Original article

Evolution of nectar spur length in a clade of *Linaria* reflects changes in cell division rather than in cell expansion

Cullen, E[1], Fernández-Mazuecos, M[1,2], Glover, BJ[1*]

[1]Department of Plant Sciences, University of Cambridge, Downing St, Cambridge CB2 3EA, UK

[2]Real Jardín Botánico (RJB-CSIC), Plaza de Murillo 2, Madrid 28014, Spain

*Email of corresponding author: bjg26@cam.ac.uk

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ABSTRACT

Background and aims

Nectar spurs (tubular outgrowths of a floral organ which contain or give the appearance of containing nectar) are hypothesized to be a 'key innovation' which can lead to rapid speciation within a lineage, because they are involved in pollinator specificity. Despite the ecological importance of nectar spurs, relatively little is known about their development. We used a comparative approach to investigate variation in nectar spur length in a clade of eight Iberian toadflaxes.

Methods

Spur growth was measured at the macroscopic level over time in all eight species, and growth rate and growth duration compared. Evolution of growth rate was reconstructed across the phylogeny. Within the clade we then focused on *Linaria becerrae* and *Linaria clementei*, a pair of sister species which have extremely long and short spurs, respectively. Characterisation at a micromorphological level was performed across a range of key developmental stages to determine whether the difference in spur length is due to differential cell expansion or cell division.

Key results

We detected a significant difference in the evolved growth rates, while developmental timing of both the initiation and the end of spur growth remained similar. Cell number is 3 times higher in the long spurred *Linaria becerrae* compared to *Linaria clementei*, whereas cell length is only 1.3 times greater. In addition, overall anisotropy of mature cells is not significantly different between the two species.

Conclusions

We found that changes in cell number and therefore in cell division largely explain evolution of spur length. This contrasts with previous studies in *Aquilegia* which have found that variation in nectar spur length is due to directed cell expansion (anisotropy) over variable timeframes. Our study adds to knowledge about nectar spur development in a comparative context and indicates that different systems may have evolved nectar spurs using disparate mechanisms.

Key words: anisotropy, cell division, cell expansion, evo-devo, *Linaria becerrae*, *Linaria clementei*, nectar spur

INTRODUCTION

The ability to vary floral traits has been key to the success and enormous speciation of the flowering plants (angiosperms). One such floral innovation is the nectar spur, a tubular outgrowth of a floral organ (petal or sepal) that contains, or gives the appearance of containing, nectar. Nectar spurs protect nectar from the environment and also enhance pollinator specificity, pollination efficiency and reproductive success (Pacini *et al.* 2003). Spurs have arisen in a wide variety of taxa, including nasturtium (Tropaeolaceae), *Aquilegia* (Ranunculaceae), many orchids (Orchidaceae) and *Linaria* (Plantaginaceae) (Hodges, 1997). However, there are substantial differences between the systems. In *Aquilegia* spurs are present on each petal, and the nectary is situated within the spur. In contrast, in *Linaria* there is only a single spur on the ventral petal, and the gynoecial disc nectary is located above the spur. This study exploits the natural variation of spur length present within the genus *Linaria* to examine the mechanistic basis for interspecific differences in spur length.

A nectar spur restricts nectar collection to specific pollinators with appropriate feeding apparatus, thereby acting to reproductively isolate plants and drive speciation. This has led to spurs being described as a 'key innovation' (Hodges and Arnold 1995; Hodges, 1997; Box et al. 2008; Bell et al. 2009). Indeed, the study of nectar spurs allows us to make inferences about the mechanisms of speciation and evolution (Bateman and Sexton 2008; Fernández-Mazuecos and Glover 2017). Darwin explained the extreme length of the *Angraecum sesquipedale* nectar spur using the 'coevolutionary race model', where both the plant and pollinator are under reciprocal selective pressure for longer spurs or longer tongues. In the case of the plant a longer spur improves the fit of the pollinator body to the flower and therefore the transfer of pollen (reproductive success), whereas in the case of the pollinator a longer tongue improves access to nectar and overall fitness. Conversely, the 'pollinator shift' model may also explain nectar spur evolution, where the plant evolves spurs better suited to

pollinators that have already adapted to other plants (Whittall and Hodges 2007). In these cases, nectar spurs can be part of a pollination syndrome – a combination of adaptations shown by a plant to a group of animals, and by that group of animals to the plant. In addition, the study of nectar spurs allows us to address evolutionary developmental (evo-devo) questions spanning the plant and animal kingdoms; for example, the extent and importance of heterochrony (when a change in the timing of a developmental process occurs). There are two main categories of heterochrony: paedomorphosis, which is where a species appears juvenilised in comparison with an ancestral species, and peramorphosis, where a species matures past adulthood to develop an extended version of a trait (Gould, 1977; Alberch et al., 1979). Extrapolating this logic, shorter spurs could be generated via paedomorphosis, and longer spurs via peramorphosis (Box and Glover 2010).

