841	Bilateral	4	1	2	3	2	1	13
	'Galahad'	P04021843	Bilateral	4	1	8	4	1	2	20
	'Astolat'	P04021844	Bilateral	5	0	7	7	1	3	23
D. × elatum	'Sky sensation'	P04021846; P04021847	Bilateral	4	1	8	5	1	1	20
Aquilegia L.				mW1			mW2		mW2'	Number of perianth organs
							Flat	Spurred		
WT			Radial	Spurless in all cases			0	5	0	10
A. vulgaris	'Leprechaun Gold'	P04021850; P04021851	Radial				0	5	10	20
	'Nora Barlow'	P04021848; P04021849	Radial				5	0	16	26
	'Green Apples'	P04021854; P04021855	Radial				5	0	33	43

	'Ruby Port'	P04021852; P04021853	Radial		0	5	27	37

The herbarium code corresponds to a representative specimen of each cultivar deposited at the Paris Herbarium (P; https://science.mnhn.fr/institution/mnhn/collection/p/item/search/form). Since a single flower of each cultivar was dissected, the information is only an indication of the possible morphological diversity within cultivars. For instance, variation in the spur presence or absence was observed in flowers of *A.* × *hybrid* 'Green Apples' (absence in this table but presence on Fig 1).

Similarly, we classified the perianth organs of the *Aquilegia* cultivars in three categories from the outside in: 1) five organs quincuncially arranged forming *A*mW1 (*Aquilegia* mutant W1); 2) five organs topologically similar to W2 organs (*A*mW2) and corresponding to the first whorl of the ortostichies (this denomination is independent of the presence or absence of spurs); and numerous supernumerary organs located between *A*mW2 and the androecium, called *A*mW2'.

Results

Floral morphology, development, and anatomy of the *Delphinium* cultivars

The anthetic flowers of all the *Delphinium* cultivars studied were zygomorphic and composed of *D*mW1, *D*mW1', *D*mW2, and *D*mW2' organs (together forming the perianth), and of sexual organs (androecium and gynoecium). The perianth was composed of 13 to 24 spirally inserted, quincuncially overlapping organs (Figs 1A-H, 2A, 3A, Table 1). *D*mW1 and *D*mW1' comprised oblong to obovate organs with acute to obtuse apex. In the flowers of all cultivars except in some flowers of *D.* × *pacific* 'Blue Jay' (Figs 1A and 3A) and *D.* × *pacific* 'Astolat' (Fig 1E), the dorsal-most *D*mW1 organ was spurred as in WT. In those varieties, spurs were present on the dorsal most *D*mW1'. *D*mW2 included 3–8 spatulate and bifurcate organs, of which one or two dorsal ones were spurred (Table 1). All the flowers presented numerous (30–41) spirally arranged stamens (Figs 2A and 3A). Between the stamens and the carpels, we found organs presenting a filament, pollen sacs-like structures and an oblate outgrowth fixed along the filament or in other cases unclosed winged structures with an oblate outgrowth (Fig 4).

Except for *D.* × *elatum* 'Sky sensation' (three carpels) and *D.* × *pacific* 'Blue Jay' (seven carpels), the other three *Delphinium* cultivars presented five carpels. Before the development of the gynoecium is completed, some *Delphinium* flowers presented groups of undifferentiated cells developing between the carpels (Fig 5D). Furthermore, in some adult flowers primordia were identified between mature developed *D*mW1' organs (Fig 5F).

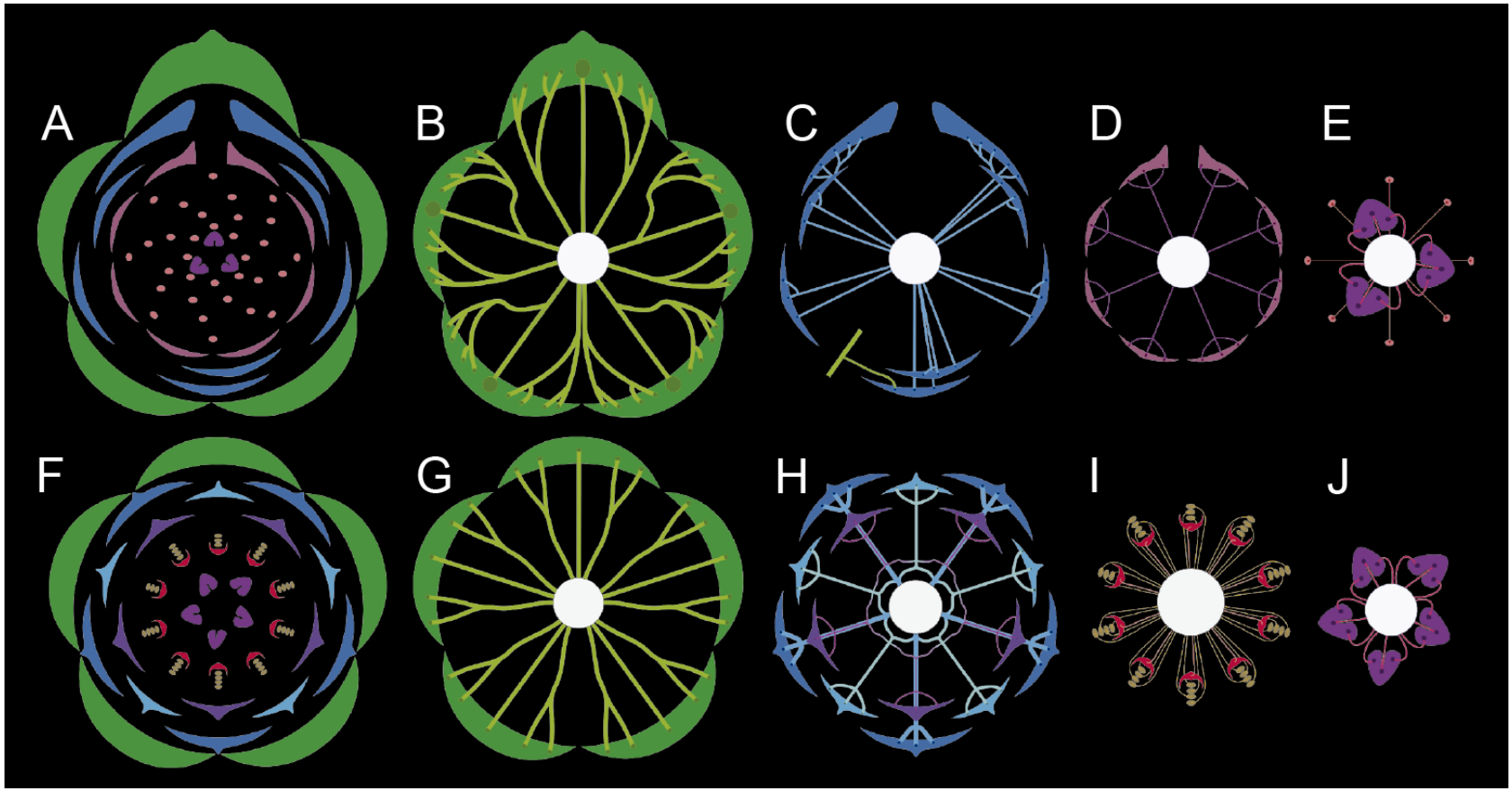
Outermost organs of the perianth in *D.* × *pacific* 'Blue Jay' are supplied by two vascular bundles, except for the dorsal organ supplied by three independent vascular bundles. The

two ventral and four lateral (two on the right side and two on the left side) inner organs are supplied by two vascular bundles each, and the two dorsal and spurred organs are supplied by a single vascular bundle each. Each of the innermost organs of the perianth is supplied by a single vascular bundle. Stamens are supplied by a single vascular bundle, and three independent bundles (Fig 2 B–E) supply each carpel.

Floral morphology, development, and anatomy of the *Aquilegia* cultivars

Anthetic flowers of the analyzed *Aquilegia* cultivars were actinomorphic (Fig 1F–I), and almost all flowers had an increased number of perianth organs compared with WT flowers (Table 1). Cultivars presented morphological variations among them, particularly linked to the presence of spurs. The *A*mW1 of all the flowers analyzed were spurless. In all the flowers of *A. vulgaris* 'Leprechaun Gold', the *A*mW2 presented spurs. On the contrary, the analyzed flowers of *A. vulgaris* 'Nora Barlow' presented *A*mW2 spurless organs and with a morphology similar to that of the *A*mW1. In the case of *A.* × *hybrida* 'Green Apples', some flowers presented *A*mW2 organs with spur and in other cases without spur, reason why the information in Fig 1H and Table 1 is contradictory. Finally, all *A.* × *hybrida* 'Ruby Port' flowers showed small spurs not visible in Fig 1I. We did not observed variations on spurs presence within the same plant.

*A*mW1 organs were ovate with an acute apex. *A*mW2 and *A*mW2' organs were arranged in three to five whorls of five organs (Fig 3B, Table 1). These whorls alternate with each other (Fig 6E–F). *A*mW2 organs were spurred and petaloid, except in *A. vulgaris* 'Nora Barlow' and some flowers of *A.* × *hybrida* 'Green apples' where the perianth was composed of non-spurred tepals. In all the cultivars analyzed, we observed *A*mW2' organs without a clear identity surrounding the outermost stamens (Figs 3B, 7 and 8). They consisted of a filament, pollen sacs-like structure and a petaloid extension at the apex. Stamens were arranged in ten orthostichies. Between the stamens and the 5–7 carpels, all analyzed flowers presented a whorl of ten staminodes (Fig 3B). During androecium development, we noticed cell masses between the orthostichies that disappeared in the latest developmental stages (Fig 6B).


A
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J

Fig 2. Floral vascularization of *Delphinium* × *pacific* 'Blue Jay' (A-E) and *Aquilegia vulgaris* 'Leprechaun Gold' (F-J).

(A) Floral diagram of *D*. × *pacific* 'Blue Jay': perianth (green, blue and red crescents), stamens (pink dots), carpels (violet reniform shapes). (B) Vascularization of *D*mW1 organs: dorsal spurred organ supplied by three bundles, the other ones by two ramified bundles. (C) Vascularization of inner perianth organs: two dorsal spurred organs supplied by a single bundle, the other ones supplied by two bundles. The ventral-most organ is supplied by two bundles and by a ramification of a bundle of one ventral *D*mW1 organ (these organs may correspond to DmW1' organs). (D) Vascularization of the most internal perianth organs: each organ is supplied by a single bundle and the dorsal-most organs are spurred (they may correspond to DmW2 organs). (E) Stamens and carpels are supplied by one and three bundles respectively. In this figure, we just make hypothesis about the *Delphinium* perianth organs denomination because the anatomical analysis did not allow us to observe the entire morphology of each organ as bud were treated before anthesis. (F) Floral diagram of *A. vulgaris* 'Leprechaun Gold': *A*mW1 (green), *A*mW2 (dark blue, light blue and violet crescents), stamens (beige dots), staminodes (red shapes), carpels (violet reniform shapes). (G) *A*mW1 organs are supplied by three vascular bundles. (H) Each *A*mW2 organ (in dark blue) is spurred and supplied by a single vascular bundle. The supernumerary perianth organs (*A*mW2') are supplied by a complex vascularization creating a sort of network between organs (bundles in violet to light blue). (I) Staminodes and stamens are supplied by a single bundle each. (J) Carpels are supplied by three bundles.

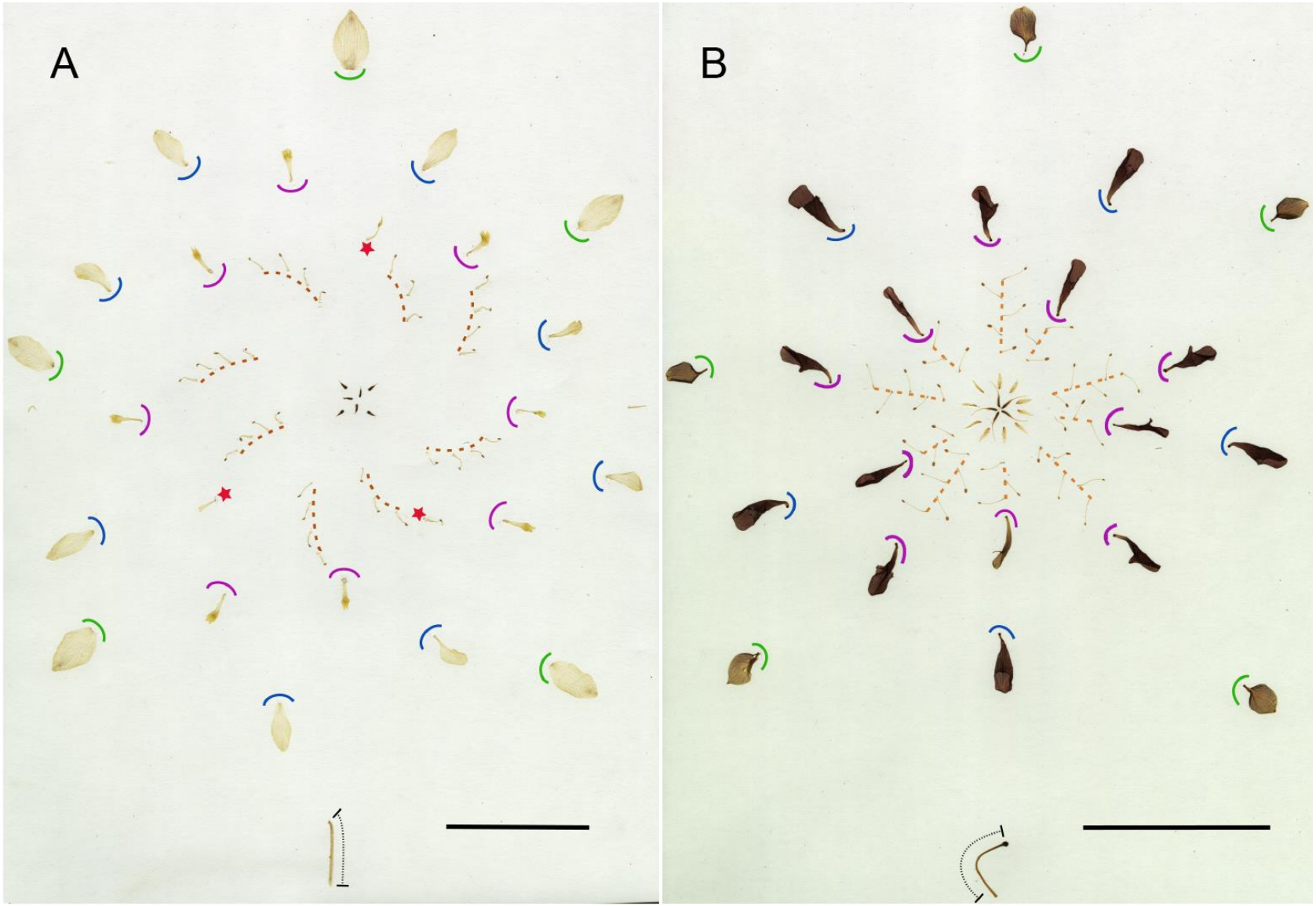
A
B

Fig 3. Dissections of *Delphinium* × *pacific* 'Blue Jay' and *Aquilegia vulgaris* 'Leprechaun Gold' flowers.

(A, B) Dissection of one flower of each cultivar, taken as an example to show floral organization. A black dotted line indicates the pedicel, scale = 5 mm. (A) Flower of *D.* × *pacific* 'Blue Jay': Green, blue, and violet arcs indicate *D*mW1, *D*mW1', and *D*mW2 organs, respectively; *D*mW2' organs are indicated with a red star and parastichies of stamens are represented by orange dotted lines. The carpels are at the center of the flower. (B) Flower of *A. vulgaris* 'Leprechaun Gold': Green arcs under the organs indicate *A*mW1 organs, blue arcs indicate *A*mW2 organs, violet arcs indicate the *A*mW2' organ. Orthostichies formed by the stamens are represented by orange dotted lines. Staminodes and carpels are arranged on whorls at the center of the flower.

