The evo-devo of plant speciation

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Abstract

Speciation research bridges the realms of macro- and microevolution. Evolutionary developmental biology (evo-devo) has classically dealt with macroevolutionary questions through a comparative approach to distantly related organisms, but the field later broadened in focus to address recent speciation and microevolution. Here we review available evidence of the power of evo-devo approaches to understand speciation in plants at multiple scales. At a macroevolutionary scale, evidence is accumulating for evolutionary developmental mechanisms giving rise to key innovations promoting speciation. At the macro-microevolution transition, we review instances of evo-devo change underlying both the origin of reproductive barriers and phenotypic changes distinguishing closely related species. At the microevolutionary scale, the study of developmental variation within species provides insight into the processes that generate the raw material for evolution and speciation. We conclude by advocating a strong interaction between developmental biology and evolutionary biology at multiple scales to gain a deeper understanding of plant speciation.

Introduction

Speciation research addresses the evolutionary processes generating the extraordinary diversity of life on Earth as well as the patterns derived from them[1]. It bridges the realms of macro- and microevolution, respectively dealing with evolutionary phenomena above and below the species level[2]. This transition is defined by the establishment of reproductive barriers restricting gene flow between populations, the prerequisite for species formation under the biological species concept. Speciation studies have classically focused on genetic and ecological mechanisms, essentially ignoring the role of developmental mechanisms. At the same time, the more recent field of evolutionary developmental biology (evo-devo), which aims to understand the developmental mechanisms of evolutionary change[3], has usually dealt with macroevolutionary questions through a comparative approach with distantly related organisms[4], paying less attention to speciation and microevolutionary processes. A broadening of focus in evo-devo to address recent speciation and microevolution has been advocated[5-6]. Indeed, new research shows the potential of a multidisciplinary approach including evo-devo to provide deeper insight into speciation.

Plants, and particularly the outstandingly diverse angiosperms, provide excellent opportunities for this approach. A number of model systems for the study of speciation have been characterised genetically, developmentally and ecologically. Moreover, several genes involved in developmental processes potentially generating pre- and post-zygotic reproductive isolation have been characterized[7-8]. Here we review available evidence for the power of evo-devo approaches to understand speciation processes and patterns in plants at multiple scales. Our focus is mainly on evolutionary changes in developmental patterns, or "developmental repatterning"[3], underlying reproductive barriers and other phenotypic differences arising during or shortly after speciation.

Although it is difficult to quantify the role of developmental changes in reproductive isolation relative to other mechanisms, available data suggest that developmental repatterning is particularly relevant to pre-zygotic barriers, and specifically to those acting at the pre-pollination level. In a review of speciation genes in plants[8], all examples of speciation genes underlying pre-pollination barriers were involved in developmental processes. These included genes causing temporal isolation and pollinator isolation, which are frequent targets of speciation studies in flowering plants (although it has also been argued that pollinator specialisation can frequently be a consequence, and not a cause, of speciation[9]). Post-zygotic barriers, on the other hand, include mechanisms of hybrid inviability and hybrid sterility[7-8] that are not described as developmental repatterning, and therefore are not reviewed here. Pre-zygotic barriers are thought to contribute more than post-zygotic barriers to reproductive isolation in plants[7], which indicates that developmental repatterning plays a crucial role in plant speciation.

Macroevolutionary scale

Speciation-promoting traits. Evo-devo research has frequently focused on comparing developmental processes across species separated by large evolutionary distances. These research lines fall within the realm of macroevolution, which is generally regarded as the long-term cumulative result of microevolutionary mechanisms[10]. However, some macroevolutionary patterns may not be entirely explained in this way, such as the differential diversification of clades (species selection) and the ways in which such differential diversification is related to morphological variety (disparity)[11-12]. Bursts of speciation (radiation) may be the result of the evolutionary acquisition of "key innovations". A key innovation can be defined as an evolutionary change in a trait

that is causally linked to an increased diversification rate in the resulting clade[13]. This may be the result of exposing the lineage to new areas of phenotypic space and new ecological opportunities[12,14]. Evo-devo can provide information on the microevolutionary developmental mechanisms by which key innovations first evolved, along with insights into their macroevolutionary effect on speciation patterns.

