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RESEARCH

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Comparative analysis of corolla tube development across three closely related *Mimulus* species with different pollination syndromes

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Abstract

Fusion of petals to form a corolla tube is considered a key innovation contributing to the diversification of many flowering plant lineages. Corolla tube length often varies dramatically among species and is a major determinant of pollinator preference. However, our understanding of the developmental dynamics underlying corolla tube length variation is very limited. Here we examined corolla tube growth in the *Mimulus lewisii* species complex, an emerging model system for studying the developmental genetics and evo-devo of pollinator-associated floral traits. We compared developmental and cellular processes associated with corolla tube length variation among the bee-pollinated *M. lewisii,* the hummingbird-pollinated *Mimulus verbenaceus*, and the self-pollinated *Mimulus parishii*. We found that in all three species, cell size is non-uniformly distributed along the mature tube, with the longest cells just distal to the stamen insertion site. Differences in corolla tube length among the three species are not associated with processes of organogenesis or early development but are associated with variation in multiple processes occurring later in development, including the location and duration of cell division and cell elongation. The tube growth curves of the small-flowered *M. parishii* and large-flowered *M. lewisii* are essentially indistinguishable, except that *M. parishii* tubes stop growing earlier at a smaller size, suggesting a critical role of heterochrony in the shift from outcrossing to selfing. These results not only highlight the developmental process associated with corolla tube variation among species but also provide a baseline reference for future developmental genetic analyses of mutants or transgenic plants with altered corolla tube morphology in this emerging model system.

1 | INTRODUCTION

The petals of many angiosperms are partially or completely united to form a corolla tube, a condition known as sympetaly. The length, width, and shape of corolla tubes vary tremendously among sympetalous species. The relative length of the corolla tube and the free lobes is a distinctive element of floral form diversity and is a major determinant of pollinator specificity (Armbruster et al., 1999; Erbar & Leins, 1996; Fenster et al., 2004; Johnson & Steiner, 1997; Nilsson, 1988; Waser et al., 1996). Sympetaly has been considered a key innovation of Asterids (a large and diverse clade within the angiosperms) and has evolved multiple times independently in other angiosperm lineages (Endress, 2001; Zhong & Preston, 2015). Despite the evolutionary and functional significance of sympetaly, our

understanding of the growth dynamics underlying corolla tube variation is very limited.

Formation of the corolla tube has long been considered a classic case of "congenital fusion" (Erbar & Leins, 1996, 2004; Erbar, 1991; Weberling, 1989), a process involving the lateral extension of the flanks of the individual petal primordia across the inter-primordial region followed by zonal growth (Erbar & Leins, 1996, 2004; Erbar, 1991; Verbeke, 1992). Following formation of the corolla tube, variation in length is associated with differences in cell division and cell elongation (Landis et al., 2016; Martin & Gerats, 1993; Stuurman et al., 2004). The interplay of cell division and elongation during floral morphogenesis has been examined in nectar spurs (tubular evaginations of the corolla) of *Linaria*; Plantaginaceae (Box et al., 2011; Cullen et al., 2018), *Aquilegia*; Ranunculaceae (Puzey et al., 2012), and *Centranthus*; Valerianaceae (Mack & Davis, 2015), and nectar tubes of *Pelargonium*; Geraniaceae (Tsai et al., 2018). Spur and nectar tube development in these taxa are associated with both cell division and elongation. However, the contribution of each process varies among taxa. Interspecific spur length variation in *Aquilegia* is mediated primarily by cell elongation (Puzey et al., 2012), whereas, in *Linaria*, differential cell number (and presumably cell division) accounts for the difference in spur length between sister species (Cullen et al., 2018). A combination of cell division and cell elongation contributes to nectar tube differences between species of *Pelargonium* (Tsai et al., 2018). In *Saltugilia* (Polemoniaceae) and *Petunia* (Solanaceae), the only taxa for which the dynamics of corolla tube variation have been studied, both cell division and cell elongation are associated with tube length variation (Landis et al., 2016; Stuurman et al., 2004). However, the primary contributor of variation is cell size in *Saltugilia* and cell number in *Petunia*. Far too few taxa have been studied to draw any generalizations about the processes that underlie length variation. Moreover, pinpointing when during development differences in cell number and/or size are established between closely related species has never been examined in any taxon.

We focus on the developmental bases of corolla tube variation in the *Mimulus lewisii* species complex, which contains several closely related species with striking variation in flower size, shape, and color that is related to their pollination strategy (Beardsley et al., 2003; Hiesey et al., 1971; Nelson et al., 2020; Schemske & Bradshaw, 1999; Vickery & Wullstein, 1987; Yuan, 2019). For example, *M. lewisii* is an outcrossing, bee-pollinated species and has flowers with long corolla tubes and flared limbs, *Mimulus verbenaceus* is pollinated by hummingbirds and has flowers with long, narrow tubes with less flare of the limb than *M. lewisii*. Flowers of *Mimulus parishii* are similar to those of *M. lewisii* but they are much smaller and are self-pollinating (Figure 1).

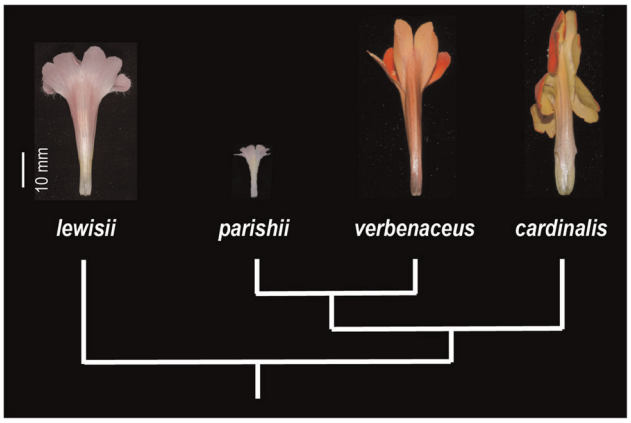

FIGURE 1 Dorsal view of the corolla of four species in the *Mimulus lewisii* species complex. *Mimulus cardinalis* was not examined in this study but is included here to highlight that *M. lewisii* is sister to a clade with more species than *Mimulus parishii* and *Mimulus verbenaceus*. The corolla images are scaled in proportion to real flower size (scale bar = 10 mm). The phylogenetic relationship is based on Nelson et al. (2020)

To identify the differences in developmental dynamics that underlie these divergent morphologies, we compare corolla tube growth and the associated patterns of cell division and elongation for *M. lewisii, M. verbenaceus,* and *M. parishii*. Among the three species, *M. lewisii* and *M. verbenaceus* have longer corolla tubes, yet have evolved different pollination strategies; have they retained similar developmental dynamics? In contrast, *M. parishii* bears self-fertilizing flowers with much shorter corolla tubes compared with the other two species. Outcrossing is ancestral in the *M. lewisii* species complex (Beardsley et al., 2003; Nelson et al., 2020). The shift from outcrossing to selfing is a frequent evolutionary transition in numerous lineages of Angiosperms (Barrett, 2002) and one of the features associated with this transition is the reduction in floral size (Barrett, 2010). Therefore, we also aim to examine the shifts in developmental processes such as change in relative timing or rates of growth, and their associated cellular processes, necessary for the transition from a large-flowered ancestor (outcrossing) to the small-flowered *M. parishii* (selfing).