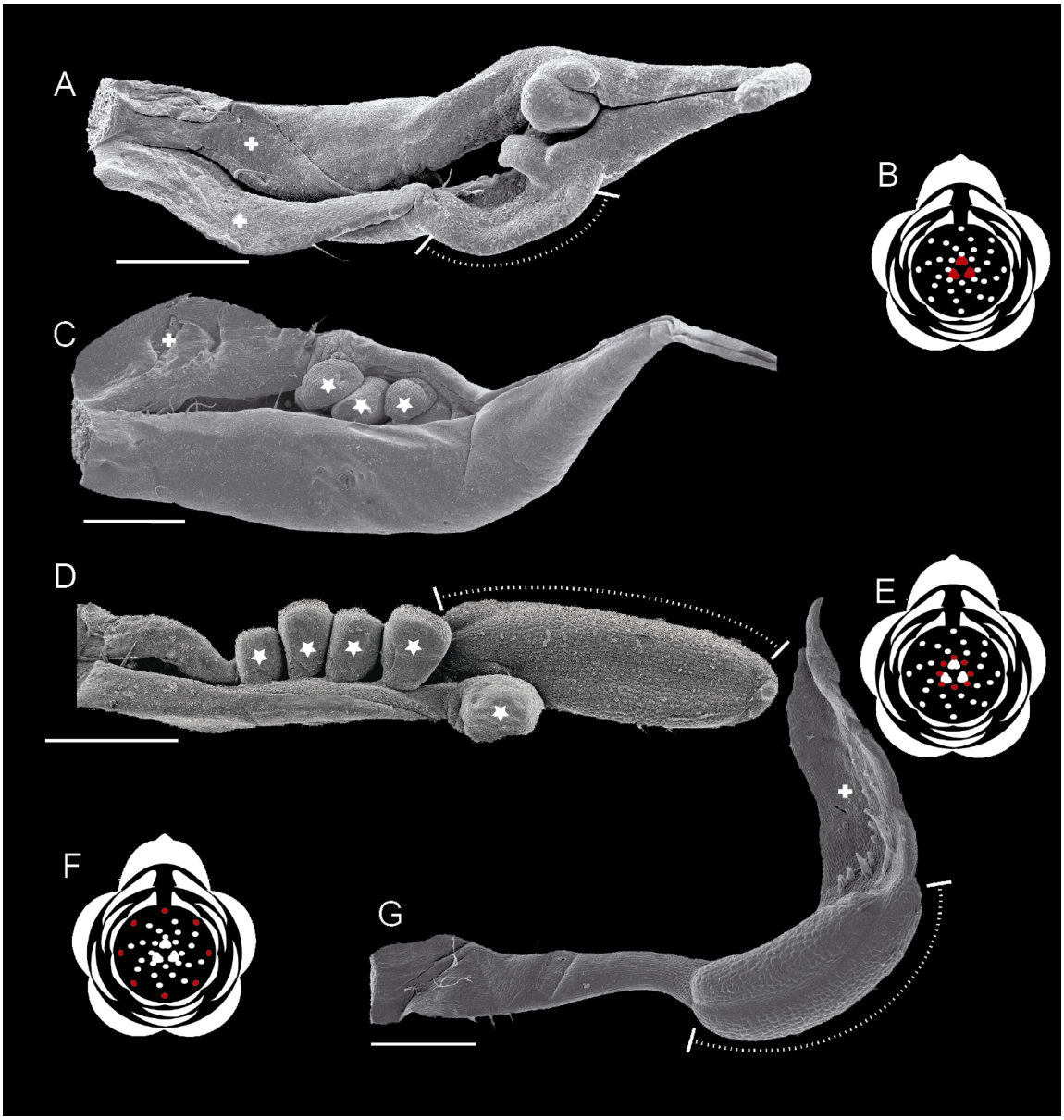
A
B
C
D
E
F
G

Fig 4. Abnormal organs of *Delphinium* × *pacific* ‘Blue Jay’ and their position within the flower.

(A and C) Abnormal organs located within the gynoecium of the flower. These organs look like open carpels with naked ovules (indicated with stars). They present abnormal structures looking like anthers (dotted line in A) and winged extensions (plus signs in A, C). (D) Abnormal organ within stamens and carpels and (G) *D*mW2’ organs surrounding typical stamens. These organs are composed of structures that look like anthers (dotted line) and outgrowths similar to naked ovules. Additionally, some of them (G) present wing petaloid extensions at the apex (plus sign), scale = 1 mm. (B, E, F) Floral diagram of *D.* × *pacific* ‘Blue Jay’, red dots indicate the position of these abnormal organs within the flower.

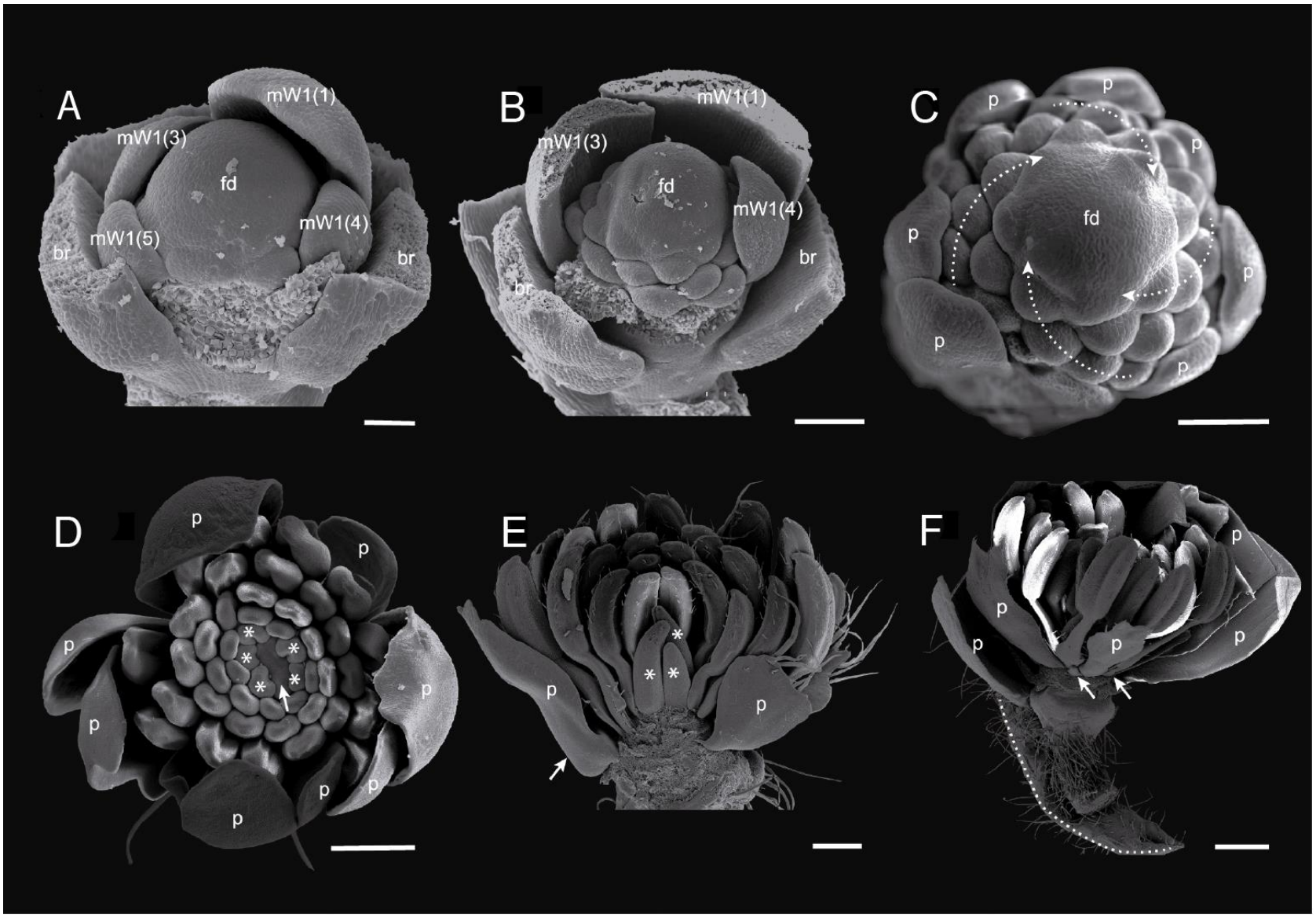
A
mW1(1)
mW1(3)
fd
mW1(4)
mW1(5)
br
br
B
mW1(1)
mW1(3)
fd
mW1(4)
br
br
C
p
p
p
fd
p
p
p
p
p
D
p
p
p
p
p
p
p
p
E
p
p
F
p
p
p
p
p

Fig 5. SEM micrographs of the floral developmental sequence of *Delphinium* × *pacific* 'Blue Jay'.

(A) Quincuncial development of *D*mW1, scale = 100 μm. (B) Before the establishment of zygomorphy, all primordia begin their differentiation on the floral dome. At this stage there is no delay in the development of any of the organs, scale = 200 μm. (C) Spirally inserted primordia producing parastichies on the floral dome (dotted arrows), scale = 200 μm. (D) Before the carpels (asterisks) are fully developed a set of undifferentiated cells remain at the top of the meristem (arrow), scale = 500 μm. (E) When organ differentiation is completed, the development of spurs begins (arrow), scale = 500 μm. (F) In horticultural flowers like in WT flowers, zygomorphy is established by the irregular development of perianth organs (arrows point to primordia with an arrested development), and by the development of spurs (highlighted with the dotted line), scale = 1mm. Abbreviations: mW1: *D*mW1 organs (numbered following the order of initiation), fd: floral dome, br: bracteoles, p: perianth organs (*D*mW1, *D*mW1', *D*mW2, or *D*mW2'), *: carpels.

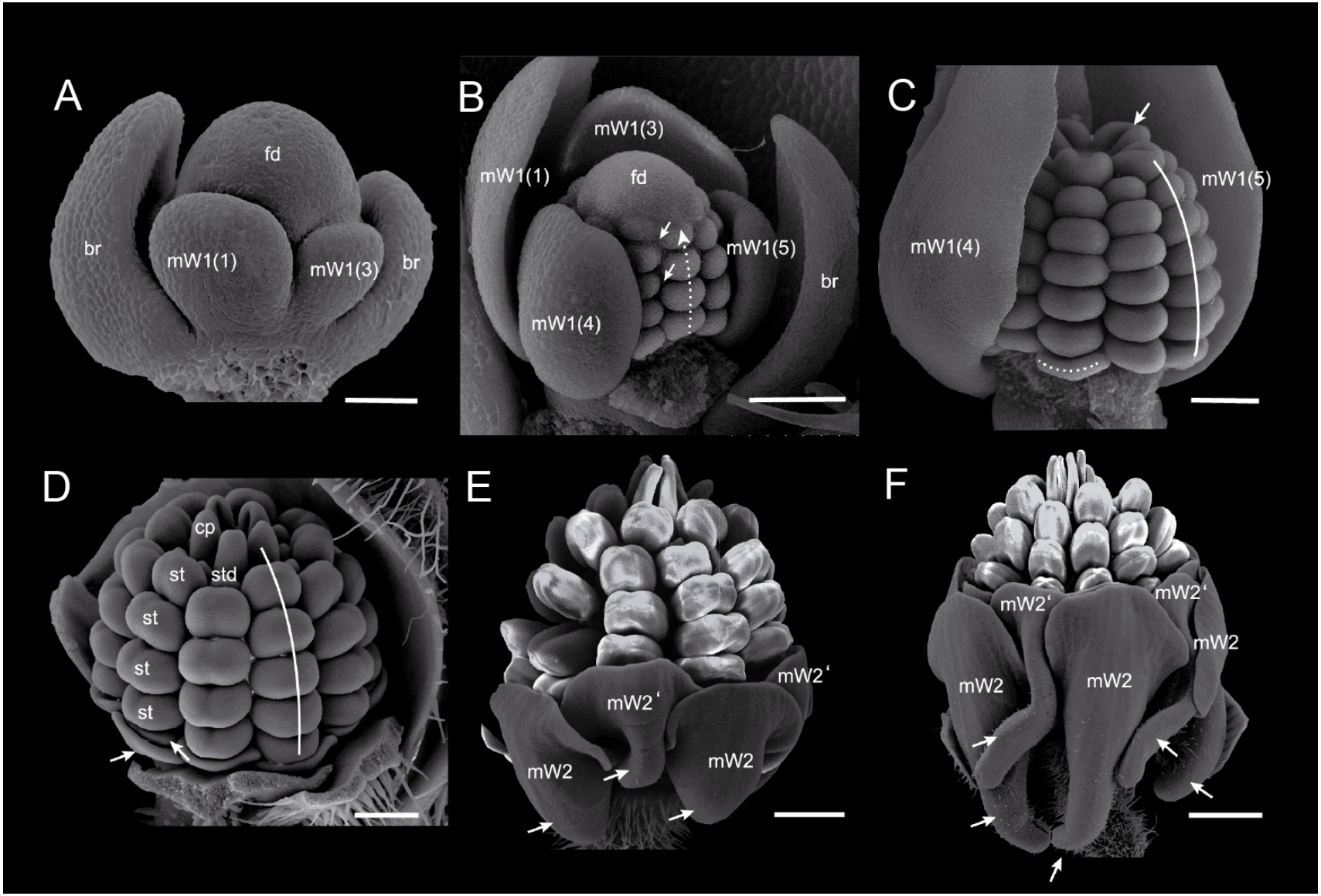
A
fd
br
mW1(1)
mW1(3)
br
B
mW1(3)
mW1(1)
fd
mW1(5)
br
mW1(4)
C
mW1(5)
mW1(4)
D
cp
st
std
st
st
st
E
mW2‘
mW2‘
mW2
mW2
F
mW2‘
mW2‘
mW2
mW2
mW2

Fig 6. SEM micrographs of the floral developmental sequence of *Aquilegia vulgaris* 'Leprechaun Gold'.

(A) Quincuncial development of *A*mW1 organs, scale = 50 μm. (B) Whorled organogenesis of *A*mW2 organs, *A*mW2' and stamens (dotted arrow showing the direction in which the differentiation of the organs occurs on the orthostichy), with sets of undifferentiated cells between abnormally distant orthostichies (pointed by arrows but not obvious in the micrograph), scale = 100 μm. (C) During carpel differentiation (arrow), there is no clear differentiation among *A*mW2' organs, and stamens or staminodes within the orthostichies (the line shows one orthostichy); only *A*mW2 primordia begin to expand and flatten (dotted line shows one flattened organ), scale = 100 μm. (D) Once all organs are differentiated, the difference in thickness and size of *A*mW2 and *A*mW2' organs and the rest of the organs on the orthostichy becomes obvious, AmW2' whorls are recognizable (arrows point to flattened organs at the base of the orthostichies; line showing an orthostichy), scale = 200 μm. (E, F) Spurs start to develop on *A*mW2 and *A*mW2' organs once floral organogenesis is completed. Successive whorls of *A*mW2 and *A*mW2' are alternate (arrows point to developing spurs), scale = 500 μm and 1 mm respectively. Abbreviations: br: bracteoles, fd: floral dome, mW1: *A*mW1 organs (numbered following the order of initiation), mW2: *A*mW2 organs, mW2': *A*mW2', st: stamens, std: staminodes, cp: carpels.

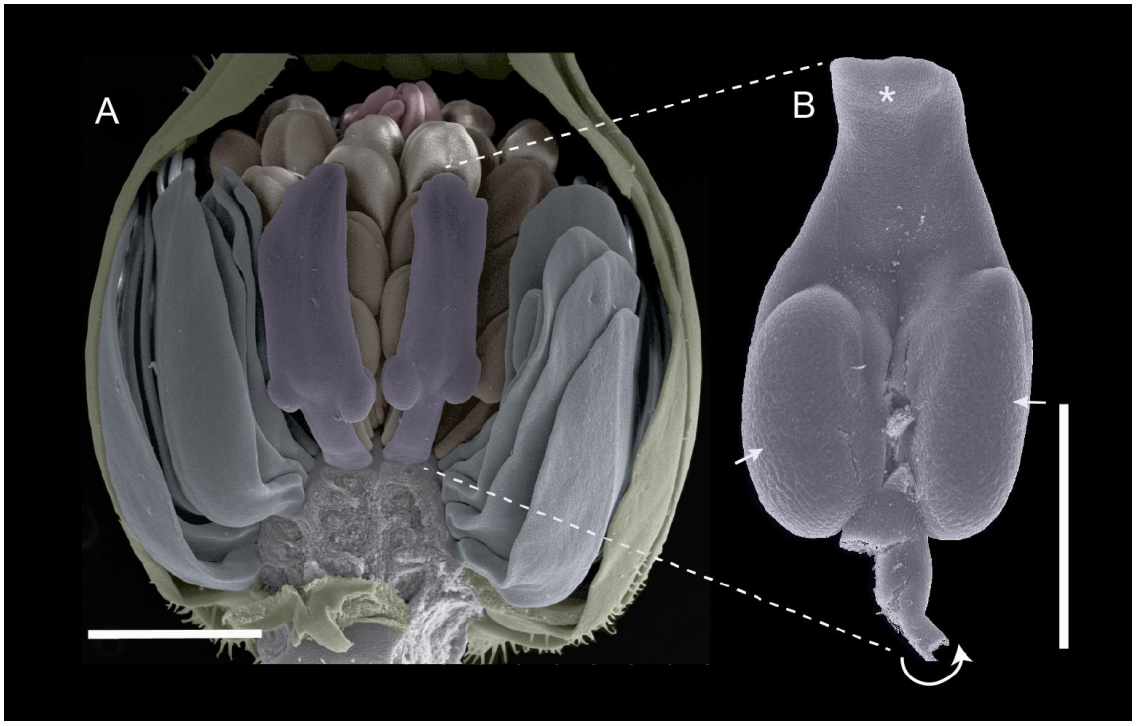

Fig 7. SEM micrographs of a floral bud and an abnormal organ of *Aquilegia vulgaris* 'Leprechaun Gold'.