The evolution of many species-rich plant clades was preceded by whole-genome duplication (WGD, or polyploidy) events that allowed the diversification of regulatory genes involved in the development of key innovations[15-16]. Frequently, however, there is a lag between innovation and radiation[17]. It has been suggested that, after an innovation first appears, strong developmental robustness needs to evolve for natural selection to efficiently explore phenotypic and ecological space, thus leading to both species diversification and an increase in morphological disparity[12]. As an outstanding example, the angiosperm flower can be considered a combination of key innovations that fostered diversification through the exploration of a brand-new array of plant-pollinator interactions.[18] Although the developmental origin of the angiosperm flower has not yet been explained, it is known that a WGD event occurred before the diversification of angiosperms, and duplication of homeotic MADS-box genes controlling the identity of floral organs was likely important in the origin of the flower[15-16]. A role for developmental robustness in the subsequent radiation is supported by the observation that early-diverging lineages of angiosperms are relatively species-poor and display a low degree of floral developmental robustness, in contrast with the high robustness found in some of the most diverse angiosperms families, such as the Orchidaceae[12].

Aside from the actual flower itself, additional floral key innovations independently acquired in multiple families are thought to have consistently enhanced diversification within the angiosperms[14]. The developmental mechanisms recruited in these

independent origins of key traits may or may not be the same between species at the organ level (e.g. different organ identity, contrasting growth patterns), cell level (e.g. patterns of cell division and cell expansion) or molecular level (e.g. changes in regulatory or coding regions of the same or different genes). Thus, recurrently acquired key innovations constitute phylogenetic replicates that can be used as "metamodels" in which to test the consistency of those developmental innovations promoting speciation across lineages[19]. For example, floral zygomorphy (bilateral symmetry) is known to have evolved many times from actinomorphic (radially symmetric) ancestors, and is thought to have promoted speciation by enabling specialized animal pollination[20]. In eudicots, the evolution of zygomorphy has recurrently involved the recruitment of *CYCLOIDEA*-like genes that are dorsally expressed during flower development[21]. In contrast to zygomorphy, diverse molecular mechanisms might underlie the repeated evolution of nectar spurs, another floral key innovation (Box 1).

In addition to key innovations, highly labile traits may be involved in numerous speciation events throughout plant lineages. The best example is probably flower colour, one of the plant traits whose developmental pathways are best understood, particularly those related to anthocyanin pigmentation[22]. Flower colour is frequently involved in pollinator-driven speciation events in distant angiosperm families[23], providing another useful example of phylogenetic replication at the macroevolutionary scale. In this case, continuing work in multiple models (*Antirrhinum*, *Petunia*, *Aquilegia*, among others; Fig. 1a,b; Box 1) reveals that evolutionary changes tend to occur in "optimally pleiotropic" components of developmental pathways, i.e. those that maximize change in the trait under selection while at the same time minimizing deleterious effects on other traits, such as seed coat development, UV resistance and pathogen defence[19,24].

The role of the fossil record. A source of macroevolutionary information that is frequently overlooked by plant evo-devo is the fossil record. Fossils provide invaluable information about evolutionary patterns that cannot be inferred from extant lineages alone, including calibrations for phylogenetic dating analyses to help estimate the timing of developmental evolution[25]. A detailed study of the fossil record also allows us to assess historical changes in the disparity (morphological variety) of clades, and their correlation, or lack thereof, with changes in diversity (number of species). Although only preliminary analyses are available for plants[26], diversity and disparity seem to be fundamentally decoupled, with maximum disparity frequently being achieved early during diversification of a clade. This is usually followed by an increase in diversity that is not accompanied by further increases in disparity. Proposed explanations for this pattern of diversification through small variations on early evolving themes, also found in animals, include developmental constraints and ecological restrictions. An eco-evo-devo approach integrating the study of fossil and extant lineages may help to distinguish these two non-mutually exclusive hypotheses.

Remarkably, fossils can preserve diagnostic features of plant development, and even structural evidence for developmental regulatory mechanisms of extinct plants[27-29], thus potentially containing information on the origin of evolutionary innovations important for diversification. This is particularly relevant for ancient evolutionary transitions whose signature in extant lineages may be limited. Examples are provided by the evolution of woody growth and tree architecture across plant lineages. Among extant plant lineages, wood is only produced by seed plants. However, the fossil record shows that woody growth has evolved several times in the course of vascular plant diversification, and there is strong developmental evidence that these multiple acquisitions were mediated by a common polar auxin regulatory pathway[29-30]. Evolution

of tree architecture is particularly intriguing in the arborescent lycopsids (Lepidodendrales), which speciated profusely in Carboniferous coal-swamp forests and whose closest living relatives are the herbaceous quillworts (*Isoetes*). Interestingly, both Lepidodendrales and *Isoetes* share a pattern of bipolar growth in which the rooting system is a highly modified shoot system known as the rhizomorph[29]. This indicates that developmental studies of *Isoetes* and comparison with other living lycopsids can provide insights into the development and diversification of the long-extinct arborescent lycopsids[31].