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Inbred lines of *M. lewisii* (LF10), *M. verbenaceus* (MvBL), and *M. parishii* (Mpar) were grown from seed in the University of Connecticut greenhouse under natural light supplemented with sodium vapor lamps, ensuring a 16-h day length.

The original source seeds for *M. lewisii*, *M. verbenaceus*, and *M. parishii* were collected from the South Fork of the Tuolumne River near Carlon, CA (Owen & Bradshaw, 2011), Oak Creek Canyon near Sedona, AZ (Stanley et al., 2020), and Deep Creek near Palm Springs, CA (Fishman et al., 2015), respectively. All inbred lines were generated by single seed descent for >10 generations.

2.2 | Comparative growth analysis

We wanted to compare developing flowers of the three species at the same ages. Size is often used as a proxy for age in such analyses (e.g., Guerrant, 1982), but this approach is only reliable if the structures are similarly sized at maturity. Because the flowers of the study species are quite different in mature size, size cannot be used as an index for comparison of development. Instead, the actual age of developing flower buds was deduced by a growth analysis that relates chronological age to corolla tube length. Fifteen flower buds of each of the three greenhouse-grown species were tagged at 2 mm tube length (three individual plants per species and five flower buds per plant). Buds with tube lengths smaller than 2 mm were enveloped by the subtending leaf and were prone to damage while taking corolla tube measurements. Therefore, they were analyzed with scanning electron microscopy (SEM; described below). Buds of 2 mm tube length were designated as age 0 and corolla tube lengths were then measured daily at the same time until anthesis (maturation). The corolla was hidden by the sepals during development, but the corolla tube was visible by shining a light through the buds. Using these data, the relationship between the developmental age and tube length was estimated for each species. Because the duration of development varied slightly among samples, we standardized the growth curves to the longest duration of growth for each species. The growth curves were then used to choose ages of development for comparison (see Results; Patterns of cell division and elongation across multiple stages of corolla tube development).

2.3 | Light microscopy

To count cell numbers and measure cell length during corolla tube development, floral buds or mature flowers ($n = 5$ per age) were dissected to expose the dorsal side of the corolla tube, placed in 70% ethanol overnight, and mounted on glass slides. Using a digital camera mounted on a compound microscope, a series of overlapping images along the entire length of the abaxial side of the corolla tube, from the base to the sinus between the two dorsal lobes, was taken. Zeiss ZEN 2.6 lite (blue edition) software was used to assemble the images into a single panoramic image that was used to trace files of cells along the length of the tube (Figure 2). The number of cells in a file, as well as cell length, were calculated. Because cell size is not uniform, even for adjacent cells, the length of successive blocks of 10 consecutive cells along the entire length of the tube rather than individual cells was recorded. Like most Asterids, *Mimulus* flowers have epipetalous stamens. That is, a portion of the filament is adnate to the corolla tube and, in the mature flower, the free portion of the filament appears to emerge from the corolla tube in a region referred to as the stamen insertion site. The position of the stamen insertion site along the length of the tube was recorded as a key morphological marker.

2.4 | SEM

Floral buds of 2 mm tube length were designated as age 0 for quantitative analyses; however, 2 mm was not the "actual day 0" of tube initiation. Therefore, buds smaller than 2 mm tube length were qualitatively compared with SEM. Buds were collected, dissected, and fixed in FAA (formaldehyde-acetic acid-alcohol; Berlyn & Miksche, 1976) overnight and dehydrated in graded ethanol series. The fixed, dehydrated samples were critical point dried with CO_2 using Tousimis 931.GL, sputter-coated using a Polaron E5100, and viewed with a FEI Nova Nano SEM 450.

2.5 | Data analysis

Growth data were fitted with an exponential regression model ($y = ae^{bx}$) using the "nls" function in R v3.6.2. Cell numbers were compared with analysis of variance and Tukey's honest significant difference (HSD) test using the package "car v3.0-10" in R (Fox & Weisberg, 2018). Graphs were generated with the R package "ggplot2 v3.3.2" (Wickham, 2016).

3 | RESULTS

3.1 | Cell number and length in mature flowers

At anthesis, the corolla tubes of *M. verbenaceus, M. lewisii,* and *M. parishii* were 37 ± 0.55, 31.5 ± 1.25, and 11.7 ± 0.15 mm long (mean $\pm$ SD; $n = 5$), respectively. To determine the cellular components of the length variation among the three species, we profiled both cell number and cell length along the entire corolla tube (from the base of the corolla to the sinus between the two dorsal petals; Figure 2)