(A) Lateral view of a bud (with some perianth organs removed in order to show the different floral organs): *A*mW1= green, *A*mW2 and outer *A*mW2'= blue, inner-most *A*mW2' with stamens characteristices = violet, stamens= orange, carpels= red; scale = 0,5 mm. (B) Inner view of an *A*mW2' organ: arrows point at structures looking like pollens sacs, the asterisk indicates a petaloid extension at the apex of the organ. A filament-like structure is found at the base of the organ; scale = 0,5 mm.

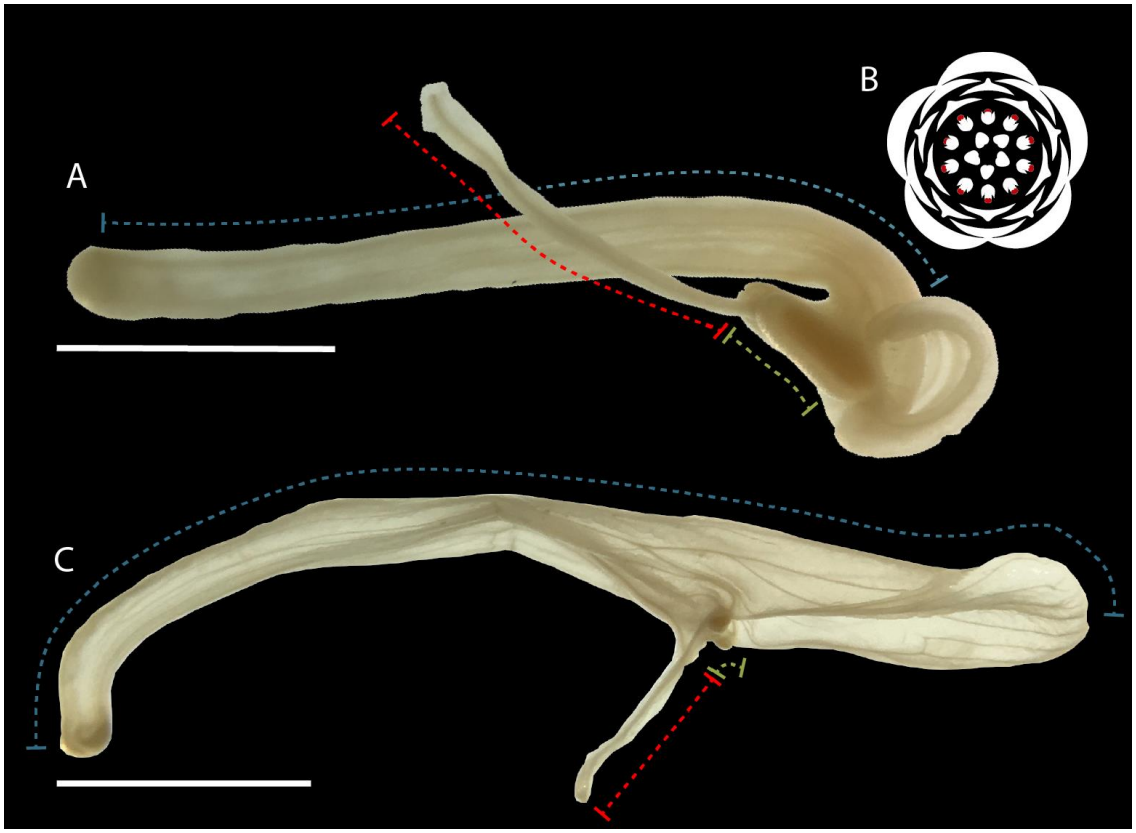

Fig 8. Abnormal organs (mW2') of *Aquilegia vulgaris* 'Leprechaun Gold' and their position within the flower.

(A, C) *A*mW2' organs presenting a spur-like structure (blue dotted line) and an anther-like structure (green dotted line), scale = 0,5 mm. The organ is fixed to the floral receptacle by a filament-like structure (red dotted line). (B) Floral diagram of *A. vulgaris* 'Leprechaun Gold', red dots indicate the localization of these type of *A*mW2' organs within the flower.

In 'Leprechaun Gold' cultivar flower, *A*mW1 organs are supplied by three independent vascular bundles; each *A*mW2 organ is supplied by one vascular bundle that ramifies in three bundles at the basis of the organ and *A*mW2' organs are supplied by a single bundle each one originating from *A*mW2 bundles. Each stamen and each staminode is supplied by a single vascular bundle and each carpel is supplied by three independent vascular bundles (Fig 2G–J).

Discussion

The cultivars of *Delphinium* and *Aquilegia* analyzed in this study presented floral variations mostly affecting the perianth, in terms of number and form. The number of stamens and carpels appeared similar to wild-types. In some cases, abnormal supernumerary organs presenting intermediary characteristics of androecium and gynoecium were observed. Additionally, major floral phenotypic variations among plants from the same cultivar were observed, indicating that floral characteristics within cultivars are not stable. In all, these variations give rise to remarkable double flowers selected by horticulturalists.

Development of supernumerary organs in cultivars of *Delphinium* and *Aquilegia*

All analyzed flowers of both *Delphinium* and *Aquilegia* presented supernumerary organs that have a morphology ranging between that of the surrounding organs. However, the wild type symmetry is conserved. *Delphinium* cultivars had zygomorphic flowers, even though not always obvious (see below), and *Aquilegia* cultivars had actinomorphic flowers. The analysis of the floral organization of the abnormal flowers in the two genera suggested that the increase in perianth organ number could be partly related to their different types of symmetry.

Supernumerary organs in *Delphinium*

In comparison with WT *Delphinium* flowers, all the analyzed flowers of cultivars presented abnormal supernumerary organs (*D*mW1') spirally arranged between *D*mW1 and *D*mW2, and with intermediate morphology and anatomy.

First, the color of these organs varies between the colors of *D*mW1 organs (blue, lilac or white) to those of *D*mW2 organs (stramineous to white or black, in some cases, the inner organs are white with some brush-strokes of black or stramineous, Fig 1).

Second, *D*mW1' organs present a gradient between the *D*mW1 and *D*mW2 morphologies, ranging from outer oblong or obovate organs with an acute apex to thinner and spatulate inner organs (Fig 3). Third, the vascularization of *D*mW1' organs differs from wild type perianth organs: the dorsal-most *D*mW1' organ presents a single vascular bundle and the other *D*mW1' organs present two vascular bundles (Fig 2B-C), while W1 typical organs are supplied with three bundless and W2 typical organs are all supplied with a single bundle [78]. Additionally, one of the ventral *D*mW1' organs shares one lateral bundle with another mW1 organ (Fig 2C), indicating a possible case of splitting within perianth organs. *D*mW2' supplementary organs present a mixted identity between perianth and fertil organs.

In this genus, zygomorphy results from two phenomena: 1) the development of nectariferous spurs in the dorsal perianth organs in W1 and W2, and 2) a restricted development of W2 organs [66]. Similar to the WT phenotype, the dorsal *D*mW1 organ is generally spurred, rendering the flowers zygomorphic (Figs 1A–E and 2A). However, in some cases of *D.* × *pacific* 'Blue Jay' and *D.* × *pacific* 'Astolat' this organ is not spurred indicating a tendency to peloria. In these cases, if *D*mW1 organs are spurless, at least one *D*mW1' or *D*mW2 spurred organ was observed. On anatomical sections of *D.* × *pacific* 'Blue Jay' flower spurs develop on the dorsal organs of *D*mW1, *D*mW1' or/and *D*mW1' and *D*mW2, creating a system of nested structures (Fig 2A–E, Table 1). In contrast to the WT *Delphinium* flower where only the four dorsal primordia are entirely developed, the abnormal homogenous development of all the *D*mW2 organs in *Delphinium* cultivars indicates a tendency to peloria and results in an increase in perianth organ number whithout affecting the type of symmetry.

Supernumerary organs in *Aquilegia*

Following our topological conventions, we interpreted the outermost whorl of these organs as *A*mW2 and the others as *A*mW2' organs (Figs 2H, 3B and 6E, F, Table 1). The flowers of *Aquilegia* cultivars presented a perianth with supernumerary organs compared to WT flowers (Fig 1F–I). There is a recognizable *A*mW1 of external organs equivalent in morphology and anatomy to W1 typical organs (Figs 2F, G, 3B and 6A, B; [79,80]). The numerous organs located between *A*mW1 and the stamens, present a morphology and a vascularization similar to typical W2 organs (spurred petaloid organs). The morphology of AmW2' organs ranges from typical W2 morphology

(petaloid spurred organs, supplied by a single bundle; [79,80]) to organs with stamen characteristics but presenting petaloid extensions at the apex (Fig 7). In some cases, this extension formed a spurred structure (Fig 8). Anatomically, *Aquilegia* supernumerary organs present the same vascularization of W2 of WT flowers (Fig 2H; [79,80]). However and in contrast with WT vascularization, the origin of the traces supplying *A*mW2 and *A*mW2' organs create a complex network of vascularization: each bundle supplying *A*mW2' organs is the union of two ramifications derived from the bundles of the nearest external whorl (Fig 2H; [79,80]).

Aquilegia vulgaris 'Nora Barlow' presented flowers with a perianth composed of more than 25 morphologically undifferentiated spurless petaloid organs (Fig 1F, Table 1), far exceeding the typical ten petaloid organs of WT flowers [24].

Putative genetic bases of perianth diversity

Different developmental mechanisms generating floral morphological diversity have been identified in this study: 1) development of supernumerary organs, 2) abnormal establishment of organ identity, 3) alterations on spur development, and 4) variations on the repression of organs development of the flower. In the following discussion, we propose potential molecular mechanisms that might account for these phenomena.

Origin of supernumerary organs in *Delphinium* and *Aquilegia* cultivars

The development of *D*mW1' and *D*mW2' organs in *Delphinium* and *A*mW2' organs in *Aquilegia* can be explained by two different mechanisms identified and studied in the model species *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae).

First, an increase in the size of a floral meristem affects the population of constituting cells. The number of cells in the floral meristem of *Arabidopsis thaliana* is controlled by the interaction between the transcription factor WUSCHEL, the *CLAVATA-1, -2* and *-3* genes encoding receptor factors [81–85] and other transcription factors such as FASCIATA-1 and -2 [86], ULTRAPETALA [87], and WIGGUM [88]. Modifications of the activity of these transcription factors result in the presence of new cellular regions producing an increased number of perianth organs. In some cases, these modifications give rise to abnormal filamentous organs [70,89]. In *Oryza sativa* (Poaceae), alterations of similar mechanisms controlled by orthologs of *CLAVATA* genes (*FLORAL ORGAN NUMBER1* and *FLORAL ORGAN NUMBER2*) [90–94] have similar consequences. In the cultivars studied here, the presence of abnormal masses of meristematic cells

between carpels in *Delphinium* (Fig 5D), and between orthostichies in *Aquilegia* (Fig 6B), could be due to an increase in size of the floral meristem, although we cannot provide any evidence for it, lacking comparative data.

Second, an alteration of spacing mechanisms determining primordia position in the floral meristem. In *Arabidopsis thaliana*, mutations of the *PERIANTHIA* [95,96] and *ETTIN*/*AUXIN RESPONSE FACTOR* (*ETT*/*ARF3*) [97] genes affect the auxin signaling pathway, and modify the organ patterning on the floral meristem, producing flowers with bigger and/or supernumerary floral organs. On an abnormally large meristem, this may lead to the presence of abnormal cell masses as in Fig 5D hence to supernumerary primordia producing new and abnormal organs [98].

Identity of supernumerary organs

The ABCDE model assumes that the combination of different functions (i.e. the interplay among different sets of transcription factors) controls the identity of the developing floral organs [99,100]. Sepal identity is established by the interaction of homeotic genes of the A and E functions; petal identity by the combination of the A, B and E functions; stamen identity by the combination of the B, C and E functions and carpel identity by combination of the functions C and E; genes of the D function are involved in ovary development [32].

In Ranunculaceae, the A function is ensured by the MADS-box genes *FRUITFUL* (*FUL*)-like and *AGL6*-like, that control the identity of W1 organs [101,102]. The B-function appears elaborated, involving at least on paralogue of the *Arabidopsis thaliana PISTILLATA* gene and three paralogs of the *APETALA3* gene. The W2 organ identity is strongly dependent on the expression of the *APETALA3-3* orthologues [103]. Interestingly in *Aquilegia*, the formation of the staminodia is controlled by the B-function paralogue *APETALA3-1* [71] and the *AqPI* promotes the petaloidy of W1 [41,70,104]. The C-function is supported by two homologs of the *Arabidopsis thaliana AGAMOUS* genes *AG-1* and *-2* and the E-function by homologs of *SEPALLATA-1, -2* and *-3* (*SEP -1*, *-2*, *-3*) [16,102]. The gradient of identity observed in the supernumerary organs *D*mW1', DmW2' and AmW2' (surrounding stamens) (Figs 2C, 3A, 4, 7 and 8) suggests a "gray zone" where identity is not stable, possibly corresponding to fluctuation in the extension of expression domains of genes involved in A- and C-functions. In this case, there is no homeosis because organs presenting an abnormal identity are supernumerary organs [105]. In some *Aquilegia* cultivars, the absence of

typical spurred W2 organs and the correlated increase of W1-like organs is reminiscent of the inactivation of the B-function *AP3-3* genes observed for example in *Nigella damascena* [103].

Spur development in *Aquilegia* and *Delphinium* cultivars

Within the family (Ranunculaceae), the formation of spurred nectariferous petals has been studied in *Aquilegia* [106–108]. It was first suggested that the development of nectariferous spurs was driven by meristematic knobs producing continuous cell divisions [69,80]. However, another study proposed a two-step model for petal spur morphogenesis in which reduction in the speed of cell divisions precedes anisotropic cell elongation [108].

More recently, a molecular investigation of the mechanisms of spur development has shown that the repression of cell division is controlled, on the one hand by a lower expression level of *KNOX* homologues and a repression of seven growth-regulating factors (GRFs), and on the other hand by the expression of *AqTCP4* [107]. Additionally, the expression of genes mediating auxin biosynthesis (such as *Cyclophilin71A* [*CYP71A*], *Auxin response factor8* [*ARF8*], *Auxin response factor3* [*ETT*], *Small auxin up RNA* [SAUR]) and affecting the brassinosteroid signaling pathway was shown to be involved in cell proliferation and expansion in the early developmental stages of the *Aquilegia* spur [107]. In our *Aquilegia* cultivars, the presence of organs with an intermediate morphology (*A*mW2' surrounding stamens) developing spurs (Figs 3B and 8), may indicate an ectopic activation of the spur development program on reproductive organs. In *A*mW2 and *A*mW2' organs in *Aquilegia vulgaris* 'Nora Barlow', the presence or absence of spurs could be linked to changes in the hormonal and/or genetic control of cell proliferation and elongation in floral organs.

Similar mechanisms of spur development could be involved in the formation of *Delphinium* spurs, even though the two genera are not closely phylogenetically related. In addition in this genus, spur development seems to interact with the floral symmetry pathway (see below), since not all organs of W1 or W2 are spurred.