The examples above show that macroevolutionary studies provide invaluable information on large-scale patterns of speciation and developmental evolution. However, they do not provide details of the mechanisms involved in particular speciation events. For that, study systems at finer evolutionary scales (closely related species and populations) are required.

The macro-microevolution transition

At the macro-microevolution transition, where separation of closely related species is studied, developmental repatterning can underlie the origin of reproductive barriers. It also plays a key role in generating further phenotypic changes that distinguish closely related species (Fig. 1). All possible types of developmental repatterning may be involved in these speciation events, including changes in timing, spacial distribution, quantity and type of developmental activities at each of the molecular, cellular and organismal levels[32]. Examples of developmental repatterning between closely related species (directly involved in reproductive isolation or not) include shifts in flowering time[33], inflorescence architecture[34], nectar spur length[35], flower colour[36], petal cell shape[37] and leaf shape[38]. Repatterning of multiple developmental traits frequently

occurs associated with a speciation event. The most conspicuous examples involve shifts in pollination syndromes, commonly studied through the developmental and genetic comparison of closely related species with contrasting pollinators (eg in *Mimulus*[39], *Petunia*[40], *Aquilegia*[41]; Fig. 1b,c; Box 1).

Shifts in pollination syndrome. Perhaps the best studied system in which evo-devo research has shed light on the separation of closely related species is the Solanaceous genus *Petunia*. The 20 species of *Petunia* originated in South America, and their radiation is considered to have occurred within the last 3 million years[42]. Major phenotypic differences between species are mostly related to the flower, and these differences underpin divergent relationships with different pollinating animals[43]. Specifically, attention has focused on understanding the differences in corolla tube length, stigma exsertion, anthocyanin production and UV-absorbing flavonol content that distinguish bee pollinated species such as *P. integrifolia* and *P. inflata* (short tube, no stigma exsertion, anthocyanin, no flavonols), moth pollinated species such as *P. axillaris* (long tube, no stigma exsertion, no anthocyanin, flavonols) and the single hummingbird pollinated species *P. exserta* (long tube, stigma exsertion, anthocyanin, no flavonols) (Fig. 1b). These studies have been facilitated by the ability to cross these different species and by the development of a range of genetic, genomic and transgenic resources. By combining these approaches to isolate individual traits of the different pollination syndromes, and by using pollinator behaviour studies, it has been possible, for several of these characters, to identify both the molecular basis of trait repatterning and the consequences for pollinator behaviour and reproductive isolation[40,44-45]. These combined studies in a single system have revealed novel conceptual insights into the developmental shifts underpinning ecological speciation. One such insight is the discovery that many of the molecular changes target transcriptional regulators of

198 developmental pathways, with the R2R3-MYB family of transcription factors being a
199 key target in *Petunia*. These proteins can be thought of as mid-level control points,
200 downstream of the essential regulation of floral organ identity but specifying the shape,
201 pattern and colour of those organs by direct activation of structural genes encoding
202 enzymes and cytoskeletal components[46]. A second insight is that multiple molecular
203 evolutionary events may underpin the same phenotypic change if that change is of
204 sufficient selective advantage. Hoballah et al.[40] reported that loss of function of AN2, a
205 MYB regulator of anthocyanin production, had occurred at least five times
206 independently in wild-sampled *P. axillaris*, a white-flowered moth-pollinated species.
207 Perhaps most striking, though, has been the unexpected discovery that many of the
208 genes controlling traits involved in pollinator specificity have become linked in *Petunia*,
209 generating a multigene "speciation locus" (or "speciation island") on chromosome II[47].
210 This is a novel feature of *Petunia* – the same genes regulating anthocyanin production,
211 UV absorption, male and female reproductive organ position and scent are distributed
212 across multiple chromosomes in other Solanaceous species. It is likely that this
213 clustering of key genes promotes linkage disequilibrium and avoids pollination
214 syndromes being disturbed by recombination that could reduce fitness.
215 ***Reproductive isolation in other ways*.** The *Petunia* system emphasizes the importance
216 of multiple trait repatterning to ensure reproductive isolation through differential
217 pollination syndromes. However, single aspects of flower development can also diverge
218 between closely related species, generating reproductive isolation more simply. One
219 classic example is the divergence of flowering time between closely related species
220 growing in the same habitat. This displacement of flowering phenology has been
221 observed in multiple systems under different conditions, and is particularly striking
222 when repeated in multiple different habitats (eg Lobo et al.[48] studying flowering time of