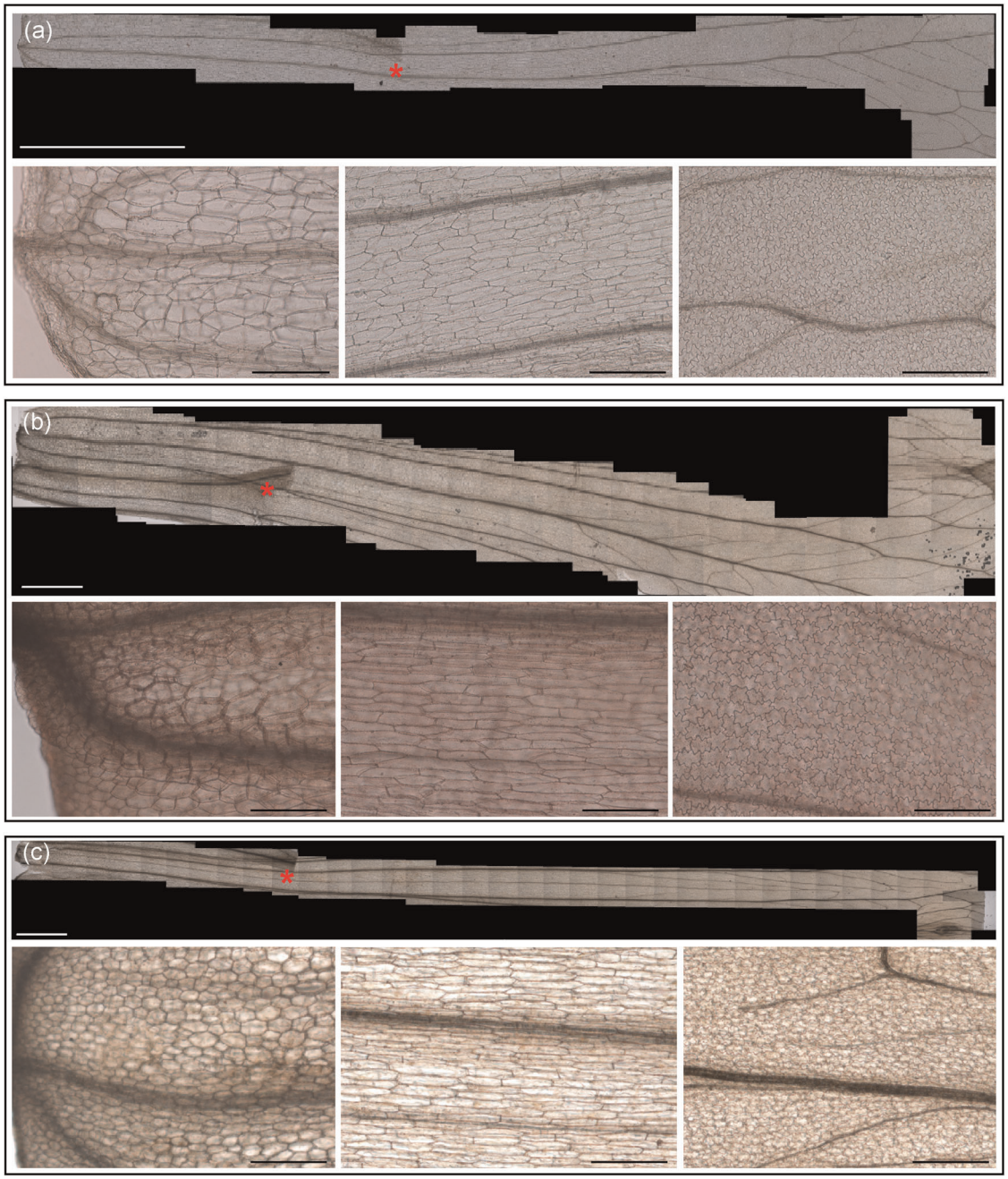

FIGURE 2 Epidermal cells of mature flowers of *Mimulus parishii* (a), *Mimulus lewisii* (b), and *Mimulus verbenaceus* (c). The top image in each panel is a panoramic image of the abaxial side of the corolla tube showing variation in cell length from the base of the corolla to the sinus between the two dorsal lobes. The asterisk marks the stamen insertion site. At the bottom of each panel are the individual images showing cell morphology at the base (left), near the stamen insertion site (center), and near the sinus (right). Scale bars: 2 mm for panoramic images and 200 μm for individual images

for each species. The corolla tube of *M. lewisii* consists of slightly fewer cells than that of *M. verbenaceus* (324 ± 8.6 vs. 343 ± 11.8; $n = 5$), but the general pattern of cell length distribution along the corolla tube is similar between the two species. In both species, cell length gradually increases from the base of the corolla, reaches a maximum just beyond the stamen insertion site (marked by the asterisks in Figures 2 and 3) and plateaus for ~50 cells, then gradually decreases distally where cells are more irregularly shaped. Notably, except in the plateau region distal to the stamen insertion site where *M. verbenaceus* has moderately longer cells than *M. lewisii*, cell length in other regions of the corolla tubes of the two species are similar (Figure 3). In sharp contrast, the corolla tubes of the selfing species, *M. parishii*, not only have far fewer cells (149.4 ± 6.23; $n = 5$) but also have much shorter cells (except at the very base of the corolla tube below

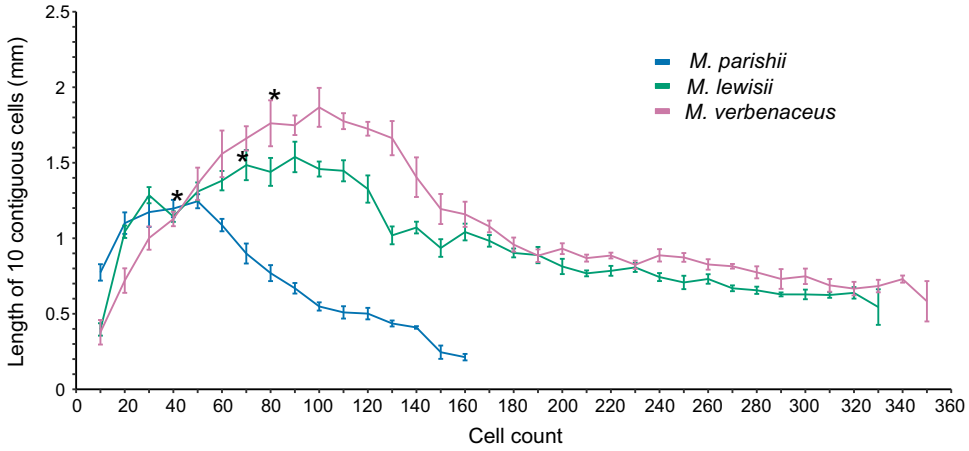

FIGURE 3 Cell number and length along the length of mature corolla tubes. The asterisks mark the stamen insertion sites. Error bars are standard error, $N = 5$ for each species

the stamen insertion site) than the two outcrossing species and appear to largely lack the plateau zone above the stamen insertion site (Figure 3).

3.2 | Comparative growth analysis

To determine when during development the cell number and length of the corolla tubes start to diverge among the three species, we compared their growth trajectories. We tagged fifteen floral buds of 2 mm tube length (designated as day 0) for each species and measured corolla tube length daily until anthesis (maturation). We found that the pattern of corolla tube growth is qualitatively similar in all three species (Figure 4). Growth is initially slow, but the rate increases over time, and is greatest in the time period just before anthesis. The growth trajectories of *M. parishii* and *M. lewisii* largely overlap (Figure 4) but flowers of *M. parishii* reach anthesis earlier than *M. lewisii* (10~12 vs. 14~16 days). Corolla tubes of *M. verbenaceus* elongate more slowly than the other two species in the beginning but continue growing for much longer, reaching anthesis ~7 days later than *M. lewisii* (21~23 vs. 14~16 days, respectively).

3.3 | Patterns of cell division and elongation across multiple stages of corolla tube development

Based on the growth curves (Figure 4), we focused on several crucial ages for detailed examination of patterns of cell division and expansion during corolla tube development. All species were sampled at 4 d when the tubes were indistinguishable, 8 d when divergence in tube length becomes apparent (*M. verbenaceus* corolla tubes are growing more slowly and are not as long as those of *M. lewisii* and *M. parishii* at this stage), and at 12 d when *M. parishii* flowers are mature but the other two species are still developing. In addition, *M. verbenaceus* was sampled at 19 d when only corolla tubes of this species are still growing rapidly (Figures 5 and S1). As noted above, flowers of all species were also sampled at anthesis (12 d for *M. parishii*, 16 d for *M. lewisii,* and 23 d for *M. verbenaceus*).

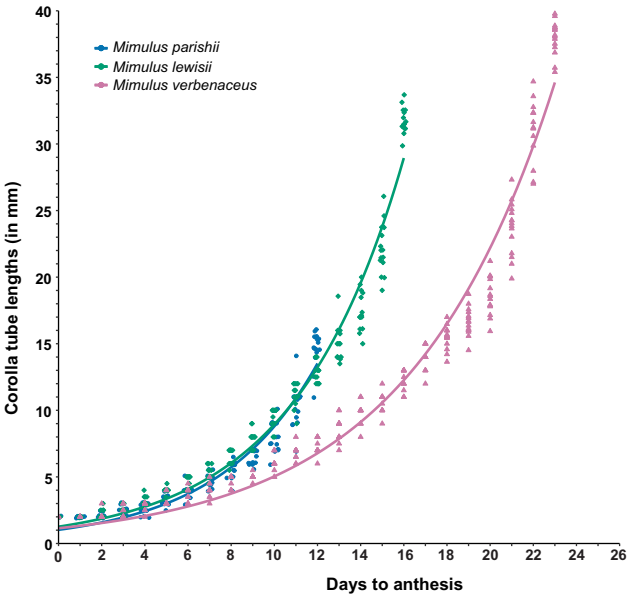

FIGURE 4 Corolla tube growth trajectories from day 0 (2 mm tube lengths) to anthesis. $N = 15$ for each species (shapes: circle for *Mimulus parishii*, diamond for *Mimulus lewisii*, and triangle for *Mimulus verbenaceus*). Growth data were fitted in an exponential model ($y = 1.03e^{0.215x}$, $y = 1.26e^{0.196x}$, and $y = 1.14e^{0.148x}$ for *M. parishii*, *M. lewisii*, and *M. verbenaceus*, respectively)