Supernumerary perianth organs and alteration in zygomorphy in *Delphinium* cultivars

The genetic mechanisms underlying the establishment of perianth zygomorphy through the differential development of organs of the same type were first studied in

Antirrhinum majus L. (Plantaginaceae). These studies demonstrated the key role of the *CYCLOIDEA* gene (*CYC*, [109]) and its paralog *DICHOTOMA* (*DICH*, [110]), both encoding TCP transcription factors that are expressed in the dorsal part of the flower during development and produce a dorso-ventral differential development of floral organs. Asymmetric expression of homologues of *CYC* have been shown to play a role in floral symmetry in diverse groups of core eudicots [10,109,111,112]. In Ranunculaceae, duplications of *CYC*-like genes coincide with the evolutionary divergence of Delphinieae and the origin of zygomorphy within the family. An asymmetric expression of *CYC*-like genes (*RANACYL*) has been observed in W1 organs of two zygomorphic Ranunculaceae species, suggesting that they may play a role in floral symmetry [27]. However, the activity of these genes can affect the development of W2 organs too. This phenomenon could thus explain the appearance of supplementary adult *D*mW2 organs in the ventral part of the flower (Fig 3A). However, among these organs we observed primordia that did not achieve a fully development (Fig 5F). The modifications of asymmetric expression of the *RANACYL* genes could occur in an irregular way creating unstable morphology between the different cultivars analyzed.

Conclusions

Cultivars result from human selection aiming at developing showy floral characteristics. *Delphinium* and *Aquilegia* cultivars present double flowers with alterations in the number and position of nectariferous spurred structures, and a visual modification of the floral symmetry with a tendency to actinomorphy in *Delphinium*. We propose that the increase in organ number might be due to an increase in meristem size that could entail an alteration in the boundaries and size of the expression domains of homeotic genes of the ABCDE model of floral organ identity. In addition, in *Delphinium* cultivars these mechanisms would be associated with alterations in the floral symmetry pathway, including in some cases the inactivation of the spur development programme. This study gives insights about possible target genes and developmental stages to study in order to understand the origin of floral diversification in Ranunculaceae.

Acknowledgments

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3. Conclusions

Cette étude m'a amené à proposer des hypothèses en génétique et en développement floral afin d'expliquer la variabilité florale au sein des cultivars étudiés.

D'une part, le développement des organes surnuméraires peut s'expliquer par deux phénomènes qui ont lieu à des stades différents du développement floral :

1) L'altération de la division cellulaire et l'accumulation de cellules dans le méristème floral peuvent provoquer l'élargissement de celui-ci, permettant ainsi le développement d'organes surnuméraires, ainsi que l'insertion de parastiches incompletes ou de verticilles anormaux. Ce phénomène aurait lieu avant l'expression des gènes d'identité florale responsables des fonctions ABCDE.

2) La modification de la distance entre les primordia sur le méristème floral peut entraîner une réorganisation des organes et induire le développement d'organes nouveaux. Ce phénomène aurait lieu après l'expression des gènes de l'identité et dépendrait de l'activité des gènes de la fonctions B.

D'autre part, l'élargissement du méristème floral peut entraîner une modification des zones de frontière entre les types d'organes floraux produisant des organes aux morphologies anormales.

De plus, le développement anormal de la corolle (produisant une modification de la symétrie du périanthe) peut indiquer des variations dans l'expression de gènes responsables, en partie, de l'établissement de la symétrie bilatérale chez les *Delphinium*.

Les altérations de certains gènes et la modification de leur expression à des moments précis dans le développement floral peuvent avoir un effet en cascade sur différents caractères morphologiques induisant des déviations importantes dans l'organisation de la fleur. A travers une sélection positive, ces altérations peuvent s'établir, créant de nouvelles variétés au sein d'une espèce. Les hypothèses formulées dans cette partie de ma thèse ont permis de cerner de façon plus précise les étapes développementales ainsi que les bases génétiques possibles menant à des nouvelles morphologies florales.

Chapitre 3 : Taxonomie vs diversité morphologique

1. Introduction

Les deux chapitres précédents m'ont permis de mettre en évidence et d'expliquer :
1) l'établissement et le maintien de variations dans la morphologie florale d'une espèce de *Delphinium*, et 2) l'origine de la diversité florale présente au sein de quelques cultivars du genre. Les déviations observées et leur analyse m'ont permis d'émettre l'hypothèse selon laquelle un nombre réduit de modifications touchant quelques gènes homéotiques pouvaient entraîner d'importantes modifications morphologiques de la fleur de *Delphinium*. De plus, ces mutations peuvent se maintenir spontanément dans la nature.

Le genre *Delphinium* est un genre connu depuis relativement longtemps et a subi de nombreuses modifications taxonomiques depuis sa publication par Linné en 1753. Ces changements taxonomiques sont dus, entre autres, à la diversité des modes de vie (vivaces, annuelles ou bisannuelles) des espèces qui le composent, ainsi qu'à la diversité des formes florales qu'il inclut. Depuis une dizaine d'années, et à l'aide de la phylogénie moléculaire, les relations au sein du genre ont été largement clarifiées. Par exemple, les genres *Consolida* (DC) Gray et *Aconitella* Spach, reconnus dans nombreuses classifications anciennes, se sont avérés être inclus, au sens phylogénétique du terme, au sein de *Delphinium* [1–4].
Par ailleurs, un travail bibliographique nous a révélé un cas assez proche de celui décrit dans le Chapitre 1 : celui de l'espèce *Chienia hoanaensis* W.T. Wang, considérée comme une population naturelle déviante de *Delphinium grandiflorum* L. (voir manuscrit suivant). Les individus de *Delphinium turcicum*, à la morphologie florale anormale, sont la preuve que des individus tératologiques peuvent survivre et se maintenir au sein du genre.

Dans ce chapitre je me suis posé la question suivante : quelles sont les conséquences taxonomiques de la diversité florale au sein du genre *Delphinium* ?
Pour répondre à cette question j'ai réalisé une revue de l'histoire pré-linnéenne du genre afin de mieux comprendre l'origine taxonomique de celui-ci. J'ai ensuite passé en revue la plupart des classifications post-linnéennes ayant entraîné des modifications dans la taxonomie du genre. Finalement, j'ai modifié la description de la fleur du genre afin de la rendre cohérente avec les caractéristiques décrites pour *Pseudodelphinium*.

Je présente ci-dessous un manuscrit en préparation destiné à être soumis à Adansonia.

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2. Manuscrit : Note historique sur *Delphinium* L. et ses variations florales : vers une nouvelle diagnose de ce genre ancien ?

An old genus in need of a new diagnosis? Historical note on *Delphinium* L. and its floral variations

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Pre-Linnean history of the genus *Delphinium* L.

The first reference to the name "*Delphinium*" (in this article, pre-Linnean Latin botanical names are written between inverted commas) is found in the translations of Dioscorides' *De Materia Medica* dating from the 16th century [1–4]. In these translations, the name is given to plants presenting prolonged, fine and dissected leaves resembling dolphins (from the Greek δελφίνι, [delphini]). One cannot exclude that the name was added to the original text at some point during the successive translations since no reference to plants called "*Delphinium*" was made in a Latin translation dating from the first half of the 9th century (*De materia medica*, Dioscorides, scanned version available at *Bibliothèque nationale de France*, classification: Latin 12995).

Interestingly, in the *Natural History* of Pliny the Elder, plants corresponding to "*Delphinium*" sensu Dioscorides are called "*Hyacintum*" [5,6]. There is no description of the plant, but the medicinal uses mentioned for "*Hyacintum*" are the same as those described by Dioscorides for "*Delphinium*". Translations of *De Materia Medica* dating from the 16th century include an illustration dating from Matthioli's translation, actually showing a specimen of *Consolida* (DC.) Gray plant, named "*Cuminum syl. alternum*" [1,4].

Later, in the first half of the 17th century, Bauhin proposed a group called "*Consolida regalis*" based on the name given by the "ancient authors" (sic) [5]. In this group, he included the "*Hyacintum*" sensu Pliny the Elder and "*Delphinium*" sensu Dioscorides [5]. At the end of the same century, Tournefort (1694) resurrected the group "*Delphinium*", considering "*Consolida regalis*" sensu Bauhin as synonym [7]. Additionally, he suggested not to use Bauhin's "*Consolida regalis*" to avoid any confusion with the groups "*Consolida major*", "*media*" and "*minor*" [7]. Tournefort found that flower buds, rather than leaves, looked like the representations of dolphins made by contemporaneous painters [1]. He described "*Delphinium*" flowers as having a pentamerous dorsally-spurred calyx and a capsule with three separate cavities [7].

Hints about the taxonomic history of the genus *Delphinium* L.

The first valid publication of the name *Delphinium* was made by Linnaeus in 1753 [8]. He placed the genus into the group called "*Polyandria trigynia*", i.e. characterized by an androecium formed of numerous stamens and a gynoecium formed of three carpels, although he also included species with a single carpel in *Delphinium*. Back then, the

genus consisted of six species: *D. consolida* L., *D. ajacis* L., *D. peregrinum* L., *D. grandiflorum* L., *D. elatum* L., and *D. staphisagria* L. The designation in the protologue of a type specimen for the genus name was missing [8].

Later, de Candolle (1818) proposed an infrageneric classification of *Delphinium* L. where the genus was divided it into four sections, one of them called *Consolida* DC., including "*Consolida regalis*" sensu Bauhin, among other species [9]. In 1821, Gray proposed to raise the section *Consolida* DC. [10] to the generic rank. But in 1839 Spach proposed a concept of the genus *Delphinium* L. including *Consolida* (DC.) Gray [11]. He considered *D. consolida* L. to be the annual representative of *Delphinium* (L.) Spach [11]. Huth (1895) conserved the name *Delphinium* L. but split it into two subgenera: subg. *Consolida* (DC.) Gray and subg. *Eudelphinium* Huth. Huth's classification was used by Dalla Torre & Harms (1901) [12].

Three lectotypifications for a single genus name

Following the automatic typification of the American Code, Britton & Brown (1913) proposed the first typification of *Delphinium* L., choosing the species listed first in the protologue: *D. consolida* L. (type: LINN 694.1[http://linnean-online.org/6495/, Figure 1.B) [13]. However, Nieuwland (1914) noted that *D. consolida* L., with a single carpel and a single petal, was a particular case of *Delphinium* L. He hence decided to separate the genus into three different subgenera, resurrecting the name *Consolida* (Brunfels) Gray. He proposed a new lectotype for *Delphinium* L.: the type specimen of *D. peregrinum* L. (BM-000628786 [http://www.nhm.ac.uk/our-science/data/linnaean-typification/search/detailimage.dsml?ID=307200], Figure 1.A). This proposition was confirmed by Hitchcock & Green [14]. In addition, and following the classification proposed by Pawłowski (1964) and Davis et al. (1965), Munz (1967) accepted the type of *D. peregrinum* L. as type of *Delphinium* L. [15–17].

The lectotypification of Linnean generic names favored the specimen of *D. peregrinum* L. [18] because, as Jarvis (1992) explained, the earlier adoption of *D. consolida* L. as type of the genus *Delphinium* L. may imply to make the combinations of all the species of *Consolida* (DC.) Gray in *Delphinium* L., and to find another generic name to the group he considers *Delphinium* L. sensu stricto. He concluded that the lectotypification of *D. peregrinum* L. was in agreement with the taxonomy of his time [18]. However, considering the genus *Consolida* (DC.) Gray, anticipating the probable adoption of the sections *Delphinellum* and *Staphysagria* as new genera, and recognizing the generic

level of two sections formed by *D. grandifl*orum and *D. elatum* respectively, Warnock (1993) decided to revise the typification of the genus and proposed as type species *D. elatum* L. (Figure 1.C) providing, according to him, "the greatest nomenclature stability"[19]. However, this proposal was rejected during the nomenclature section held at the XV International Botanical Congress Tokyo (2009) [20]. Blanché (1997) considered the second lectotypification (based on *D. peregrinum* L.) as a "traditional" typification.

Nowadays, in the Code, although Heath (1990) proposed the deletion of this example [21], *Delphinium* L. continues to be cited as a case of typification following the American code, later supplanted by a traditional typification in order to be in agreement with Art. 10.5 [22].

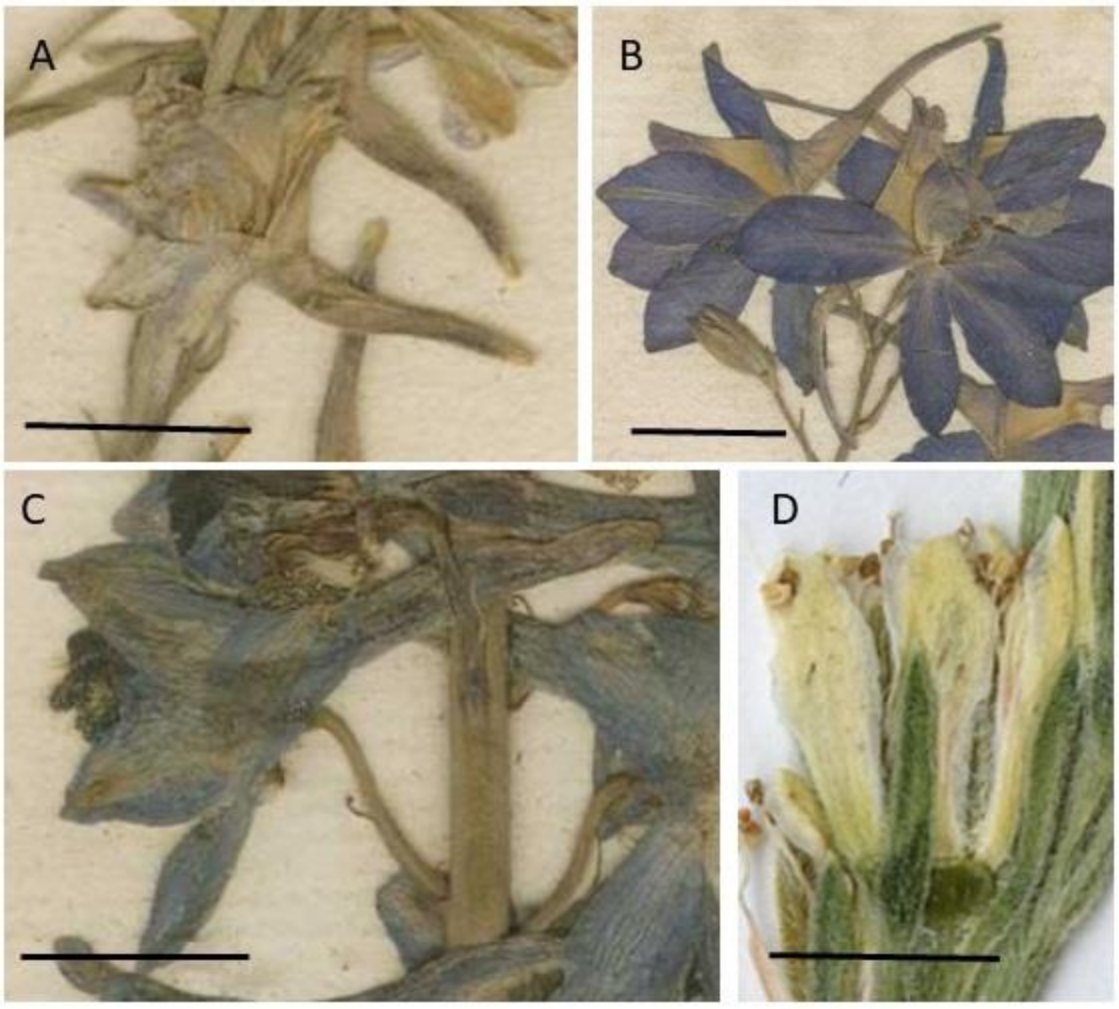

Figure 1. Détail de fleurs des spécimens de : A- *D. peregrinum* (LINN 694.6); B- *Consolida regalis* (LINN 694.1); C- *D. elatum* (LINN 694.8); échelle = 1cm; D- *Delphinium turcicum* (P04021863).

Current taxonomy of the genus *Delphinium* L.

Morphological, cytological and molecular characters provided an important background to better understand the evolutionary history of *Delphinium* L. Comparing molecular data with morphological data, Jensen et al. (1995) proposed *Delphinium* L. and *Consolida* (DC) Gray as independent genera. He proposed that *Delphinium* L. and *Aconitum* L. together form the subtribe Delphiniinae Benth. [23]. In addition, he included this subtribe into the tribe Delphinieae Warm., within the subfamily Ranunculoideae Hutch. in Ranunculaceae [23]. Using 65 morphological characters and plastid and ribosomal molecular data, Wang et al. (2009) placed the genera *Delphinium* L. *Consolida* (DC.) Gray and *Aconitum* L. within the tribe Delphinieae [24]. However, Jabbour & Renner (2011) found that the genus *Consolida* (DC.) Gray was nested within *Delphinium* L. making the latter genus paraphyletic. For this reason, they proposed the inclusion of the genus *Consolida* (DC.) Gray into the genus *Delphinium* L. rather than splitting *Delphinium* L. into several genera [25]. Later, based on the results of a molecular phylogenetic analysis, they decided to resurrect the genus *Staphisagria* J. Hill, including *D. staphisagria* L. [26]. They recognized three genera in Delphinieae: *Delphinium* L., *Aconitum* L. and *Staphisagria* J. Hill. [27]. More recently, the genus *Aconitum* L. was split into the genera *Aconitum* L. and the monotypic *Gymnaconitum* (Stapf.) Rapaics [28]. Taking these results into consideration, and among the species cited in Linnaeus' protologue, the genus *Delphinium* L. now contains the species *D. consolida* L., *D. ajacis* L., *D. peregrinum* L., *D. grandiflorum* L. and *D. elatum* L.