223 bat pollinated Bombaceae in three different habitats with different rainfall patterns).
224 Displacement of flowering can occur to minimize competition for pollinator attention
225 and interspecific hybridisation between established species, or it may occur as part of
226 the process of reproductive isolation as species diverge. Ellis et al.[33] observed
227 displacement of flowering time over a 14-week winter rainfall season for species of the
228 stone plant *Argyroderma*, and interpreted this displacement as an adaptation to isolate
229 populations that had diverged in their tolerance for different soil conditions, facilitating
230 full speciation. The processes that determine when a plant flowers are well described in
231 *Arabidopsis thaliana*, with multiple environmental and endogenous pathways
232 converging on the activity of a set of floral meristem identity genes (reviewed by Holt et
233 al.[49], Glover[50]). To fully understand the molecular basis of the developmental transitions
234 in flowering time, observed in various plant radiations, multi-species studies will need
235 to be connected to the micro-evo-devo work currently exploring variation in flowering
236 time in different ecotypes of *Arabidopsis*[51-53].
237 Reproductive isolation between close relatives can also result from shifts in breeding
238 system. Shifts from outcrossing to selfing in flowering plants are commonly associated
239 with the evolution of a set of phenotypic traits known as the "selfing syndrome":
240 smaller flowers, reduced pollen production and loss of scent and nectar production[54].
241 The developmental changes producing the selfing syndrome are being studied in the
242 sister species *Capsella grandiflora* (outcrossing, large flowers) and *C. rubella* (selfing,
243 small flowers). Reduced petal size in *C. rubella* results from a reduction in the number
244 of petal cells caused by a shortening of the cell division period. Allelic variation in the
245 intron of a general growth regulator, affecting the levels of STERILE APETALA (SAP)
246 protein in developing petals, has contributed to this change[55]. Interestingly, it seems that
247 the small-petal allele of SAP was already present in the ancestral outcrossing

population, explaining the rapid evolutionary reduction of petal size during speciation. In addition, *C. rubella* has lost a major component of floral scent present in *C. grandiflora* (benzaldehyde) as a result of repeated inactivations of the *CNL1* gene (encoding the enzyme cinnamate:coA ligase), caused by independent mutations in its coding sequence[56].

Another example of reproductive isolation generated through an evolutionary change to a developmental programme is the specialisation of plant species on pollinators with particular lengths of feeding apparatus, through transitions in the length of floral tubes and nectar spurs (see Box 1). This sort of change can be associated with major shifts of pollinator, and therefore with other changes to flower morphology and colour, or it can occur in isolation of other traits and simply select between insects of different proboscis length. In a classic study of nectar spur evolution in North American species of *Aquilegia*, Whittall and Hodges[57] demonstrated that the evolution of increasingly long spurs is driven by speciation events involving shifts between pollinators with increasingly long mouth parts (bees, hummingbirds and hawkmoths). Other studies have focused on simpler transitions in spur length between closely related species of orchid[58] and *Linaria*[59]. Current advances in our understanding of nectar spur development are allowing analysis of the molecular evolutionary processes underpinning these speciation events[35,60-61] (see Box 1).