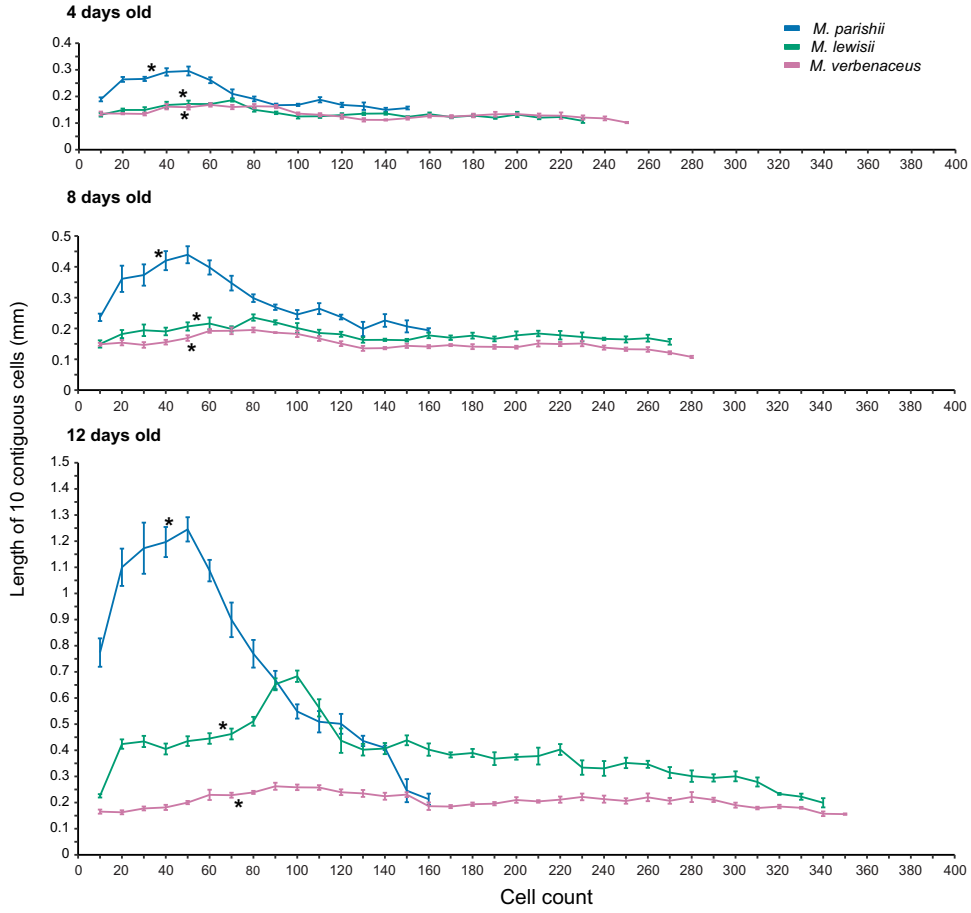

FIGURE 5 Corolla tube cell number and length at different developmental ages. $N = 5$ for each species and each stage. Error bars are 1SE. Asterisks mark stamen insertion sites. See Figure S1 for comparison between different developmental ages of the same species

3.3.1 | Cell number

At 4 d, the cell number along the lengths of *M. verbenaceus* and *M. lewisii* corolla tubes is 242 ± 13.22 and 226 ± 18.12 (mean $\pm$ SD; $n = 5$), respectively (Figure S2). For both species, cell number increases by ~40%–45% from 4 d to 12 d but does not change significantly after this sampling time (Figures 5 and S2). In contrast, the tubes of *M. parishii* at 4 d have fewer cells than the larger flowered species and cell number does not change from 4 d to anthesis (Figures 5 and S2), indicating that cell division is already completed in the corolla tube of *M. parishii* at this stage.

3.3.2 | Cell size

At 4 d, the corolla tube cell lengths of *M. verbenaceus* and *M. lewisii* do not appear to differ and are relatively uniform along the length of the tube (Figure 5). In contrast, the pattern of cell size variation for *M. parishii* at 4 d is similar to that of the mature flowers, with small cells at the base, the longest cells in the region of stamen insertion, and decreased length distally, indicating that the 4 d corolla of *M. parishii* is developmentally more advanced than that of the other two species. These patterns persist through 8 d; cell elongation was relatively uniform along the length of the tube for *M. lewisii* and *M. verbenaceus*, while for *M. parishii,* more cell elongation occurred near the stamen insertion site than other regions of the tubes. At 12 d, *M. parishii* flowers reached anthesis and growth ceased; cell elongation was differentially enhanced in the region of the stamen insertion for *M. lewisii*, but remained relatively uniform along the length of corolla tubes of *M. verbenaceus*, which started to show preferential elongation near the stamen insertion site at 19 d (Figure S1). For all species, cell elongation continues throughout development, but most of the elongation occurs during the interval just before anthesis (Figure S1).

3.4 | Early flower morphogenesis

We designated floral buds of 2 mm tube length as day 0 for practical reasons. However, the cell division and elongation patterns (Figure 5) suggest that *M. parishii* flowers may have already reached a more advanced developmental stage than the other two species at day 0. To test this hypothesis, we studied early flower morphogenesis using scanning electron microscopy. Overall early flower morphogenesis is similar for the three species. The five petals are initiated as separate primordia (Figure 6a–c). The stamen primordia are antipetalous except that there is no evidence of initiation in the dorsal most position. The central gynoecium consists of two fused carpels. Following the initiation of all floral organs, the corolla tube forms congenitally as the flanks of petal primordia spread laterally across the inter-primordial region (Figure 6d–f), followed by elongation of the nascent tube both above and below this region (the stamen insertion site; Figure 6g-i). When corollas are ~1 mm long, differences among developing flowers are apparent (Figure 6j–l). Although the gynoecia of all three species appear to be similar in size at this stage, the anthers of *M. parishii* flowers are smaller and the filaments are longer compared to the other species (Figure 6j–l). When corollas are ~2 mm in length (Figure 6m-o), the style of *M. parishii* becomes distinct from the ovary, and the stamen filaments are nearly as long as the ovary. That is, morphogenesis in both the stamens (anther vs. filament) and gynoecia (style vs. ovary) is more advanced in *M. parishii* than the other two species, indicating that flowers of *M. parishii* are indeed in a more advanced developmental stage than those of *M. verbenaceus* and *M. lewisii* at day 0. Developing flowers of *M. verbenaceus* and *M. lewisii* are indistinguishable from one another at these early developmental stages.