Description of the typical *Delphinium* L. flower

The genus *Delphinium* L. sensu lato (including *Consolida* (DC.) Gray and *Aconitella* Spach) [25] belongs to the tribe Delphinieae (Ranunculoideae, Ranunculaceae, Ranunculales). This tribe is the only clade within the family Ranunculaceae presenting flowers with a bilateral symmetry.

Based on *D. peregrinum* L. flowers, *Delphinium* typical flowers are composed of four different types of organs: two types forming the perianth, W1 (outer perianth organs) and W2 (inner perianth organs) [29], and two types of sexual organs, the stamens and the carpels [30]. From the outside in, W1 organs are 5 free petaloid organs quincuncially arranged: 2 ventral, 2 lateral and a dorsal one that is spurred. W2 organs are 4 free petaloid organs located in the dorsal half of the flower: 2 dorsal organs

forming nectariferous spurs inserted into the spur of the dorsal W1 organ and with an exserted limb, and 2 lateral organs with a wide limb and a narrow claw. The other W2 organs (corresponding to 4 ventral primordia) stop developing shortly after organogenesis [29,31]. Stamens are arranged in 8 spiral series, and the gynoecium is composed of 3(–5) free follicles [17]. In *Delphinium*, bilateral symmetry is established through two phenomena: 1) the development of dorsal spurs, and 2) the arrested development of the ventral W2 organs [32].

Amending *Delphinium* L. description to account for exceptions in floral morphology

On the one hand, the inclusion of *Consolida* (DC.) Gray into *Delphinium* L. [25] implies an enlargement of the floral typical characters of the genus. The perianth of *Consolida* (DC.) Gray is bilaterally symmetrical and is composed of 5 W1 petaloid organs which arrangement and morphological characteristics are identical to those of *Delphinium* L. W1. However, the inner perianth organs of *Consolida* (DC.) Gray consist of only 2 fused dorsal organs forming a single organ with a nectariferous spur inserted into the W1 spur. The other W2 organs (in this case 6 primordia) stop developing shortly after initiation [29,31]. *Consolida* (DC.) Gray flowers are bisexual, with 5 spirals of stamens, 3 less than the typical *Delphinium* L. flowers and, a single follicle, 2 less (at least) than *Delphinium* typical gynoecium [17]. *Consolida* (DC.) Gray flowers exemplify a case of reduction in floral organ numbers (W2, stamens and carpels) compared with the typical *Delphinium* L. flowers.
Including the genus *Consolida* (DC) Gray into *Delphinium* L. resulted in the publication of several new combinations [33].

On the other hand, Vural et al. (2012) described the genus *Pseudodelphinium* H.Duman, Vural, Aytaç & Adıgüzel including a single species, *Pseudodelphinium turcicum* H.Duman, Vural, Aytaç & Adıgüzel [34]. The description of the new species is based on a single population reported since 1997 in central Turkey. Plants of this species are herbaceous with radially symmetrical flowers presenting a perianth composed of 5 petaloid W1 (considered as petals by the authors), numerous stamens, and three free follicles (Figure 1.D). Authors noted its probable taxonomic proximity with the genus *Delphinium* L., but based on its morphological particularities (no dorsal spurs, radial

symmetry, perianth composed of a single type of organs) they decided to create a new genus. Later, the genus was placed in *Delphinium* L. subg. *Delphinium* by Xiang et al (2017) based on molecular analyses [35]. Espinosa et al. (2017) carried out morphological, molecular, anatomical, and palynological analyses in order to better understand the taxonomical position of the genus [36]. The authors proposed that in this species the perianth is composed by W1 organs, and that W2 organs stopped their development at a very early stage. They proposed the new combination *Delphinium turcicum* (H.Duman, Vural, Aytaç & Adigüzel) Espinosa. More analyses relying on herbarium material and particularly living material are needed to better understand the origin of the morphological deviations. Having access to seeds of the species may allow conducting caryological analysis in order to identify possible recent hybridization events, and testing the stability of the phenotype on other substrates (the only known population of this species grows in the basin of the hypersaline lake Tuz Gölü, known for high levels of plant endemism) [34,37].

In 1964, W. T. Wang published the new species *Chienia honanensis* W. T. Wang (Ranunculaceae) [38], based on a single specimen bearing flowers with bilateral symmetry and a biseriate perianth. W1 are 5 free petaloid organs quincuncially arranged and there are 5(–6) W2 organs, all in the dorsal half of the flower. The flowers present numerous stamens and the gynoecium is composed by three free follicles. Even if the author recognized the proximity of *C. honanensis* with the genus *Delphinium*, the higher number of W2 organs (5–6 vs 4 in the typical *Delphinium* flower) and the absence of dorsal spurs led him to propose the new genus *Chienia* W.T. Wang [38]. The species was later considered as a single teratological specimen of *Delphinium grandiflorum* L. by Warnock (1993).

As far as floral morphology is concerned, and considering that the former genus *Consolida* (DC.) Gray is now included in *Delphinium* L., we state that the *Delphinium* flower is zygomorphic and characterized by a perianth consisting of 5 spirally-initiated W1 organs (the dorsal one being spurred) and 4 (2 lateral, 2 dorsal spurred) or 1 (spurred) W2 organs, all in the dorsal half of the flower. The spurred dorsal W2 organs are nectariferous, and their spurs are nested within the spur of the W1 dorsal organ. The gynoecium consists of a single carpel, or of 3(–5) carpels. The recent inclusion of *Delphinium turcicum* (H.Duman, Vural, Aytaç & Adigüzel) Espinosa into the genus

Delphinium L. [35] implies amending the description of the genus indicating that there is an exception to the typical floral morphology. The major diagnostic floral characters of *D. turcicum* are: 1) radial symmetry, 2) an uniseriate perianth composed of W1 organs, and 3) the absence of spurs.

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3. Conclusions

Cette revue nous a permis de constater et de discuter :

1) L'ancienneté des observations documentées du groupe *Delphinium* ainsi que l'ambigüité des classifications pré-linnéennes le concernant.
2) Les nombreuses modifications taxonomiques subies par le genre après sa publication valide, ainsi que les difficultés rencontrées par les botanistes lors de sa lectotypification.
3) L'organisation typique de la fleur du genre ainsi que les possibles variations naturelles que celle-ci peut présenter.

De nouvelles questions se posent :

1) La nouvelle espèce *Delphinium turcicum* a-t-elle une validité taxonomique ? Correspond-t-elle uniquement à une population tératologique de *D. virgatum* ou de *D. venulosum*, ou constitue-t-elle un cas d'espèce en cours de formation ?
2) Serait-il nécessaire de proposer une description complète du genre *Delphinium* afin d'inclure les caractéristiques florales de l'espèce *Delphinium turcicum* ?

Discussion

La fleur de *Delphinium* L. est communément décrite comme étant composée d'un périanthe bisérié composé de cinq organes W1 dont le dorsal est éperonné, et de quatre organes W2 dont les deux dorsaux présentent des éperons nectarifères inclus dans l'éperon de l'organe W1 dorsal. L'androcée est composé de nombreuses étamines et le gynécée est formé de trois à cinq carpelles libres [1–3]. Toutefois, même si cette composition de la fleur est partagée par une majorité des espèces du genre (les espèces composant les sous-genres *Delphinastrum, Oligophyllon* et *Delphinium*), certaines variations sont présentes au sein d'autres groupes (le clade *Consolida* (DC.) Gray incluant le clade *Aconitella* Spach).

Dès 1753, Linné indiquait certaines variations dans le nombre d'organes nectarifères et de carpelles : un seul carpelle ainsi qu'un seul organe nectarifère pour les espèces *D. consolida* et *D. ajacis*, et trois carpelles et deux organes nectarifères pours les espèces *D. peregrinum, D. grandiflorum, D. elatum, D. staphisagria*. Il indiquait aussi ces variations dans la description présentée dans son *Genera Plantarum*. Toutefois, la fleur ayant une place prépondérante dans les classifications botaniques, ces variations dans la morphologie florale du genre ont depuis lors induit de nombreuses modifications taxonomiques [4–8].

La principale ambiguïté concernant le statut du clade *Consolida* par rapport au reste des *Delphinium* est due à ce qui peut être interprété comme une réduction du nombre d'organes W2 (de quatre organes dont deux éperons nectarifères à deux organes fusionnés et constituant une structure nectarifère unique), et du nombre de carpelles (de trois-cinq à un seul). Payer [9] avait interprété les réductions de la corolle comme dues à un avortement de certains organes W2 du *Delphinium* typique. Toutefois une étude récente a démontré qu'il s'agissait d'une fusion postgénitale des deux organes dorsaux produisant un seul organe éperonné [10]. Par contre, pour le reste des organes W2, il s'agit bien d'un arrêt dans leur développement. La fleur typique de *Delphinium* présente au début de son développement huit primordia d'organes W2 dont les quatre dorsaux seulement arriveront à se développer entièrement, tandis que dans le cas des fleurs de *Consolida* seuls les deux dorsaux fusionnés arrivent au terme de leur développement [9,10]. Comme indiqué précédemment, le genre *Delphinium*, comme le reste des genres de la tribu des Delphinieae, présente naturellement une pause dans le développement des organes W2 [11].

1. Implications des variations morphologiques d'un phénotype déviant

1.1 Origine évolutive de la symétrie bilatérale florale

La symétrie bilatérale est considérée comme un état dérivé étant apparu à de nombreuses reprises de façon indépendante dans l'évolution des plantes à fleurs [12] (les premiers fossiles présentant cet état de caractère datant du Crétacé supérieur [13]). Celle-ci peut parfois mettre en jeu la synorganisation de structures florales (par exemple le gynostème des orchidées) dans la mesure où elle nécessite l'établissement d'une organisation supplémentaire des organes floraux faisant intervenir différents verticilles de la fleur à la fois, indépendamment de l'identité propre à chaque organe [11,12]. C'est pourquoi son apparition au cours de l'évolution implique une fixation préalable du méristème floral en termes de nombre d'organes et de la disposition de ceux-ci [12]. Dans la famille des Ranunculaceae, la fleur ancestrale est considérée comme une structure à phyllotaxie spiralée [14–16], à méristème non fixé (en nombre et disposition des organes) et à symétrie radiale [11,16]. Au sein de cette famille, au moins neuf épisodes différents de fixation du méristème ont eu lieu, celui concernant la tribu des Delphinieae impliquant en plus une réduction partielle dans le nombre des organes W2 due à un développement inégal des organes (condition qui entraîne l'établissement de la symétrie bilatérale [17]) [11]. Toutefois, même si la fleur des Delphinieae est décrite comme ayant une symétrie bilatérale, les organes sont initiés sur une spirale [9,11], ce qui correspondrait à strictement parler à un cas d'asymétrie. La réduction de la corolle correspondrait aussi à un cas dérivé à partir d'une fleur ancestrale dotée d'un périanthe entièrement développé. En effet, on peut segmenter le développement des fleurs de *Delphinium* en trois étapes principales : 1) l'initiation en spirale des primordia sur le méristème floral jusqu'au début de la morphogenèse ; 2) l'arrêt du développement de certains organes W2 ; 3) l'établissement de la structure tridimensionnelle des organes du périanthe (le développement des éperons).

1.2. Apparition d'un périanthe unisérié sans éperons nectarifères

Des modulations dans le retard de développement de certains organes floraux peuvent entraîner des modifications plus importantes au sein de la fleur. Dans le cas de l'espèce

Delphinium turcicum, un arrêt total de développement de l'ensemble des organes W2 entraîne une modification importante du plan de base de la fleur. Cette modification donne naissance à des fleurs adultes avec un périanthe unisérié composé uniquement d'organes W1.

En plus de la dorso-ventralisation liée à la différence de développement des organes W2, la symétrie bilatérale est établie grâce au développement d'éperons dorsaux (celui de l'organe dorsal W1 qui recouvre et protège les éperons nectarifères des organes dorsaux W2) [11,18]. Toutefois leur développement peut se voir affecté par des causes génétiques telles que la répression dans l'expression de certains gènes (particulièrement des homologues des gènes *KNOX*) par l'expression des gènes de type TCP, ainsi que par l'action de gènes participant à la biosynthèse de l'auxine [19]. Dans l'étude des cultivars de *Delphinium*, nous avons observé la modification de la position des éperons dans la fleur liée au développement d'organes surnuméraires, avec tout de même une conservation de la zygomorphie. Dans le cas particulier de *Delphinium turcicum*, la fleur est modifiée par l'absence de développement des organes W2, résultant en un périanthe unisérié. A cette perte apparente d'organes s'ajoute l'absence de l'éperon sur l'organe W1 dorsal. Ces deux modifications ont pour conséquence la radialisation ou pélorisation de la fleur par un phénomène de ventralisation [20].

2. Conséquences des variations morphologiques

2.1. Interprétation des variations dans un cadre évolutif

Le cadre développemental décrit antérieurement pour la fleur de *Delphinium* implique de nombreuses difficultés d'interprétation de la fleur de *Delphinium turcicum*. En effet, l'analyse de la morphologie de cette dernière requiert une décomposition des caractéristiques florales de l'espèce :

1) La fixation des méristèmes floraux est établie : il y a un nombre constant d'organes du périanthe disposés de façon invariante sur le méristème. Ce caractère est considéré comme un caractère dérivé [11].

2) L'initiation des organes de la fleur se fait en spirale : comme dans le reste des fleurs de *Delphinium*, les organes W1 ainsi que les étamines et les carpelles sont initiés sur une spirale. Les vestiges des organes W2 non développés sont disposés à la base de chaque spirale et sont au nombre de huit [20]. Cette disposition en spirale des organes floraux est supposée avoir été présente dans la fleur ancestrale des Delphinieae [11,16].

3) A différence de la fleur typique de la tribu des Delphinieae, où il existe une polarisation dès le début de la morphogénèse avec un développement plus marqué de l'organe W1 dorsal, qui recouvre toute la partie dorsale du bouton [9,11], les organes W1 de *Delphinium turcicum* présentent un développement homogène empêchant l'établissement de la dorso-ventralisation de la fleur. Ce caractère peut être relié à l'état actinomorphe de la fleur ancestrale des Delphinieae.

4) Il y a une réduction importante du nombre d'organes du périanthe : à la différence des fleurs des Delphinieae avec une réduction partielle du nombre d'organes W2, les fleurs de *Delphinium turcicum* présentent une réduction totale du nombre d'organes W2 due à l'arrêt précoce du développement des pétales/organes W2. Au même titre que la corolle de *Delphinium* dont le développement est inégal, on peut considérer ce cas comme étant dérivé d'une corolle primitive entièrement développée.

5) Le périanthe des fleurs de *Delphinium turcicum* ne présente pas de structures complémentaires (éperons) impliquant une modification tridimensionnelle tardive de l'organe. Le développement de telles structures est lié à la symétrie bilatérale des fleurs typiques de Delphinieae. Jabbour et al. [11] considèrent même ces deux états (zygomorphie et développement d'éperons) de façon indissociable dans les fleurs des

Delphinieae. L'absence d'éperons pourrait correspondre à un retour à l'état ancestral des organes W1.
6) La combinaison des caractères présentés produit une fleur adulte à symétrie radiale.

Cette analyse nous donne à penser que l'originalité de la morphologie florale de cette espèce ne correspond pas à un cas de réversion totale (c'est à dire, retour à l'état primitif d'un caractère étant passé par un état dérivé) mais à une combinaison de modifications produisant une fleur avec une combinaison de caractères rappelant les états primitifs (organes initiés en spirale, symétrie radiale, absence d'éperons) et de variations du plan ancestral, comme la répression du développement des organes de la corolle.