Speciation by hybridisation and polyploidisation. Hybridisation and polyploidy can rapidly generate reproductive isolation and therefore lead to speciation in plants[62]. In the genus *Tragopogon*, for example, new allopolyploid species (*T. miscellus*, *T. mirus*) have evolved in the last century in North America as a result of hybridisation between three naturalised Eurasian species (Fig. 1d). Each allopolyploid has been produced multiple times in independent hybridisation events, and they can also be generated synthetically,

273 providing excellent opportunities for comparative analysis. Upon allopolyploidisation,
274 genes inherited from the progenitor species can be differently expressed, silenced or
275 even lost[63]. This may lead to developmental variation, such as that found between
276 populations of *T. miscellus*, which display long or short ligules depending on the
277 identity of the maternal and paternal parents.
278 ***Taxonomically diagnostic traits.*** While it is tempting to focus on traits directly
279 involved in reproductive isolation, phenotypic changes resulting from developmental
280 repatterning are frequently used to taxonomically delimit species, even if their
281 involvement in the initial stages of reproductive isolation is uncertain. One example is
282 evolution of leaf shape, which has been studied in the genera *Antirrhinum*[38] (Fig. 1a)
283 and *Solanum*[64]. *Antirrhinum* comprises around 25 species, originating around 4 million
284 years ago in the Mediterranean region[65]. Analysis of QTLs associated with leaf size and
285 shape following crosses between small-leaved and large-leaved species suggested that
286 the species had diverged in response to fluctuating selection regimes, consistent with a
287 radiation in a period of climate and vegetation cycles[38]. Leaf size and shape are
288 understood as a product of the combined amount of cell division and cell expansion that
289 occurs throughout organ development, and these processes are controlled in a
290 coordinate way. Molecular evolution of these processes is often developmentally
291 constrained, causing leaves and other organs to evolve together[38], generating major
292 phenotypic differences that can be used in taxonomic species description as well as
293 underpinning selection in different environmental conditions. In *Solanum*, closely
294 related species display contrasting levels of complexity of compound leaves. Variation
295 in expression of the BLADE-ON-PETIOLE (BOP) transcription factor seems to explain
296 this diversity through dynamic rewiring of interactions in the gene regulatory network
297 for leaf development[64]. This includes the alteration of the transcript levels of *KNOX*

genes, which have been recurrently recruited to generate leaf diversity during plant evolution.

The examples in this section illustrate evolutionary developmental changes involving recently diversified species, usually with reproductive barriers already in place. To better understand how traits involved in speciation first evolve, developmental variation can be investigated at an even finer scale, within species and populations.

Microevolutionary scale

Genetically based intraspecific variation. Relatively little attention has been paid to microevolution in the plant evo-devo literature. Microevolutionary processes have been traditionally studied from two interacting perspectives: (1) population genetics, including the study of genetic variation in populations and allele frequency changes due to mutation, selection, migration and drift; and (2) evolutionary ecology, which investigates the biotic and abiotic interactions underlying the selective pressures that lead to evolutionary change in populations. A deeper understanding of plant speciation emerges from the integration of these approaches with developmental biology. The evo-devo approach to microevolution (micro-evo-devo[5]) examines evolvability, the ability of species and populations to produce heritable phenotypic variation, as determined by genetic architecture and developmental constraints[6,66]. In this way, it provides insight into the processes that supply the raw material for adaptation, evolution and speciation[5,67]. This generally involves the study of developmental variation across populations of the same species and within populations, particularly those polymorphisms that may underlie local adaptation, divergence between populations and, potentially, the establishment of reproductive barriers (Fig. 2).

A fertile field for microevolutionary research in plants is the study of flower colour polymorphisms. Flower pigmentation is involved in pollinator specialisation[23,68], and its molecular and developmental basis has been well studied[22,24]. In addition, intraspecific colour polymorphisms are relatively common in nature, and they frequently involve few genetic changes. A link between intraspecific flower colour variation and incipient diversification has been demonstrated in the sticky monkey-flower (*Mimulus aurantiacus*). A number of studies[69-71] have addressed the genetic basis of flower colour variation, population genetics, pollinator interactions and isolating barriers in two closely related morphs of *M. aurantiacus* distributed in southwestern California: a red-flowered ecotype preferentially pollinated by hummingbirds and a yellow-flowered one preferred by hawkmoths (Fig. 2a). This multi-disciplinary approach has revealed incipient ecological speciation in the face of gene flow, primarily resulting from pollinator preferences causing divergent selection on an R2R3-MYB transcription factor (*MaMyb2*) involved in the regulation of the anthocyanin biosynthetic pathway[71]. A *cis*-regulatory change in *MaMyb2* is responsible for the colour change underlying incipient pre-mating isolation between ecotypes. This example reveals microevolutionary mechanisms by which traits involved in pollination syndrome shifts first evolve.