4 | DISCUSSION

The *M. lewisii* species complex is emerging as a powerful model system to study the developmental genetics and evo-devo of pollinator-associated floral traits (Yuan, 2019), including the formation and elaboration of corolla tubes (Ding et al., 2017, 2020). Yet, little is known about the developmental dynamics of corolla tube growth in these species. In this study, we characterized the patterns of cell division and elongation during corolla tube growth in three closely related species that have distinct pollination syndromes. We found that in all three species, cell length is non-uniformly distributed along the length of the mature tube, with the longest cells just above the stamen insertion site, and that variation in both cell division and cell elongation contribute to differences in corolla tube length among species. These results not only highlight the developmental process associated with corolla tube variation among species but also provide a baseline reference for future developmental genetic analyses of mutants or transgenic plants with altered corolla tube morphology in this emerging model system.

Differences in corolla tube length among the three *Mimulus* species are not associated with processes of organogenesis and early development; tube formation begins when buds of all species are 0.4–0.5 mm in diameter (Figure 6). Rather, differences are associated with variation in multiple processes occurring later in development. The rate and duration of overall tube growth, as well as the location and duration of cell division and cell elongation, all differ among the species. The hummingbird-pollinated *M. verbenaceus* has the longest corolla tube among the three species, yet early tube growth is far slower. In addition to slightly more cells of greater length, it is the longer duration of growth that ultimately results in the greater tube length of *M. verbenaceus* at maturity (Figure 4). Conversely, the self-pollinated *M. parishii* has the shortest corolla tubes, with substantially reduced duration of growth and fewer cells that are shorter (except in the region below the stamen insertion point) compared to the outcrossing species (Figures 3 and 4).

The evolution of small flower size in self-pollinating species is one of the most common evolutionary transitions in flowering plants (Barrett, 2002) and has been associated with heterochrony, changes in the relative rate and/or timing of developmental events (e.g., Diggle, 1992; Kostyun et al., 2017; Li & Johnston, 2000). Our data are consistent with a progenetic origin (precocious termination of development with no change in rate; sensu Alberch et al., 1979) of the small flowers of *M. parishii*. The overall morphology of *M. parishii* flowers is strikingly similar to that of the bee-pollinated *M. lewisii* (Figure 1), which most likely represents the ancestral state of the entire species complex (Nelson et al., 2020). The tube growth curves of *M. parishii* and *M. lewisii* are essentially indistinguishable, except that *M. parishii* tubes stop growing earlier at a smaller size (Figure 4). The SEM analysis shows that sexual maturation of *M. parishii* flowers likely begins far earlier than for *M. lewisii*. When corolla tubes are 1 mm in length, development of the stamens and ovary of *M. parishii* appear advanced relative to the other species. This precocious sexual maturation may trigger the early truncation of corolla tube growth, a pattern consistent with the heterochronic pattern of progenesis. A progenetic origin of small self-pollinating flowers is associated with the evolution of shorter life cycles in environments where the growing season is limited, for example by drought. The decreased duration in flower development may contribute to earlier seed-set before the onset of conditions unfavorable for growth (Diggle, 1992; Runions & Geber, 2000). Plants of *M. parishii* grow near ephemeral streams and desert environments (Fishman et al., 2015) and may have experienced selection for rapid

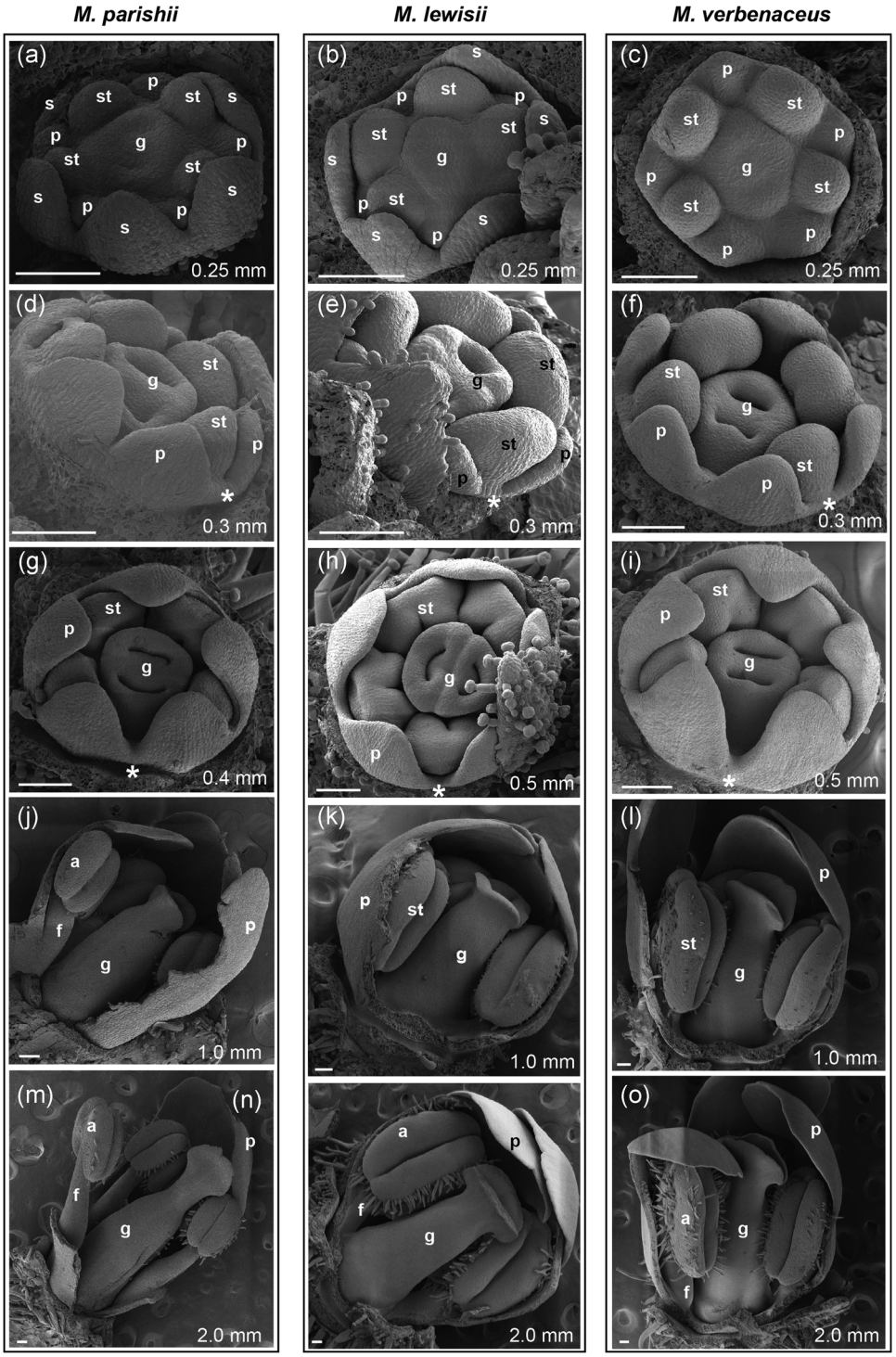

FIGURE 6 Scanning electron micrographs of early flower development. (a–c) Initiation of floral organs. (d–f) Lateral extension of the flanks of the petal into the inter-primordial region (indicated by the asterisks). (g–i) Synchronized growth of the petal primordium base and the inter-primordial region leading to corolla tube formation. (j–l) Differentiation of stamen into anther and filament. (m–o) Buds of 2 mm corolla tube length. Calyces and ventral petals (j–o) have been removed to reveal the inner organ whorls. Left column: *M. parishii*; center: *M. lewisii*; right: *M. verbenaceus*. Diameter of the corolla is marked on the bottom right of each image. a, anther, f, filament, g, gynoecium, p, petal, s, sepal, st, stamen. Scale bars = 100 μm

reproduction. Small self-pollinating flowers of *Solanum pimpinellifolium* (Georgiady & Lord, 2002) and *Capsella rubella* (Sicard et al., 2011) similarly appear to be progenetic via precocious maturation, whereas those of *Mimulus micranthus* (Fenster et al., 1995) and *Clarkia xantiana* ssp *parviflora* (Runions & Geber, 2000) have truncated development as well as increased growth rate suggesting that multiple developmental changes can underlie apparently progenetic morphology.