L'étude des cultivars a mis en évidence une combinaison d'états de caractères ancestraux et dérivés mais n'aboutissant pas à une morphologie équivalente à celle de *Pseudodelphinium turcicum*, notamment concernant la symétrie En effet, tous les cultivars présentaient des fleurs à symétrie bilatérale, issue du développement d'éperons, en accord avec la relation établie par Jabbour et al. [11]. Toutefois à certaines occasions cette symétrie est difficilement reconnaissable, d'une part à cause du développement de l'intégralité des organes W2, et d'autre part, à cause du développement d'organes surnuméraires du périanthe. A première vue, les fleurs des cultivars pourraient ressembler à des fleurs à symétrie radiale ou asymétriques. Je les ai considérées tout de même comme étant à symétrie bilatérale à cause du développement plus marqué de l'organe W1 dorsal durant la morphogénèse, ainsi que du développement des éperons nectarifères dans certains organes dorsaux de la fleur. Il est important de remarquer que cette étude a permis de montrer la possibilité du développement de fleurs déviantes, qui ne possèdent pas de potentiel évolutif dû à l'origine artificielle des cultivars (par sélection artificielle ou hybridation forcée), dont les caractéristiques morphologiques visent essentiellement à satisfaire la recherche de variétés horticoles aux fleurs plus exubérantes.

2.2. Conséquences écologiques

Les particularités dans la morphologie florale de *Delphinium turcicum*, plus particulièrement ce qui peut être qualifié comme la perte des structures nectarifères visant un groupe de pollinisateurs en particulier, nous amènent à nous questionner quant

à la stratégie de pollinisation utilisée par ces plantes. Dans l'histoire évolutive des plantes à fleurs, la modification de structures impliquées dans la reproduction (pollinisation, dispersion) peut entraîner la diversification en termes de nombre d'espèces au sein d'un clade. Ainsi des changements dans la symétrie de la fleur ou le développement de structures spécialisées pour la production et le stockage du nectar entraînant une sélection dans le type de pollinisateur peuvent être une cause majeure de d'isolement reproducteur et de spéciation [21–24]. En effet, le développement des éperons nectarifères au sein des Delphinieae est liée à une pollinisation effectuée principalement par des insectes du genre *Bombus* (Apidae) [10,18,25,26]. Toutefois, Tamura (1993) indique la présence de nombreuses espèces de Ranunculaceae au périanthe à symétrie radiale sans structures additionnelles destinées à une pollinisation particulière (aussi appelées fleurs allophiles), permettant l'accès à la fleur d'une variété d'insectes non spécialisés [18]. Les fleurs de *Delphinium turcicum,* au périanthe actinomorphe et sans structures tridimensionnelles, pourraient être pollinisées par des insectes généralistes, ce qui pourrait, dans un certain contexte environnemental, représenter un avantage reproducteur par rapport au reste des espèces de *Delphinium*.

2.3. Conséquences taxonomiques

Delphinium turcicum existe bien en tant qu'entité taxonomique. La publication du nom et celle de la nouvelle combinaison suivent les règles édictées par le code international de nomenclature botanique. Toutefois, il est légitime de questionner le statut de cette espèce en tant qu'entité biologique. D'emblée la réponse à cette question se voit affectée par l'information très limitée que l'on a concernant la population qui constitue l'espèce (pas de données sur la reproduction de l'espèce, pas de données sur le nombre chromosomique, peu de données moléculaires). Néanmoins nous disposons de données qui peuvent nourrir notre réflexion :

1) Les fleurs de l'espèce présentent des modifications importantes (absence de structures nectarifères qui récompensent les pollinisateurs) entraînant des possibles conséquences biologiques, notamment en ce qui concerne la reproduction.

2) L'aire de répartition de l'espèce est réduite à une zone aux conditions particulières : le bassin d'un des plus grands lacs salés dans le monde, zone présentant un important endémisme végétal [27].

3) L'analyse phylogénétique place les individus analysés de l'espèce dans un clade en position de groupe frère de l'espèce *Delphinium venulosum* Boiss. (présente dans l'aire de répartition de *Delphinium turcicum*) au sein du sous-genre *Delphinium*.

4) L'espèce présente de l'hétéromorphisme pollinique qui pourrait indiquer une origine hybride de celle-ci.

Ces données nous indiquent que cette espèce pourrait exploiter une large gamme de polinisateurs dû à ses particularités florales, et que ses pollinisateurs pourraient être différents de ceux des espèces apparentées à cause de l'absence d'organes nectarifères [18]. Ainsi cette espèce pourrait correspondre à un cas de spéciation sympatrique, dans le cas où les espèces proches phylogénétiquement peuvent se développer dans le même écosystème (des données indiquent la présence de *D. venulosum* et *D. virgatum* Poir. dans la région mais leur présence autour du lac Tuz Gölü n'est pas précisément attestée). Ainsi, elle pourrait correspondre à une espèce en voie de formation, probablement par hybridation, et à cause de l'existence de deux types de barrières [28] : une possible barrière reproductive liée au comportement des pollinisateurs [21] et une possible barrière écologique liée aux caractéristiques extrêmes de son environnement physique. Cela ne correspondrait pas au premier cas d'hybridation naturelle chez les *Delphinium* donnant naissance à une nouvelle espèce. Lewis et Epling [29] ont décrit le phénomène de spéciation par hybridation du *Delphinium gypsophilum* Ewan à travers un isolement écologique, sans changement du nombre de chromosomes.

3. Lier théorie et empirisme dans la délimitation d'une espèce nouvelle

L'étude de *Delphinium turcicum* m'a amené à me poser de nombreuses questions sur la pertinence de la publication de la nouvelle combinaison. En effet le peu de donnés disponibles sur l'espèce, son aire de répartition restreinte et les importantes transformations morphologiques observables dans la fleur m'ont fait me poser la question suivante : doit-on la considérer comme une simple population tératologique sans valeur taxonomique ou, au contraire, la considérer comme une entité biologique distincte nécessitant un nom botanique au rang d'espèce ?

Pour m'aider à y voir plus clair et prendre une décision taxonomique, j'ai décidé de demander à quelques chercheurs du Muséum national d'Histoire naturelle travaillant en taxonomie de différents organismes, de définir le concept d'espèce qu'ils manipulent ainsi que les outils qu'ils utilisent afin de délimiter les espèces. Leurs avis sont consignés dans le tableau 1.

Tableau 1 - Concepts d'espèces utilisés de façon théorique et empirique par quelques chercheurs (C) du Muséum national d'Histoire naturelle.

C1	Epistémologie / zoologie	Théorie : regroupement d'individus participant du même flux généalogique non scindé. Cela implique une vision diachronique.
		Pratique : hypothèse de reconnaissance d'individus apparentés avec l'hypothèse d'une lignée cohérente. Pour cela, différents types de caractères peuvent être utilisés.
C2	Bryologie	Théorie : le General Lineage Concept (une espèce correspond à un groupe d'individus faisant partie d'une lignée qui évolue indépendamment des autres).
		Pratique : convention pratique qui permet l'identification d'un groupe d'individus qui diffèrent du reste. Pour essayer de rendre le groupe le plus robuste possible, treize types de caractères différents sont considérés.
C3	Ornithologie	Théorie : concept biologique des espèces (un groupe d'individu qui présente un isolement reproductif par rapport au reste des individus)

		Pratique : identifier l'isolement reproductif à travers trois types de caractères principaux : la génétique, la biométrie et l'éthologie, en donnant des poids différents aux types différents de caractères.
C4	Entomologie	Ne fait pas de la délimitation d'espèces mais se base sur les espèces déjà proposées. Généralement, C4 se base sur des données moléculaires pour l'identification des spécimens, même s'il donne une certaine place à la morphologie.
C5	Entomologie	Théorie : concept phylogénétique de l'espèce (correspond à un groupe d'individus monophylétique)
		Pratique : délimitation empirique de groupes d'individus qui peut varier dans le temps en fonction des données disponibles. Actuellement, C5 se fonde sur des données moléculaires, morphologiques et éthologiques.
C6	Epistémologie	Théorie : l'espèce n'existe pas, C6 préfère parler de taxon (défini par lui comme un groupe monophylétique) qui diffère du reste des taxa
		Pratique : C'est une convention de langage qui permet la délimitation d'un groupe d'organismes à travers certains caractères relatifs et de façon empirique.

Les entretiens avec les chercheurs m'ont permis de comprendre plusieurs points relatifs au concept d'espèce et aux méthodes de délimitations des espèces. Il ne s'agit pas dans cette partie de faire une discussion sur le concept d'espèce lui-même mais de voir comment les différents concepts sont utilisés.

Dans un premier temps, il a été nécessaire de comprendre qu'il y a une distinction fondamentale à faire quand on aborde ce sujet, et c'est le fait de séparer la théorie et la pratique.

La théorie permet de mettre en place un cadre qui donne les limites à ce que l'on considérera comme une espèce. On distingue généralement les concepts biologique (groupe défini par l'isolement reproducteur [30]), morphologique (groupe morphologiquement distinguable des autres), phénétique (groupe établi sur des différences quantitatives mesurées [31]), phylogénétique (groupe monophylétique [32]) ou écologique (groupe dont les constituants partagent la même niche écologique [33]),

entre autres [34]. La mise en évidence de l'évolution du groupe et de ses proches requiert un recours à ces différents concepts sur une certaine échelle de temps. Dans de nombreux cas, le choix du concept dépendra de l'objectif des études à mener.

Dans un deuxième temps, la pratique permet de mettre en place une délimitation qui puisse être en accord avec le concept théorique. Pour cela on choisira les différents outils capables de qualifier ou de quantifier les relations (par exemple caryologiques, moléculaires, éthologiques, morphologiques) entre taxa.

La délimitation empirique des espèces permet de manipuler et de nommer un groupe qui puisse être clairement identifié, selon des intérêts précis (par ex. pour la conservation, ou en pharmacologie), et selon les outils conceptuels et méthodologique disponibles. En raison des modifications affectant très probablement la pollinisation, le cas de *Delphinium turcicum* pourrait correspondre à un cas d'espèce biologique en formation avec l'établissement d'une barrière reproductive. Il demeure toutefois indispensable de réunir plus d'informations pour tester cette hypothèse. Morphologiquement, l'espèce présente des états de caractères suffisamment éloignés de ceux des espèces phylogénétiquement (et géographiquement) proches pour que son identification soit évidente. Le maintien de l'espèce sur un temps non négligeable (20 ans au moins) fait que ses caractéristiques florales atypiques dans le genre *Delphinium* peuvent être considérées comme fixées. C'est pourquoi, dans le cas précis de *D. turcicum*, j'ai décidé de publier la nouvelle combinaison et de maintenir le rang d'espèce pour le taxon.

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Conclusion

Cette thèse montre l'importance de l'étude des phénotypes floraux déviants en tant qu'outils dans la compréhension de l'évolution des plantes à fleur. En effet, l'étude de l'origine des déviations de la morphologie florale par rapport à une morphologie standard a mis en évidence que des variations dans le développement des organes floraux ainsi que dans leur organisation et morphologie peuvent entraîner des modifications importantes de l'ensemble du phénotype floral, et avoir des effets directs dans la reproduction de la plante. Ces déviations semblent être la conséquence directe de modifications dans l'expression de gènes homéotiques responsables de l'établissement de l'identité florale et de la symétrie florale. De plus, l'étude du développement floral nous a montré que ces modifications doivent avoir lieu à des moments clés du développement tels que les premières étapes de l'organogénèse ou de l'établissement de l'identité des organes floraux. A travers des changements de symétrie florale et du développement de structures spécialisées tels que les éperons nectarifères, il est possible que des barrières reproductives se mettent en place par la modification des organismes impliqués dans la pollinisation. De telles barrières peuvent donner lieu à la naissance de nouvelles espèces qui participeront à la diversification d'un clade. Toutefois, le traitement de ce type d'espèces issues des modifications importantes dans la morphologie florale est délicat en taxonomie. L'apport des données génétiques, complémentant les données morphologiques, ainsi que celui des données anatomiques et palynologiques est indispensable pour l'interprétation de l'évolution d'un clade, la délimitation d'une espèce nouvelle ainsi que pour l'interprétation de sa biologie. Cette étude ouvre la voie à des études de type évo-dévo testant le lien entre les phénotypes déviants et les gènes candidats dont l’effet potentiel a été ici discuté. Mon travail pourra aussi inspirer des études visant à vérifier ou tester des hypothèses d’espèces, en particulier quand celles-ci sont susceptibles d’être en cours de formation.

ANNEXES

Annexe 1- Capture d'écran de la page web avec les informations concernant l'article "Recircumscription of *Delphinium* subg. *Delphinium* (Ranunculaceae) and implications for its biogeography" publié en collaboration avec l'équipe du professeur Wei Wang, en Chine

TAXON
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Systematics and Phylogenys

Recircumscription of *Delphinium* subg. *Delphinium* (Ranunculaceae) and implications for its biogeography

Kun-Li Xiang, Zeki Aytaç, Yang Liu, Felipe Espinosa, Florian Jabbour, James W. Byng, Cai-Fei Zhang, Andrey S. Erst, Wei Wang

First published: 23 June 2017 | https://doi.org/10.12705/663.3

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Abstract

The monophyly of Delphinium subg. Delphinium remains unresolved, owing to the controversial systematic position of the monotypic, eastern Asian endemic D. sect. Anthriscifolium. The other section of the subgenus, D. sect. Delphinium, is distributed in the Irano-Turanian (IT) region extending westward to the Mediterranean Basin. Recently, a new genus endemic to Turkey, Pseudodelphinium, was described and considered related with Delphinium (Delphinieae) or Garidella (Nigelleae). In this study, we first conducted a broad phylogenetic analysis within Ranunculaceae using matK sequences and placed Pseudodelphinium in Delphinieae. We then performed a series of analyses using four molecular markers (trnK-matK, trnS-G, trnL-F, ITS) focused on the tribe. The phylogenetic analyses based on the four-marker dataset indicate that Pseudodelphinium is embedded within sect. Delphinium. The Swofford-Olsen-Waddell-Hillis tests also reject the exclusion of Pseudodelphinium from Delphinium and sect. Delphinium. The monophyly of subg. Delphinium is not recognized because sect. Anthriscifolium unites with D. subg. Delphinastrum and Consolida. Based on molecular, morphological, and karyological data, we raise sect. Anthriscifolium to subgeneric status, whereas sect. Delphinium composed subg. Delphinium s.str. An integration of phylogenetic, molecular dating, and biogeographical methods indicates that the subgenus s.str. originated in East Asia during the latest Oligocene and began to diversify in the IT region at 8.45 Ma. Subsequently, the westward colonization events occurred at 7.06 Ma from the IT region to the Italian and Balkan Peninsulas and at 5.4 Ma from Italy to North Africa. A dispersal from North Africa to the Iberian Peninsula was inferred in the late Pliocene, supporting a hypothesis of trans-sea dispersal. Within the Mediterranean Basin, climate aridification and eustatic sea-level changes could have initiated the westward stepwise expansion of Delphinium.

Supporting Information

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Delphinium anthriscifolium
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Annexe 2- Réponse reçue de la part des relecteurs concernant le manuscrit "Insights into the floral morphological diversity of cultivars of *Delphinium* L. and *Aquilegia* L. (Ranunculaceae): organogenesis and potential molecular clues"

PONE-D-19-07534
Insights into the floral morphological diversity of cultivars of Delphinium L. and Aquilegia L. (Ranunculaceae): organogenesis and potential molecular clues
PLOS ONE

Dear Felipe Espinosa Moreno,

Thank you for submitting your manuscript to PLOS ONE. After careful consideration, we feel that it has merit but does not fully meet PLOS ONE's publication criteria as it currently stands. Therefore, we invite you to submit a revised version of the manuscript that addresses the points raised during the review process.

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Kind regards,

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Academic Editor
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Reviewers' comments:

Reviewer's Responses to Questions

Comments to the Author

1. Is the manuscript technically sound, and do the data support the conclusions?

The manuscript must describe a technically sound piece of scientific research with data that supports the conclusions. Experiments must have been conducted rigorously, with appropriate controls, replication, and sample sizes. The conclusions must be drawn appropriately based on the data presented.
Reviewer #1: Yes
Reviewer #2: Partly

2. Has the statistical analysis been performed appropriately and rigorously?
Reviewer #1: N/A
Reviewer #2: N/A

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3. Have the authors made all data underlying the findings in their manuscript fully available?

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Reviewer #1: Yes
Reviewer #2: Yes

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4. Is the manuscript presented in an intelligible fashion and written in standard English?