Another trait relevant to speciation that is amenable to population-level research is floral symmetry. Floral zygomorphy is known to have evolved in several genera of Brassicaceae in correlation with differences in expression of *CYC2* during corolla development[72]. To understand how zygomorphy has evolved at a microevolutionary scale, an ideal system is provided by *Erysimum mediohispanicum*, a member of the Brassicaceae displaying heritable intraspecific variation in floral symmetry, from actinomorphic to zygomorphic[73] (Fig. 2b). Evolutionary ecological approaches show that plants bearing zygomorphic flowers have the highest fitness, and that strong

347 selection on corolla shape is exerted by pollinators[74]. By analyzing the developmental
348 genetic basis of floral symmetry variation in *E. mediohispanicum*, the
349 microevolutionary process by which zygomorphy evolves would be more fully
350 understood, and this would in turn enhance our understanding of macroevolutionary
351 patterns in Brassicaceae.

352 Many other traits potentially involved in speciation are being investigated using
353 polymorphic target species, including the following examples: flowering time
354 differentiation between locally adapted populations in *Arabidopsis thaliana*[51];
355 continuous variation in pollination by sexual deception in the South African beetle daisy
356 (*Gorteria diffusa*; Fig. 2c)[75]; the recurrent parallel divergence of morphologically
357 distinct ecotypes adapted to contrasting habitats in the Australian groundsel *Senecio*
358 *lautus*[76]; and adaptive variation in the production of leaf trichomes, involved in
359 resistance to herbivory, in *Arabidopsis lyrata*[77]. There is the exciting potential for the
360 comparative microevolutionary study of similar traits in distant lineages to provide
361 metamodels linking the macro- and microevolutionary scales.

362 ***Beyond genetic variation.*** While intraspecific phenotypic variation discussed thus far is
363 considered to be the result of genetic changes, recent research has highlighted a
364 potential role in speciation for other components of variation. Phenotypic plasticity, the
365 capacity of a genotype to produce alternative phenotypes in response to environmental
366 variation, has been suggested as a facilitator of adaptive divergence and speciation[78-79].
367 Intraspecific phenotypic differences initially generated by plasticity may be fixed in
368 different populations by natural selection in the process of genetic assimilation, and can
369 then contribute to potentially rapid genetic divergence, reproductive isolation and
370 eventually speciation[78]. As a result, the developmental mechanisms responsible for
371 plasticity may parallel those underlying interspecific diversity. For example,

heterophylly in the North American lake cress (*Rorippa aquatica*), involving morphological differences between leaves developing under submerged and terrestrial conditions, is the result of environmentally induced changes in the expression of *KNOX1* genes, which are also implicated in the diversification of leaf shape across species of the same family[80].

Related to phenotypic plasticity, there is speculation that heritable epigenetic variation, shaped by the environment and natural selection, might also aid evolutionary change and speciation[81-83]. Epigenetic diversity, triggered by environmental changes, may enable genetically depauperate populations to quickly adapt until genetic assimilation fixes phenotypic differences. Interestingly, one of the first naturally occurring morphological mutants to be genetically characterized, the peloric mutant of *Linaria vulgaris* (showing radially symmetrical flowers instead of the zygomorphic flowers that are characteristic of *Linaria*), was found to be an epimutant resulting from extensive methylation of the *CYC* gene[84]. Although the evolutionary significance of this particular mutant is probably limited given its compromised reproductive success, evidence has since been found of heritable intraspecific epigenetic variation correlated with phenotypic differences and potentially subject to natural selection[85]. For example, in the yellow monkeyflower (*Mimulus guttatus*), epigenetically inherited variation in trichome density is induced by herbivore damage, and is correlated with differential regulation of a *MYB MIXTA-like* transcription factor[86]. The emerging field of population epigenetics, combined with ecological and developmental approaches, can provide insights into this still largely hypothetical link between epigenetics and speciation.

Concluding Remarks

The processes of speciation have puzzled evolutionary biologists for over 150 years. Many models to explain how new species emerge have been proposed and many systems developed in which to test those models. It is clear that speciation events result from a combination of multiple molecular, environmental and stochastic factors. However, the recent input of evolutionary developmental biology into this field has generated new insights. It has allowed both the crystallisation of novel concepts surrounding speciation processes and the revisiting of old questions (Box 2). We conclude that a strong interaction between developmental biology and evolutionary biology (including phylogenetics, population genetics, evolutionary ecology, paleontology; Box 3) is crucial to retain momentum in the drive to gain a deeper understanding of plant speciation.