Along much of the corolla tube, the cells of *M. parishii* are conspicuously shorter than those of the other two species; however, the region below the stamen insertion site is an exception (Figure 3). This observation suggests that the portions below versus above the stamen insertion site (i.e., "lower vs. upper corolla tube"; Boke, 1948; Nishino, 1978) may be distinct developmental modules. Whereas in our study system the upper corolla tube is the major contributor to corolla tube length variation among species (Figure 3), in *Petunia*, the corolla tube cells below the stamen insertion site are longer than those above and the dramatic corolla tube length variation between the hawkmoth-pollinated *Petunia axillaris parodii* and the bee-pollinated *P. integrifolia inflata* is almost entirely attributed to the region below the stamen insertion site (i.e., lower corolla tube; Stuurman et al., 2004). These contrasting patterns of variation in the upper and lower corolla tube are consistent with independent regulation of the two regions, and a comparison of *Mimulus* and *Petunia* suggests that evolutionary divergence in corolla tube length can be mediated through either developmental module, or possibly a combination of both.

The distinctive pattern of cell length variation documented in *Mimulus* arises relatively late in the development of the two larger flowered species. The cells are relatively uniform in length at the 4 d stage, and remain so until differential elongation begins between 8 and 12 d (50%–75% of the total duration of development and 16%–41% of mature size) for *M. lewisii* and between 12 and 19 d (52%–83% of the total duration of development and 19%–52% of mature size) for *M. verbenaceus* (Figures 5 and S1). Similarly, in *Petunia axillaris parodii* and *P. integrifolia inflata*, cell length is uniform at early stages (when tubes are about 50% and 20% of final length, respectively; Stuurman et al., 2004). However, the timing of differential elongation is not known for these species. In *Petunia hybrida,* cell length is also uniform until flowers are approximately 25%–50% of their final length when cells in the tube begin to elongate; greater elongation occurs in the basal than distal region (the stamen insertion site was not specified; Reale et al., 2002). The differential elongation observed in *Mimulus* and *Petunia* is also consistent with putative upper and lower corolla tube modules, and suggests that the stamen may be a source of growth signals (e.g., auxin, gibberellic acid; Greyson & Tepfer, 1967; Song et al., 2013; Weiss et al., 1995) that forms a proximodistal gradient and induces cell elongation when its concentration is higher than a threshold.

The growth of all land plant lateral organs is associated with both cell division and cell expansion. These processes have been described for leaves as largely sequential; during early development, cell division predominates, followed by a shift from cell division to cell expansion. The transition moves as a wave from one end of the organ to the other (Walcher-Chevillet & Kramer, 2016) and the arrest of cell division in leaves occurs before they are 10% of their final size (Lenhard & Czesnick, 2015). Far less is known about these processes in petal development in general and corolla tube development in particular. Our data suggest that cell division and expansion occur simultaneously and that cell division persists in corolla tubes of the large-flowered species later than reported for leaves. Total cell number continues to increase beyond 8 d, arresting before 12 d in both *M. lewisii* (~40% of mature size) and *M. verbenaceus* (20% of mature size). Moreover, the number of cells both below and above the stamen insertion site increases between 8 and 12 d (Figures 5 and S1), suggesting that cell division may be diffuse along the length of the tube. Cell expansion does occur during the early stages of corolla tube development, albeit relatively slowly, and only becomes rapid and highly regional (i.e., distal to the stamen insertion site) close to anthesis. In contrast to the large-flowered *Mimulus* species, corolla tube cell numbers of *M. parishii* were established before our first sampling stage (4 d) when corolla tubes were 3 mm long, 27% of their final size, and the cells in the stamen insertion region were already slightly longer than more proximal and distal regions (Figure 5), indicating that arrest of the cell cycle and initiation of regional cell elongation occurred earlier in the small-flowered species.

Because of its obvious importance in flower-pollinator interactions, corolla tube length variation has been subjected to quantitative trait locus (QTL) mapping analyses in several plant lineages, including *Mimulus* (Bradshaw et al., 1998; Fishman et al., 2015), *Petunia* (Stuurman et al., 2004), *Leptosiphon* (Goodwillie et al., 2006), *Ipomopsis* (Nakazato et al., 2013), *Penstemon* (Wessinger et al., 2014), and *Jaltomata* (Kostyun et al., 2019). These studies show that tube length is clearly a polygenic trait. However, no causal genes have been identified for any of the QTLs. Given the recent success of identifying causal genes underlying natural flower color variation in the *M. lewisii* species complex through fine-scale genetic mapping and transgenic validation (Yuan et al., 2013, 2016), and the dramatic difference in corolla tube length between *M. parishii* and its outcrossing relatives, these species represent a promising experimental system to dissect the genetic basis of corolla tube length variation. The patterns of variation in the duration of growth as well as cell number and cell length during corolla tube growth, as reported in this study but rarely characterized in other plant

systems, will be critical information to link allelic variation in gene sequences to morphological differences between species. Furthermore, many chemically induced mutants with altered corolla tube attributes in both *M. lewisii* and *M. verbenaceus* are being characterized to understand the molecular basis of corolla tube development (Yuan, 2019). The results reported here will provide the baseline reference to facilitate interpretation of the various mutant phenotypes and to help infer gene function in corolla tube development.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

AUTHOR CONTRIBUTIONS

Designed the research: Vandana Gurung, Pamela K. Diggle, and Yao-Wu Yuan. *Implemented the research, analyzed the data, and designed the figures*: Vandana Gurung. All authors contributed to the writing of the manuscript.

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REFERENCES

Alberch, P., Gould, S. J., Oster, G. F., & Wake, D. B. (1979). Size and shape in ontogeny and phylogeny. *Paleobiology*, *5*(3), 296–317. https://doi.org/10.1017/s0094837300006588

Armbruster, W. S., Di Stilio, V. S., Tuxill, J. D., Flores, T. C., & Velásquez Runk, J. L. (1999). Covariance and decoupling of floral and vegetative traits in nine neotropical plants: A re-evaluation of Berg's correlation-pleiades concept. *American Journal of Botany*, *86*(1), 39–55. https://doi.org/10.2307/2656953

Barrett, S. C. H. (2002). The evolution of plant sexual diversity. *Nature Reviews Genetics*, *3*(4), 274–284. https://doi.org/10.1038/nrg776

Barrett, S. C. H. (2010). Understanding plant reproductive diversity. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, *365*(1537), 99–109. https://doi.org/10.1098/rstb.2009.0199

Beardsley, P. M., Yen, A., & Olmstead, R. G. (2003). AFLP phylogeny of *Mimulus* section *Erythranthe* and the evolution of hummingbird pollination. *Evolution*, *57*(6), 1397–1410.