PLOS ONE does not copyedit accepted manuscripts, so the language in submitted articles must be clear, correct, and unambiguous. Any typographical or grammatical errors should be corrected at revision, so please note any specific errors here.
Reviewer #1: Yes
Reviewer #2: Yes

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5. Review Comments to the Author

Please use the space provided to explain your answers to the questions above. You may also include additional comments for the author, including concerns about dual publication, research ethics, or publication ethics. (Please upload your review as an attachment if it exceeds 20,000 characters)

Reviewer #1: The authors chose cultivated samples of Delphinium and Aquilegia to study the morphogenesis of their floral organs. Basically, this study was well organized and the manuscript was also well written. Experiment data of this study was robust for their discussion and their finding is important for the further study concerning evo-devo issue for the flower evolution.
I only propose some minor questions that I have to mention here.
First, Table 1 was not designed well. It is hard to read.
Second, there is a very important floral character of Delphinium missing in this study, the two petaloid staminodes.
This is important because that is the main character to discriminate Delphinium and Aconitum, which is also a large genus in Ranunculaceae to have zygomorphic flowers.
What I really curious about is how this petaloid staminodes getting evolved.
The authors should discuss, or at least mention about this character.
Why there are no staminodes in the Delphinium samples of this study?
If all the sampled flowers lacked this structure. The sampling of the study was of some problem because there is no actual wild type of (typical) Delphinium flower sampled.
The results and discussion cannot represent normal condition of the genus.
So, I suggest a minor revision of this paper.

Reviewer #2: PONE-D-19-07534
Insights into the floral morphological diversity of cultivars of Delphinium L. and Aquilegia L. (Ranunculaceae): organogenesis and potential molecular clues

In this manuscript, the authors seek to describe the development and morphology of several floral homeotic mutants from Aquilegia and Delphinium. While I think the topic has some interest, the data are rather poorly presented, leave many important questions unaddressed, and are inaccurately interpreted in several regards.

Introduction:

The Introduction provides no background on the considerable amount of molecular data already available concerning the genetic control of floral organ identity, both in Aquilegia and the relatively close Delphinium relative, Nigella. This context is critical to the proper interpretation of the presented data.

Results:

Table 1: This should be reoriented in landscape so that the column and cultivar labels can be fully expanded. It is difficult to read and interpret in this format. Also, there are aspects of the data that I find confusing. They note whether the second whorl organs are flat or spurred by they don't note whether the mW2' organs are flat or spurred. 'Ruby Port''s additional whorls of sterile organs are usually sepals, but sometimes there are

sepal/petal chimeras. Panel H is supposed to be ‘Green Apples’, which they report as have sepals in the second whorl, but the photo clearly shows that some of the organs are spurred. Are those in inner whorls? And if they really want to understand homeosis and indeterminacy, they should be counting all the floral organs.

Figure 1: This is odd. Some of the images appear to be from living flowers but others are from pressed herbarium sheets (which is noted in the text), and therefore rather difficult to interpret. In some case (e.g., panels H and I), the image is either too bright or too dark to clearly discern the morphology of the flowers.

Figure 3. I also found this schematic quite odd and thought it added relatively little relative to Table 1. Why not present simple floral diagrams showing the transformed organs?

Overall, the results are limited and do not present full developmental SEM series, histology, or SEM of all individual types of transformed organs to confirm their identity. They clearly did histology to evaluate the vascular patterns but only the conclusions are presented. Developmental histology would be informative. The greatest weakness is that no genetic data of any kind is presented, even the results of simple crosses to determine allelism of similar phenotypes or whether some of these phenotypes might be due to single vs. double mutants.

Discussion:

This is the most problematic part of the manuscript. The discussion is unnecessarily speculative and seems to ignore the simplest explanation of the phenotypes. As noted above, we know quite a lot about organ identity in these taxa or their close relatives, including that a paralog of AP3 specifically controls petal identity in Aquilegia. Studies of Nigella demonstrate that this same paralog promotes petal identity and promotes the maintenance of AG expression in the outer whorls of stamens. More generally, we know from broader studies that mutants of AG result in sterilization of the reproductive organs as well as loss of floral meristem determinacy. Weak alleles of AG give rise to a range of chimeric organs and variable degrees of indeterminacy. Thus, the authors are mistaken when they suggest that the supernumerary organs cannot experiencing homeosis because they are the products of indeterminacy (line 493-494). If they are chimeric, they are experiencing homeosis. Weak expression of AG can produce both phenotypes simultaneously – chimerism of identity and indeterminacy.

There is a very simple explanation for the Aquilegia mutants, which does not require the invocation of the long list of disparate candidate genes cited in the Discussion. In the horticultural trade there are three common homeotic mutants of Aquilegia: 1) the ‘clematiflora’ class, which are AP3-3 mutants yielding sepal to petal transformation

(not covered here); 2) the ‘Winky’-type, in which the AG expression domain is contracted, yielding transformation of outer stamens into petals and some degree of indeterminacy, here exemplified by ‘Leprechaun Gold’, 3) and double mutants of 1 and 2, resulting in the transformation of petals into sepals as well as outer stamen whorls into sepals, again with some degree of indeterminacy. Because there are a variety of ap3-3 and ag alleles in the trade, they often exhibit semi-penetrance such that chimeric organs are common. The authors need to be more explicit about when they observe petal to sepal transformation (usually indicated by a lanceolate apex) vs. sepal/petal chimeras, which typically have a rounded apex but weak spur development. This is due to variable expression of AP3-3. SEM of the transformed organs would indicate whether the epidermal cell types typical of Aquilegia sepals are present.

For the Delphinium, the situation is more complex and, really, much more interesting because of the zygomorphy and different types of spurred organs. We understand much less about what controls the differential organ identity between whorls in Delphinium but based on what we know from Aquilegia and Nigella, it is still most likely that the phenotypes observed here are simple ABC class mutants that are simply variably penetrant. What is most interesting about Delphinium is how the zygomorphy interacts with organ identity. In the core eudicots, the CYC-based zygomorphy program generally functions in parallel to organ identity. There are instances in which identity and zygomorphy interact, such as the dorsal stamen abortion in Antirrhinum, but CYC does not control organ identity genes. In contrast, there are several instances in the monocots (rice, orchids) that suggest that whatever controls zygomorphy acts upstream of organ identity – the dorsal organs actually have different identity than the lateral and ventral. This seems likely in Delphinium as well, where the dorsal spurred sepal and petals have dramatically different morphology. This section, therefore, deserves more careful consideration and explaination.

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6. If you would like your identity to be revealed to the authors, please include your name here (optional).

Your name and review will not be published with the manuscript.

Reviewer #1: (No Response)

Reviewer #2: (No Response)

[NOTE: If reviewer comments were submitted as an attachment file, they will be

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Annexe 3- Poster présenté à Euro Evo Devo 2016 (26-29 juillet, 2016; Uppsala, Suède)

RANUNCULACEAN FLOWER TERATA: MORPHO-ANATOMICAL CHARACTERIZATION AND CLUES ABOUT FLORAL DEVELOPMENTAL GENETICS AND EVOLUTION

Felipe ESPINOSA[1], Thierry DEROIN[1], Catherine DAMERVAL[2], Sophie NADOT[3], Florian JABBOUR[1]

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The study of teratologic floral phenotypes is a way to address the origin and the building of morphological variations (1). Horticultural varieties are an appropriate biological material in this respect. The characterization of teratologic floral phenotypes of horticultural varieties of *Delphinium* and *Aquilegia* allowed us to describe the development of atypical phenotypes and to analyze the corresponding changes in the organ vascularization. In this study, anomalies affected the identity, the shape and the number of organs. We formulate hypotheses to explain the molecular control of the observed deviant phenotypes. These assumptions will be useful for future evo-devo studies aiming at deciphering the diversity and the evolution of forms and floral organizations in Ranunculaceae.

INTRODUCTION AND OBJECTIVES

The angiosperm family Ranunculaceae presents a great floral diversity. Within this group, the tribe Delphinieae, including the genus *Delphinium*, is the only lineage which presents zygomorphic flowers. The flowers have a nectariferous spurs in dorsal position. The genus *Aquilegia* also presents nectariferous spurs, but the flowers are actinomorphic. In order to study the potential morphological variation of flower body plans in both genera, and to identify the possible genetic bases generating these variations, a morpho-anatomical study of these flowers is currently conducted. Our objectives are:

* Identify the organs most subject to anomalies
* Define the organogenesis and morphogenesis stages at which the anomalies arise
* Suggest hypotheses to explain the genetic determinism of teratological phenotypes

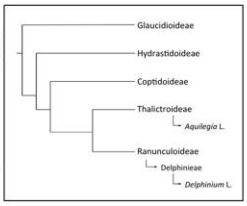

Figure 1. Simplified phylogeny of the Ranunculaceae (2)

MATERIALS AND METHODS

* Horticultural varieties were chosen: 5 for *Delphinium* and 4 for *Aquilegia*.
* For all the selected varieties, floral buds were sampled at various stages of development, dissected and observed with SEM (scanning electron microscope)
* For each genus, one bud was chosen before anthesis, treated and cut with a microtome in order to carry out the necessary analysis to understand its anatomy.

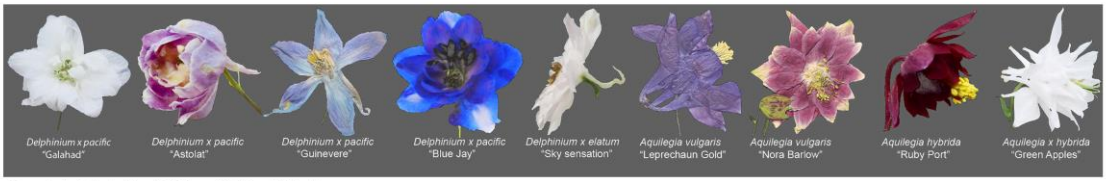

Figure 2. The horticultural varieties of *Delphinium* and *Aquilegia* used in this study

RESULTS

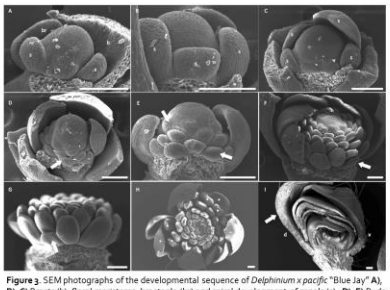

Figure 3. SEM photographs of the developmental sequence of *Delphinium x pacific* "Blue Jay" **A), B), C)** Bracts (b), floral meristems, bracteole (br) and spiral development of sepals (s) ; **D), E)** Buds with primordia and differentiating organs (arrow) ; **F)** and **G)** Delay in the development of petals (arrow) compared to the other organs. **H)** Perianth (+) and stamens (*) fully differentiated. **I)** Dorso (d) – ventral (v) polarization of the bud, established by a pronounced development of the sepal 2 (arrow). Scale = 200 µm.

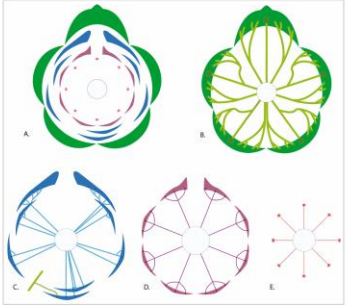

Figure 7. Anatomical diagram of the *Delphinium x pacific* "Blue Jay" bud. **A)** Floral diagram. **B)** Sepal vascularization. **C)** Supernumerary organ vascularization. **D)** and **E)** Petal and stamen vascularization. **Sepals** in green, **supernumerary organs** in dark blue, **petals** in dark pink and **stamens** in orange.

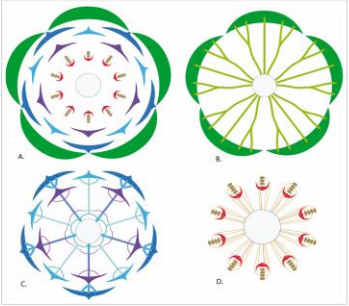

Figure 8. Anatomical diagram of the *Aquilegia vulgaris* "Leprechaun Gold". **A)** Floral diagram. **B)** Sepal vascularization. **C)** Vascularization of the three petal whorls **D)** Stamen and staminode vascularization. **Sepals** in green, **petals** in dark blue (whorl 1), light blue (whorl 2) and purple (whorl 3), **stamens** in brown and **staminodes** in red.

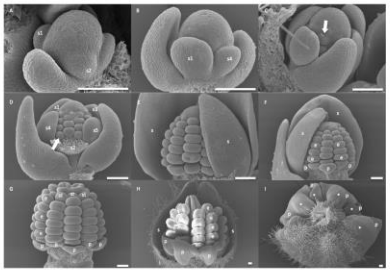

Figure 4. SEM photographs of the developmental sequence of *Aquilegia vulgaris* "Leprechaun Gold". **A), B)** Quinconcial development of sepals (s) numbered following the order of initiation. **C), D), E), F)** Emergence of orthostichies and differentiation of the petals (p) at the base of the orthostichies. **G)** All organs are fully developed and differentiated: carpels, on top of the bud, staminodes (st), stamens (e) and petals (p). **H), I)** Development of the petal (p) spurs (*). Scale = 100 µm.

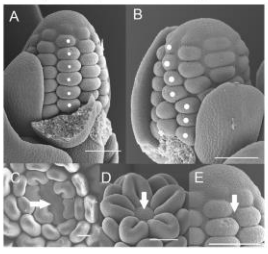

Figure 6. SEM photographs showing developmental anomalies in *Aquilegia x hybrida* "Ruby Port" **A), B), E)** and *Delphinium x pacific* "Blue Jay" **C), D). A)** Typical orthostichies compared to **B)** Abnormal orthostichies (stamens marked by white spots). Atypical zones constituted by undifferentiated cells at the top of the meristem **B), C)** and between the orthostichies of stamens **E).** Scale = 100 µm.

Morpho-anatomical changes in the studied flowers compared to the wild-type		
	Delphinium	*Aquilegia*
Number of organs	Increase	
Perianth	Abnormal morphology linked to the atypical development of spurs	
Androecium- Gynoecium	No change	
Organ identity	Supernumerary organs with no clear identity	Supernumerary organs with petal characteristics
Spurs	Always present in at least one of the perianth whorls	Present or absent according to the variety
Symmetry	No change	
Vascularization	Complexified or modified for some organs of the perianth	Complexified for all the bud but not independently for each perianth organ

DISCUSSION AND CONCLUSIONS

* The studied morphotypes may originate from changes in the expression profile of homeotic genes.
* Modifications in the expression pattern of the A- and B-function genes (identity) (3), of *CYCLOIDEA* homologs (symmetry) (4), and of the auxin biosynthesis genes (spurs) (5) are hypothesized to be at play during the development of such deviant flowers.
* Our results suggest that the expression of the genes in charge of floral symmetry and that of the genes that establish the floral organ identity and number in the both are independent.
* The development of the spur may be linked to the expression of one or several genes in the dorsal zone of the floral meristem which corresponds to the sepal, petal and supernumerary organ formation zone.
* The phenotype of the flowers investigated has been under strong human selection to produce horticultural varieties. The morphological changes are localized in the perianth, and seem not to affect the sexual organs. Whether these varieties could survive in the wild (i.e. be pollinated, reproduce, and have a fertile progeny) is unknown, but they provide a good biological model to study the genetic determinism of teratologic phenotypes as they may happen in the wild.

1) Jabbour F, Nadot S, Espinosa F, & Damerval C (2015). Ranunculaceae flower terata: records, a classification, and some clues about floral developmental genetics and evolution. *Flora, Morphology, Distribution, Functional Ecology of Plants, 217*, 64-74.
2) Cossard G, Sannier J, Sauquet H, Damerval C, de Craene LR, Jabbour F, & Nadot S (2016). Subfamilial and tribal relationship of Ranunculaceae: evidence from eight molecular markers. *Plant Systematics and Evolution, 302*(4), 419-432.
3) Causier B, Schwarz-Sommer Z, & Davies B (2010). Floral organ identity: 20 Years of ABCs. *Seminars in Cell & Developmental Biology, 21*, 73-79.
4) Jabbour F, Cossard G, Le Guilloux M, Sannier J, Nadot S, & Damerval C (2014). Specific duplication and dorsoventrally asymmetric expression patterns of *CYCLOIDEA*-Like genes in zygomorphic species of Ranunculaceae. *Plos One, 9*(4), e95727.
5) Yant L, Collani S, Puzey J, Levy C, & Kramer E M (2015). Molecular basis for three-dimensional elaboration of the *Aquilegia* petal spur. *Proceedings of the Royal Society of London B: Biological Sciences, 282*(1083), 20142778.