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Figures

Figure 1 | Model systems for the evolutionary developmental study of plant speciation. a, *Antirrhinum* spp.: variation in flower and leaf morphology in a recent radiation, represented by *A. majus* (left), *A. braun-blanquetii* (centre) and *A. charidemi* (right). **b**, *Petunia* spp.: transitions in flower tube length, stigma exsertion and colour associated with pollinator shifts, represented by *P. inflata* (bee-pollinated, left), *P. exserta* (hummingbird-pollinated, centre) and *P. axillaris* (moth-pollinated, right). **c**, *Mimulus* spp.: differences in floral morphology between two sister species with contrasting pollination syndromes, *M. lewisii* (bee-pollinated, left) and *M. cardinalis* (hummingbird-pollinated, right). **d**, *Tragopogon* spp.: capitula of the allopolyploid hybrid *T. mirus* (centre) and its parent species *T. dubius* (left) and *T. porrifolius* (right). Photos by A. Hudson (a), H. Sheehan (b), H.D. Bradshaw (c), E. Mavrodiev (d, left and centre) and A.N. Doust (d, right).

Figure 2 | Species showing intraspecific variation in developmental traits relevant to plant speciation. a, *Mimulus aurantiacus*: floral colour variation associated with pollinator preferences between a red-flowered ecotype (preferred by hummingbirds, left) and a yellow-flowered ecotype (preferred by hawkmoths, right). **b**, *Erysimum mediohispanicum*: variation in floral symmetry correlated with fitness differences, including radial (left), dissymmetric (centre) and zygomorphic (right) flowers. **c**, *Gorteria diffusa*: variation in presence of petal spots and degree of sexual deception between three morphotypes (from left to right: Steinkopf, Cal and Buffels). Photos by M.A. Streisfield (a), J.M. Gómez (b) and G. Mellers (c).

Box 1 | Nectar spurs and speciation: from macro- to microevolution.

Floral nectar spurs constitute one of the best examples of a trait involved in plant speciation that is being studied at multiple evolutionary scales, integrating evolutionary, developmental and ecological perspectives. These spurs are tubular outgrowths of floral organs usually containing a nectar reward for pollinators, and they have evolved multiple times during the diversification of flowering plants, within families as distantly related as Ranunculaceae, Orchidaceae, Violaceae and Plantaginaceae[87]. Phylogenetic comparative analyses suggest bursts of diversification associated with clades with nectar spurs, leading to the consideration of this trait as a "key innovation" promoting speciation through pollinator specialisation[14,87].

At the ***macroevolutionary scale***, the comparative study of nectar spurs in different families allows us to test the degree to which similar ontogenetic and genetic mechanisms have been recurrently recruited to produce a morphologically and functionally convergent trait. Thus far, ontogenetic mechanisms producing nectar spurs seem to be broadly similar across families, with an initial phase of cell division followed by a phase of cell elongation[35,60,88]. However, recent evidence suggests a diversity of molecular mechanisms recruited to achieve this, with *KNOX* and *TCP4* genes respectively proposed as regulators of spur development in toadflaxes (*Linaria*, Plantaginaceae)[60] and columbines (*Aquilegia*, Ranunculaceae)[61].

At the ***macro-microevolution transition***, recent speciation potentially driven by nectar spurs has been studied mainly in the North American clade of the genus *Aquilegia*. In this lineage, changes in spur length are associated with shifts in the main pollinators (bees, hummingbirds or hawkmoths)[57], and spur length differences contribute to the reproductive isolation between co-occurring species[89]. Ontogenetically, variation in spur length across species is the result of changes in the duration of the phase of cell