Berlyn, G. P., & Miksche, J. P. (1976). *Botanical microtechnique and cytochemistry*. Iowa State University Press.

Boke, N. H. (1948). Development of the perianth in *Vinca rosea* L. *American Journal of Botany*, *35*(7), 413–423.

Box, M. S., Dodsworth, S., Rudall, P. J., Bateman, R. M., & Glover, B. J. (2011). Characterization of *Linaria* KNOX genes suggests a role in petal-spur development. *Plant Journal*, *68*(4), 703–714. https://doi.org/10.1111/j.1365-313X.2011.04721.x

Bradshaw, H. D., Otto, K. G., Frewen, B. E., McKay, J. K., & Schemske, D. W. (1998). Quantitative trait loci affecting differences in floral morphology between two species of monkeyflower (*Mimulus*). *Genetics*, *149*(1), 367–382.

Cullen, E., Fernández-Mazuecos, M., & Glover, B. J. (2018). Evolution of nectar spur length in a clade of *Linaria* reflects changes in cell division rather than in cell expansion. *Annals of Botany*, *122*(5), 801–809. https://doi.org/10.1093/aob/mcx213

Diggle, P. K. (1992). Development and the evolution of plant reproductive characters. In R. Wyatt (Ed.), *Ecology and evolution of plant reproduction: New approaches* (pp. 326–355). Chapman and Hall.

Ding, B., Mou, F., Sun, W., Chen, S., Peng, F., Bradshaw, H. D., & Yuan, Y. W. (2017). A dominant-negative actin mutation alters corolla tube width and pollinator visitation in *Mimulus lewisii*. *New Phytologist*, *213*(4), 1936–1944. https://doi.org/10.1111/nph.14281

Ding, B., Xia, R., Lin, Q., Gurung, V., Sagawa, J. M., Stanley, L. E., Strobel, M., Diggle, P. K., Meyers, B. C., & Yuan, Y.-W. (2020). Developmental genetics of corolla tube formation: role of the tasiRNA-ARF pathway and a conceptual model. *The Plant Cell*, *32*, 3452–3468. https://doi.org/10.1105/tpc.18.00471

Endress, P. K. (2001). Origins of flower morphology. *Journal of Experimental Zoology*, *291*, 105–115. https://doi.org/10.1002/jez.1063

Erbar, C. (1991). Sympetaly—A systematic character. *Botanische Jahrbücher für Systematik, Pflanzengeschichte und Pflanzengeographie*, *112*, 417–451.

Erbar, C., & Leins, P. (1996). Distribution of the character states "Early sympetaly" and "Late sympetaly" within the "Sympetalae Tetracydicae" and presumably allied groups*. *Botanica Acta*, *109*(5), 427–440. https://doi.org/10.1111/j.1438-8677.1996.tb00593.x

Erbar, C., & Leins, P. (2004). Sympetaly in Apiales (Apiaceae, Araliaceae, Pittosporaceae). *South African Journal of Botany*, *70*(3), 458–467. https://doi.org/10.1016/S0254-6299(15)30230-1

Fenster, C. B., Armbruster, W. S., Wilson, P., Dudash, M. R., & Thomson, J. D. (2004). Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics*, *35*, 375–403. https://doi.org/10.1146/annurev.ecolsys.34.011802.132347

Fenster, C. B., Diggle, P. K., Barrett, S. C. H., & Ritland, K. (1995). The genetics of floral development differentiating two species of *Mimulus* (Scrophulariaceae). *Heredity*, *74*, 258–266. https://doi.org/10.1038/hdy.1995.39

Fishman, L., Beardsley, P. M., Stathos, A., Williams, C. F., & Hill, J. P. (2015). The genetic architecture of traits associated with the evolution of self-pollination in *Mimulus*. *New Phytologist*, *205*(2), 907–917. https://doi.org/10.1111/nph.13091

Fox, J., & Weisberg, S. (2018). *An R companion to applied regression* (3rd ed.). Sage.

Georgiady, M. S., & Lord, E. M. (2002). Evolution of the inbred flower form in the currant tomato, *Lycopersicon pimpinellifolium*.

International Journal of Plant Sciences, *163*(4), 531–541. https://doi.org/10.1086/340542

Goodwillie, C., Ritland, C., & Ritland, K. (2006). The genetic basis of floral traits associated with mating system evolution in *Leptosiphon* (Polemoniaceae): An analysis of quantitative trait loci. *Evolution*, *60*(3), 491–504. https://doi.org/10.1554/05-471.1

Greyson, R. I., & Tepfer, S. S. (1967). Emasculation effects on the stamen filament of *Nigella Hispanica* and their partial reversal by Gibberellic acid. *American Journal of Botany*, *54*(8), 971–976. https://doi.org/10.1002/j.1537-2197.1967.tb10722.x

Guerrant, E. O. (1982). Neotenic evolution of *Delphinium nudicaule* (Ranunculaceae): A hummingbird-pollinated larkspur. *Evolution*, *36*(4), 699–712.

Hiesey, W. M., Nobs, M. A., & Bjorkman, O. (1971). Experimental studies on the nature of species. Biosystematics, genetics, and physiological ecology of the *Erythranthe* Section of *Mimulus*. *Carnegie Institute of Washington Publications*, *628*, 1–213.

Johnson, S. D., & Steiner, K. E. (1997). Long-tongued fly pollination and evolution of floral spur length in the *Disa draconis* Complex (Orchidaceae). *Evolution*, *51*(1), 45–53.

Kostyun, J. L., Gibson, M. J. S., King, C. M., & Moyle, L. C. (2019). A simple genetic architecture and low constraint allow rapid floral evolution in a diverse and recently radiating plant genus. *New Phytologist*, *223*(2), 1009–1022. https://doi.org/10.1111/nph.15844

Kostyun, J. L., Preston, J. C., & Moyle, L. C. (2017). Heterochronic developmental shifts underlie floral diversity within *Jaltomata* (Solanaceae). *EvoDevo*, *8*, 17. https://doi.org/10.1186/s13227-017-0080-z

Landis, J. B., O'Toole, R. D., Ventura, K. L., Gitzendanner, M. A., Oppenheimer, D. G., Soltis, D. E., & Soltis, P. S. (2016). The phenotypic and genetic underpinnings of flower size in Polemoniaceae. *Frontiers in Plant Science*, *6*, 1144. https://doi.org/10.3389/fpls.2015.01144

Lenhard, M., & Czesnick, H. (2015). Size control in plants—Lessons from leaves. *Cold Spring Harbor Perspectives in Biology*, *7*, a019190. https://doi.org/10.1101/cshperspect.a019190

Li, P., & Johnston, M. O. (2000). Heterochrony in plant evolutionary studies through the twentieth century. *Botanical Review*, *66*(1), 57–88. https://doi.org/10.1007/BF02857782

Mack, J. L. K., & Davis, A. R. (2015). The relationship between cell division and elongation during development of the nectar-yielding petal spur in *Centranthus ruber* (Valerianaceae). *Annals of Botany*, *115*(4), 641–649. https://doi.org/10.1093/aob/mcu261