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Annexe 4- Poster présenté au XIXème Congrès International de Botanique (23-29 juillet, 2017; Shenzhen, Chine)

XIX International Botanical Congress

July 23-29, 2017 | Shenzhen China

Poster number T2-54-16

The Turkish endemic *Pseudodelphinium turcicum* (Ranunculaceae): an unusual population of *Delphinium* with peloric flowers

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Background information

Vural et al. (2012) reported the occurrence of a population of plants showing features of annual species of *Delphinium* (Ranunculaceae) in central Turkey, in the basin of the Salt Lake Tuz Gölü in Konya Province (earliest collections in 1997). In addition to traits from *Delphinium*, the flowers present a series of original traits. The authors considered that these plants should be attributed to a new genus, *Pseudodelphinium* (Ranunculaceae), and a new species, *Pseudodelphinium turcicum*.

Figure 1 – Restored flower of *P. turcicum*

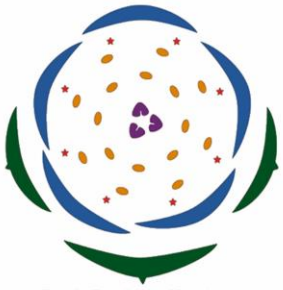

Figure 2 – Floral diagram of *P. turcicum*

Methods

1- Phylogeny: Plastid (*trn*L-F) and nuclear (ITS) markers were sequenced from 3 specimens of *P. turcicum*. The phylogenetic reconstruction was performed using maximum likelihood (ML).
2- Morphology: Dissected adult flowers of *P. turcicum* were observed using a stereomicroscope. Dissected buds and pollen grains were observed using scanning electron microscopy (SEM). In order to compare *P. turcicum* flowers with related species, we did the same study with *Delphinium venulosum* and *D. virgatum* based on the phylogeny.
3- Anatomy: Floral vascularization was reconstructed by the interpretation of serial cross sections of buds of *P. turcicum*, *Delphinium venulosum*, and *D. virgatum*.

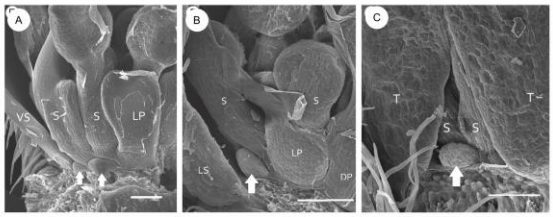

Figure 5 – SEM micrographs of perianth organs with an arrested development of A) *D. venulosum*, B) *D. virgatum*, C) *P. turcicum*

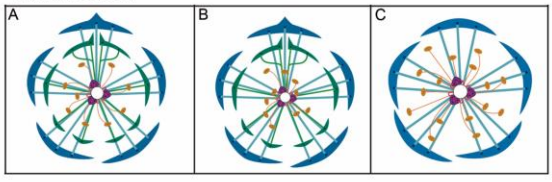

Figure 6 – Floral vascularization of A) *D. virgatum*, B) *D. venulosum*, C) *P. turcicum*

Objectives

→ **Improve our knowledge about the genus *Pseudodelphinium* and better understand its morphological specificities and its evolutionary origin.**

1. Place the genus in the phylogenetic tree of Ranunculaceae.
2. Describe the particular floral organization in the genus.
3. Update the taxonomic treatment of the genus.

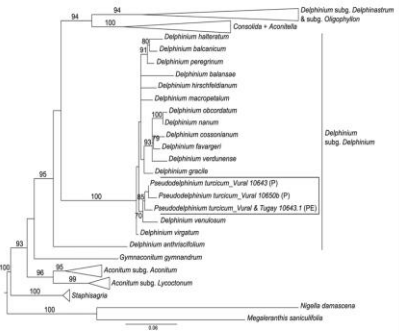

Figure 3 – ML tree of Delphinieae based on the combined *trn*L-F and ITS dataset.

Figure 4 – Specimen of *P. turcicum* kept at P

Main results

1- Phylogeny: *Pseudodelphinium turcicum* accessions formed a well-supported clade sister to *Delphinium venulosum* and nested within *Delphinium* subgenus *Delphinium*. Therefore, *P. turcicum* should be considered as a member of *Delphinium*.
2- Morphology and anatomy: The perianth of *P. turcicum* is composed of a single pentamerous whorl of petaloid organs. From the comparison of the arrangement and the morphological characteristics of these organs, we consider that they are equivalent to *Delphinium* sepals and that adult flowers lack a fully developed corolla. This results are confirmed by the anatomy. Additionally, a whorl of a second type of perianth organs with an arrested development was evidenced, located between the tepals and the stamens. *Pseudodelphinium turcicum* pollen grains are heteromorphic.
3- Flower symmetry: In *P. turcicum*, the expression of *CYC*-like genes could affect all perianth organs identically by repressing their development, hence resulting in the ventralization of the flower, which becomes actinomorphic.

Conclusions

The Turkish endemic *P. turcicum* is nested within *Delphinium*.
***Pseudodelphinium* should not to be considered as a distinct genus.**
Flowers of *P. turcicum* are spurless peloric flowers with an aborted corolla compared to Delphinium flowers.

Delphinium L.
= *Pseudodelphinium* H.Duman, Vural, Aytaç & Adigüzel
Delphinium turcicum (H.Duman, Vural, Aytaç & Adigüzel) Espinosa, comb. nov.
≡ *Pseudodelphinium turcicum* H.Duman, Vural, Aytaç & Adigüzel

Literature

Vural M, D Hayri, Z Aytaç, N Adigüzel 2012 A new genus and three new species from Central Anatolia, Turkey. Turk J Bot 36:427–433.
Citerne HL, RT Pennington, QCB Cronk 2006 An apparent reversal in floral symmetry in the legume *Cadia* is a homeotic transformation. PNAS 102:12017–12020.

Annexe 5- Poster présenté au 5th Young Natural History Scientists' meeting (mars, 2018; Paris, France)

RANUNCULACEAN FLOWER TERATA: MORPHOLOGICAL - ANATOMICAL CHARACTERIZATION AND CLUES ABOUT FLORAL DEVELOPMENTAL GENETICS AND EVOLUTION

Felipe ESPINOSA[1], Thierry DEROIN[1], Catherine DAMERVAL[2], Sophie NADOT[3], Florian JABBOUR[1]

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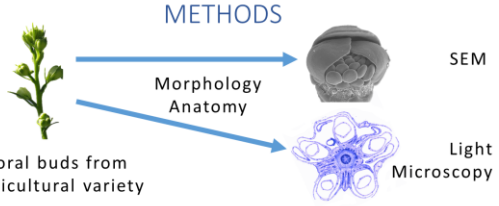
INTRODUCTION

In order to study the morphological variations of flower body plans in Ranunculaceae, and further identify the possible genetic bases generating these variations, a morphological and anatomical study of the flowers of two genera (*Delphinium* L. and *Aquilegia* L.) of the Ranunculaceae is currently conducted.

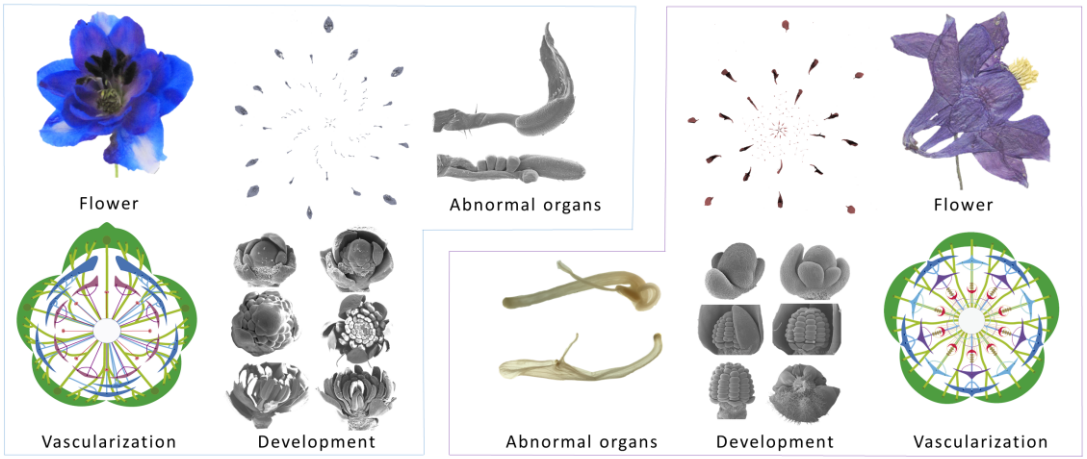
OBJECTIVES

- Identify the organs most subject to anomalies
- Define the organogenesis and morphogenesis stages at which anomalies arise
- Suggest hypotheses for the genetic determinism of teratological phenotypes

METHODS



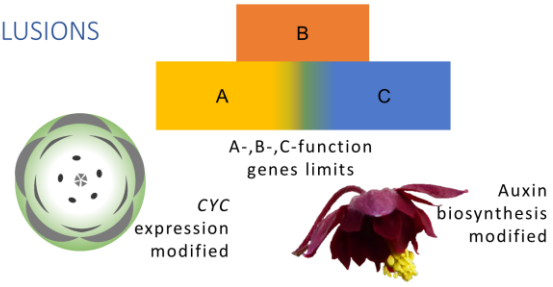
MAIN RESULTS



	Number of organs	Perianth	Androecium Gynoecium	Organ identity	Spurs	Symmetry	Vascularisation
Delphinium L.	Increase	Abnormal morphology	Abnormal organs	Organs with no clear identity	Always present	No change	Modified
Aquilegia L.					Present or absent		

CONCLUSIONS

- The studied morphotypes may originate from changes in the expression profile of homeotic genes.
- Our results suggest that the expression of the genes involved in the establishment of floral symmetry and that of the genes that establish the floral organ identity and number in both genera are independent.



Annexe 6- Résumé de la conférence présentée au "2nd International Symposium on Floral Ecology and Evolution, in honour of Stefan Vogel" (24-25 novembre, 2018; Zurich, Suisse)

Floral variation in *Delphinium* L. (Ranuncuclaceae): Morphological-anatomical and teratology to understand their origin, establishment and evolutionary potential

Felipe Espinosa; Thierry Deroin; Sophie Nadot; Florian Jabbour

Delphinium genus has been subject to numerous taxonomic rearrangements having its typical floral morphology modified. Molecular phylogenetics, morphological-anatomical studies and evo-devo can help us to clarify the origin of the rapid diversification of the genus, still misunderstood. During my PhD, I brought some answers to the following questions: 1) What are the molecular bases underlying the floral diversity of horticultural *Delphinium*?; 2) What are the developmental bases leading to an actinomorphic *Delphinium*?; 3) Are the larkspur flowers reduced *Delphinium* flowers? I found that: 1) Changes in the expression pattern of key genetic factors can explain abnormal perianths; 2) The perianth of *D. turcicum* can be explained by an undeveloped corolla, and a generalized expression of *CYCLOIDEA*-like genes; and 3) larkspur flowers are the result of undeveloped floral organs and of a synorganization with postgenital fusion. I propose a knowledge synthesis about floral evolution in this genus.

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Titre : Etude sur l'origine et l'évolution des variations florales chez *Delphinium* L. (Ranunculaceae) à travers la morphologie, l'anatomie et la tératologie

Mots clés : anatomie, botanique, développement, évolution, morphologie, tératologie

Résumé : Les plantes à fleurs se caractérisent par une diversification rapide au cours de l'évolution des plantes à graines, en partie grâce à l'émergence de la structure florale. L'étude des phénotypes tératologiques se présente comme un outil important pour mieux comprendre l'apparition et le maintien des innovations florales. Le genre *Delphinium* L. appartient à la famille des Ranunculaceae, une des premières familles ayant divergé parmi les Eudicotylédones (clade le plus diversifié au sein des Angiospermes) et présentant une grande diversité florale.

Dans un premier temps, nous avons réalisé une étude phylogénétique, morpho-anatomique et pollinique de l'espèce *Delphinium turcicum* (H.Duman, Vural, Aytac & Adiguzel) Espinosa, espèce endémique turque connue depuis plus de 20 ans dans la nature et présentant des déviations par rapport à la fleur typique du genre *Delphinium*. Cette étude nous a permis de comprendre l'organisation de la fleur de cette espèce ainsi que de mettre en évidence des modifications de symétrie, du type d'organes formant le périanthe, et du développement des éperons. Des anomalies dans le développement des organes floraux, potentiellement liées à des modifications dans l'expression des gènes responsables de l'établissement de l'identité florale, sont à l'origine de ce phénotype particulier. Dans un deuxième temps, nous avons réalisé une étude de la morpho-anatomie florale de cultivars, organismes présentant des variations florales importantes issues d'une sélection artificielle, des genres *Delphinium* L. et *Aquilegia* L. (ce dernier appartenant également aux Ranunculaceae et un des genres modèles pour les études évo-dévo). Cette étude nous a permis d'identifier les étapes cruciales du développement floral pendant lesquelles ces variations prennent place. Chez les deux genres nous avons trouvé que la symétrie florale et la phyllotaxie sont conservées, tandis que l'organisation florale et la vascularisation de la fleur sont modifiées par rapport à la morphologie des fleurs typiques. La plupart des déviations morphologiques et anatomiques se situent au niveau du périanthe, et concernent notamment le nombre et l'identité des organes qui le forment. Nous avons émis l'hypothèse que les phénotypes floraux des cultivars d'*Aquilegia* L. et *Delphinium* L. résultent d'altérations génétiques affectant la taille du méristème, les frontières entre les organes d'identité différente, l'organogenèse et le développement des structures nectarifères. Dans un troisième temps, une recherche historique sur la taxonomie du genre *Delphinium* L. a permis de mettre en évidence les possibles ambiguïtés induites par les variations florales au sein du genre. L'inclusion de l'espèce *D. turcicum* dans le genre *Delphinium* requiert d'amender la description du genre afin d'inclure les particularités florales de l'espèce.

Cete thèse permet de mettre en évidence les étapes cruciales du développement pendant lesquelles les variations morphologiques prennent place, ainsi que de viser les gènes potentiellement à l'origine de ces variations chez une espèce sauvage et chez des variétés issues d'une sélection artificielle. Ces nouvelles informations permettront d'orienter des études en évo-dévo visant la compréhension de l'évolution florale au sein des Angiospermes.

Title: Studying the origin and evolution of floral variations in *Delphinium* L. (Ranunculaceae) through morphology, anatomy and teratology

Keywords: anatomy, botany, development, evolution, morphology, teratology

Abstract: In the evolution of seed plants, flowering plants are characterized by an especially rapid, partly due to the emergence of the floral structure. The study of teratological phenotypes is an interesting tool to better understand the origin and maintenance of floral innovations. The genus *Delphinium* L. belongs to the family Ranunculaceae, one of the earliest-diverging families among Eudicots and characterized by an outstanding floral.

First, we carried out phylogenetic, morphological, anatomical and palynological studies of *Delphinium turcicum* (H.Duman, Vural, Aytac & Adiguzel) Espinosa, a Turkish endemic species known for more than 20 years in the wild and presenting unusual floral features. This study allowed us to understand the organization of the flower of this species, as well as to characterize the deviations to the typical morphology (modification of symmetry, of the type of organs composing the perianth and of the spur development). Changes in floral organ development, possibly related to changes in the expression of genes involved in floral identity, are responsible for this particular phenotype. Second, I carried out a morphological-anatomical study of cultivars (organisms with major floral variations resulting from artificial selection) of the genera *Delphinium* L. and *Aquilegia* L. (the latter belongs to Ranunculaceae and has been proposed as model genus for evo-devo studies). This study allowed me to identify the crucial stages of floral development where morphological variations take place. In both genera, floral symmetry and phyllotaxis were found to be conserved, while flower arrangement and vascularization were altered with respect to the typical flower morphology. Most morphological and anatomical deviations affect the perianth, especially on the number and identity of the organs. We hypothesized that the floral phenotypes of the cultivars of *Delphinium* L. and *Aquilegia* L. result from genetic alterations affecting meristem size, boundaries between sets of organs of different identity, organogenesis and development of nectariferous structures. Third, a historical research on the taxonomy of the genus *Delphinium* L. highlighted the taxonomical ambiguities induced by the floral morphological variations recorded within the genus. The inclusion of the species *Delphinium turcicum* implies amending the genus description in order to include the floral features of the species.

This thesis, through the study of a wild species and of varieties undergoing artificial selection, allowed to highlight the crucial developmental stages during which the morphological floral variations take place, as well as to target the genes possibly responsible for these variations. These results will guide future evo-devo studies aiming at understanding floral evolution within angiosperms, and better understand the evolution of angiosperms as a whole.