665 elongation during spur development[35]. It remains to be determined whether this
666 heterochronic mechanism of spur length evolution also characterises other unrelated
667 spurred lineages.
668 The ***microevolutionary scale*** provides the best opportunities to test evolutionary models
669 of spur length change, such as the coevolutionary race and pollinator shift hypotheses[57],
670 and the degree to which spur evolution contributes to incipient divergence. In
671 Scandinavian populations of the orchid *Platanthera bifolia*, geographic variation in spur
672 length is correlated with the proboscis length of distinct local pollinators, suggesting
673 that intraspecific changes in spur length are driven by pollinator shifts[90]. The
674 developmental mechanisms behind these changes, as well as their potential contribution
675 to incipient divergence and eventual speciation, remain to be studied.
676
677 **[Box 1 Figure] Model systems to study the evolution of nectar spurs.** Ordinal-level
678 phylogeny of flowering plants, with red branches indicating orders in which nectar
679 spurs have evolved[87]. Three phylogenetically disparate genera in which evolution of
680 nectar spurs is being investigated at different scales are shown: (a) *Aquilegia*: species
681 with different pollination syndromes display contrasting spur lengths, as exemplified by
682 *A. sibirica* (c. 10 mm, bee-pollinated, top), *A. formosa* (c. 20 mm, hummingbird-
683 pollinated, centre) and *A. chrysantha* (c. 70 mm, hawkmoth-pollinated, bottom). (b)
684 *Linaria*: *L. salzmannii* (c. 13 mm, left) and *L. clementei* (c. 3 mm, right) are two closely
685 related species with nearly identical floral morphology but contrasting spur lengths. (c)
686 *Platanthera*: intraspecific variation in spur length in *P. bifolia* (c. 15-40 mm) correlates
687 with the proboscis length of local pollinators. Nectar spurs are indicated by arrow heads
688 in all photos. Photos by E.S. Ballerini (a), M. Fernández-Mazuecos (b) and J. Quiles (c).
689

Box 2 | Hopeful monsters?

The evolutionary relevance of large-effect mutations in evolution and speciation is at the centre of a long-standing debate in evolutionary biology[10]. While widely accepted evolutionary models regard gradual change as the most likely mode of evolution, it has been frequently argued by developmental biologists that homeotic mutations, changing the identity of whole organs, may have played a role in some major evolutionary transitions[2-3]. For example, evolutionary changes in floral organ identity have been hypothesised to be the result of homeotic mutations involving changes in the expression domains of genes in the ABC model of flower development[91]. While this hypothesis is intriguing, systems in which the feasibility of such changes can be studied at the microevolutionary scale are required to test it. The *Stamenoid petals* (*Spe*) mutant of the shepherd's purse (*Capsella bursa-pastoris*) has been proposed as a suitable model for this[92]. It is a naturally occurring floral homeotic mutant in which petals have been transformed into stamens. Unlike other known homeotic mutants, it forms stable populations in the wild, mixed with wild-type plants. The mutation has been shown to be heritable and involves a single locus, hypothesized to be a class C floral identity gene[93]. Both morphs seem to have similar fitness, and a degree of genetic differentiation and reproductive isolation between them has been detected in a German locality, suggesting incipient speciation[94-95]. Even if considered a rarity, this system nicely bridges microevolutionary processes and macroevolutionary outcomes, and hints at the feasibility of saltational changes giving rise to "hopeful monsters" of potential long-term evolutionary relevance[96].

[Box 2 Figure] A hypothetical "hopeful monster". Flowers of wild-type *Capsella bursa-pastoris* (left) and the naturally ocurring *Spe* mutant of the same species (right). Photos by G. Theißen.

Box 3 | The need for a robust phylogenetic context.

The role of phylogenetics in evolutionary developmental biology has been highlighted since the origins of evo-devo[97], and it is particularly crucial when the focus is on speciation. Indeed, phylogenetic relationships have to be known if the sequence and direction of developmental changes in the course of speciation are to be understood[98], including, for example, the detection of instances of parallelism that may result from developmental biases. However, integration of phylogenetic and developmental data is often lacking, and the use of new analytic tools to achieve it is desirable[99-100]. In addition, speciation studies frequently involve recently diverged species or populations whose phylogenetic relationships cannot be easily resolved using conventional phylogenetic approaches. To that end, high throughput sequencing methods capable of providing genome-wide markers are required. In their study of flower colour divergence during incipient diversification in the *Mimulus aurantiacus* complex, for example, Stankowski & Streisfeld[70] provide a good example of the use of a robust phylogenetic framework, developed using RAD-Seq markers, to reconstruct evolutionary developmental changes. According to phylogenetic analyses, red flowers have been acquired in two independent lineages with yellow flowered ancestors. In both cases, the red pigmentation is the result of a *cis*-regulatory mutation in the gene *MaMyb2*. Interestingly, population genetic analyses suggest that a single red allele may have evolved and subsequently been transferred between the two red-flowered morphs by introgression.