Martin, C., & Gerats, T. (1993). Control of pigment biosynthesis genes during petal development. *The Plant Cell*, *5*(10), 1253–1264. https://doi.org/10.1105/tpc.5.10.1253

Nakazato, T., Rieseberg, L. H., & Wood, T. E. (2013). The genetic basis of speciation in the Giliopsis lineage of *Ipomopsis* (Polemoniaceae). *Heredity*, *111*, 227–237. https://doi.org/10.1038/hdy.2013.41

Nelson, T. C., Stathos, A. M., Vanderpool, D. D., Finset, F. R., & Yuan, Y. (2020). Ancient and recent introgression shape the evolutionary history of pollinator adaptation and speciation in a model monkeyflower radiation (*Mimulus* section *Erythranthe*). *BioRxiv*, 287151. https://doi.org/10.1101/2020.09.08.287151

Nilsson, L. A. (1988). The evolution of flowers with deep corolla tubes. *Nature*, *334*(6178), 147–149. https://doi.org/10.1038/334147a0

Nishino, E. (1978). Corolla tube formation in four species of Solanaceae. *The Botanical Magazine Tokyo*, *91*, 263–277. https://doi.org/10.1007/BF02488940

Owen, C. R., & Bradshaw, H. D. (2011). Induced mutations affecting pollinator choice in *Mimulus lewisii* (Phrymaceae). *Arthropod-Plant Interactions*, *5*(3), 235–244. https://doi.org/10.1007/s11829-011-9133-8

Puzey, J. R., Gerbode, S. J., Hodges, S. A., Kramer, E. M., & Mahadevan, L. (2012). Evolution of spur-length diversity in *Aquilegia* petals is achieved solely through cell-shape anisotropy. *Proceedings of the Royal Society B: Biological Sciences*, *279*(1733), 1640–1645. https://doi.org/10.1098/rspb.2011.1873

Reale, L., Porceddu, A., Lanfaloni, L., Moretti, C., Zenoni, S., Pezzotti, M., Romano, B., & Ferranti, F. (2002). Patterns of cell division and expansion in developing petals of *Petunia hybrida*. *Sexual Plant Reproduction*, *15*(3), 123–132. https://doi.org/10.1007/s00497-002-0150-8

Runions, C. J., & Geber, M. A. (2000). Evolution of the self-pollinating flower in *Clarkia xantiana* (Onagraceae). I. Size and development of floral organs. *American Journal of Botany*, *87*(10), 1439–1451. https://doi.org/10.2307/2656870

Schemske, D. W., & Bradshaw, H. D. (1999). Pollinator preference and the evolution of floral traits in monkeyflowers (*Mimulus*). *Proceedings of the National Academy of Sciences of the United States of America*, *96*(21), 11910–11915. https://doi.org/10.1073/pnas.96.21.11910

Sicard, A., Stacey, N., Hermann, K., Dessoly, J., Neuffer, B., Bäurle, I., & Lenhard, M. (2011). Genetics, evolution, and adaptive significance of the selfing syndrome in the genus *Capsella*. *The Plant Cell*, *23*, 3156–3171. https://doi.org/10.1105/tpc.111.088237

Song, S., Qi, T., Huang, H., & Xie, D. (2013). Regulation of stamen development by coordinated actions of jasmonate, auxin, and gibberellin in *Arabidopsis*. *Molecular Plant*, *6*(4), 1065–1073. https://doi.org/10.1093/mp/sst054

Stanley, L. E., Ding, B., Sun, W., Mou, F., Hill, C., Chen, S., & Yuan, Y. W. (2020). A tetratricopeptide repeat protein regulates carotenoid biosynthesis and chromoplast development in monkeyflowers (*Mimulus*). *The Plant Cell*, *32*(5), 1536–1555. https://doi.org/10.1105/tpc.19.00755

Stuurman, J., Hoballah, M. E., Broger, L., Moore, J., Basten, C., & Kuhlemeier, C. (2004). Dissection of floral pollination syndromes in *Petunia*. *Genetics*, *168*(3), 1585–1599. https://doi.org/10.1534/genetics.104.031138

Tsai, T., Diggle, P. K., Frye, H. A., & Jones, C. S. (2018). Contrasting lengths of *Pelargonium* floral nectar tubes result from late differences in rate and duration of growth. *Annals of Botany*, *121*(3), 549–560. https://doi.org/10.1093/aob/mcx171

Verbeke, J. A. (1992). Fusion events during floral morphogenesis. *Annual Review of Plant Physiology and Plant Molecular Biology*, *43*(1), 583–598. https://doi.org/10.1146/annurev.pp.43.060192.003055

Vickery, R. K. J., & Wullstein, B. (1987). Comparison of six approaches to the classification of *Mimulus* Sect. *Erythranthe* (Scrophulariaceae). *Systematic Botany*, *12*(3), 339–364.

Walcher-Chevillet, C. L., & Kramer, E. M. (2016). Breaking the mold: Understanding the evolution and development of lateral organs in diverse plant models. *Current Opinion in*

Genetics and Development, *39*, 79–84. https://doi.org/10.1016/j.gde.2016.06.005

Waser, N. M., Chittka, L., Price, M. V., Williams, N. M., & Ollerton, J. (1996). Generalization in pollination systems, and why it matters. *Ecology*, *77*(4), 1043–1060. https://doi.org/10.2307/2265575

Weberling, F. (1989). *Morphology of flowers and inflorescences*. Cambridge University Press.

Weiss, D., van der Luit, A., Knegt, E., Vermeer, E., Mol, J. N. M., & Kooter, J. M. (1995). Identification of endogenous gibberellins in *Petunia* flowers: lnduction of anthocyanin biosynthetic gene expression and the antagonistic effect of abscisic acid. *Plant Physiology*, *107*, 695–702. https://doi.org/10.1104/pp.107.3.695

Wessinger, C. A., Hileman, L. C., & Rausher, M. D. (2014). Identification of major quantitative trait loci underlying floral pollination syndrome divergence in *Penstemon*. *Philosophical Transactions of the Royal Society, B: Biological Sciences*, *369*(1648), 20130349. https://doi.org/10.1098/rstb.2013.0349

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag. https://ggplot2.tidyverse.org

Yuan, Y. W. (2019). Monkeyflowers (*Mimulus*): New model for plant developmental genetics and evo-devo. *New Phytologist*, *222*(2), 694–700. https://doi.org/10.1111/nph.15560

Yuan, Y. W., Rebocho, A. B., Sagawa, J. M., Stanley, L. E., & Bradshaw, H. D. (2016). Competition between anthocyanin and flavonol biosynthesis produces spatial pattern variation of floral pigments between *Mimulus* species. *Proceedings of the National Academy of Sciences of the United States of America*, *113*(9), 2448–2453. https://doi.org/10.1073/pnas.1515294113

Yuan, Y. W., Sagawa, J. M., Young, R. C., Christensen, B. J., & Bradshaw, H. D. (2013). Genetic dissection of a major anthocyanin QTL contributing to pollinator-mediated reproductive isolation between sister species of *Mimulus*. *Genetics*, *194*(1), 255–263. https://doi.org/10.1534/genetics.112.146852

Zhong, J., & Preston, J. C. (2015). Bridging the gaps: Evolution and development of perianth fusion. *New Phytologist*, *208*(2), 330–335. https://doi.org/10.1111/nph.13517

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