The modification of plant form in non-model plant species is currently of great interest. The study of spurs also allows us to examine how organ outgrowth can occur from a planar surface (Monniaux and Hay 2016). Organ outgrowth in plants requires the interplay of genetic and mechanical forces (Rebocho *et al.* 2017). First, cell division is required, which is followed by cell expansion (Teale *et al.* 2006). Once cell division has taken place, plant cells remain fixed in place. It is the cell wall that remains plastic and allows further growth to occur (Cosgrove, 2005; Dupuy *et al.* 2016). In order for directed cell expansion (anisotropy) to occur, stress occurs in the cell walls and microtubules direct cellulose synthase enzymes in the direction of cell growth (Braybrook and Jönsson 2016). Growth hormones such as auxins and cytokinins are involved in cell division and expansion, so it is likely they are also involved in spur development (Yant *et al.* 2015).

Studies in species of both *Aquilegia* and *Linaria* have provided some insight into how nectar spurs develop. There is cell division followed by cell elongation in both species. However, the importance of each phase and whether variation in spur length is achieved by varying cell

division or cell elongation is debated. Correlative evidence indicates that cell division is the more important phase in *L. vulgaris* and several orchid species (Bateman & Sexton 2008; Box *et al.* 2008; Box *et al*. 2011). However, research in *Aquilegia* indicates that nectar spur development may be largely due to anisotropic (directional) cell elongation, with more anisotropic growth occurring in longer spurred species (Puzey *et al.* 2012). Data from Mack and Davies (2015) on *Centranthus ruber* (Red Valerian) also indicates that nectar spur development is due to anisotropy. Given that these are different systems in which nectar spurs have evolved independently, it is possible that nectar spur development and interspecific variation are driven by different mechanisms in each system.

To analyse the natural variation in spur length among toadflax species we examined the Iberian clade of *Linaria* subsect. *Versicolores*, which contains eight species with contrasting spur lengths. We focused at a micromorphological level on *Linaria clementei* and *L. becerrae* (fig. 1) – sister species which have extremely short and long spurs, respectively – to probe how two species that are so closely related can acquire such dramatically different spur lengths.

MATERIALS AND METHODS

Study species

To analyse the natural variation in spur length amongst toadflax species we examined the Iberian clade of *Linaria* subsect. *Versicolores*, containing eight species: *Linaria algarviana* Chav., *Linaria clementei* Haens., *Linaria incarnata* (Vent.) Spreng., *Linaria onubensis* Pau, *Linaria becerrae* Blanca, Cueto & J. Fuentes, *Linaria spartea* (L.) Chaz., *Linaria salzmannii* Boiss., *Linaria viscosa* (L.) Chaz. (Fernández-Mazuecos *et al*. 2013; Blanca *et al*. 2017).

There now exist relatively well-resolved phylogenies for the Antirrhineae, including *Linaria* (Oyama and Baum 2004; Guzmán *et al*., 2015), and the detailed phylogeny of this particular eight-species *Linaria* clade has recently been investigated (fig. 1B) (Fernández-Mazuecos *et al.* 2017). This recent phylogenetic analysis used genome-wide DNA sequences generated by genotyping by sequencing, and identified *L. clementei*, with the shortest spur in the group, as sister to *L. becerrae*, with one of the longest spurs. It is also known that the clade diversified very recently, within the Quaternary (Fernández-Mazuecos *et al*., 2013).

Plant growth conditions

Plants were grown from seeds collected in wild populations (see supplementary table 1). Glasshouse conditions were maintained at 18-25°C, with 16-18hr daylight, depending on the month when the plants were grown. Plants were grown in Levington's M3 (UK) compost at the Department of Plant Sciences, or at the Plant Growth Facility at the University of Cambridge, UK.

Images of spur growth captured over 13 consecutive days

A Dino-Lite digital microscope (Am400/AD4000 series, AM4113T(R4)) was used to take *in vivo* images of developing spurs for 13 consecutive days. A lateral view of the spur was taken. Five replicates of each species were taken, from two or three biological replicates. Spurs were measured from the calyx-corolla insertion to the tip using ImageJ (Schindelin *et al.* 2012), and growth curves were plotted on linear and logarithmic scales.

Digital microscopy

Appropriate and equivalent developmental stages for *L. becerrae* and *L. clementei* were determined by observing the spur growth curves over 13 days. Five biological replicates from two or three individuals were imaged for each developmental stage (table 1). Material was dissected to ensure it was as flat as possible, then mounted on slides covered with double-

sided sticky tape. Imaging was performed under standard settings with a digital microscope, VHX-5000 (KEYENCE, America).

Image analysis

Image analysis was performed in ImageJ (Schindelin *et al.* 2012). To examine cell length and width, 30 cells were randomly chosen within the field of view. The 30 replicates were imaged at the base, middle and tip of the spur for each developmental stage and biological replicate (apart from developmental stage one spurs, where only 10 replicates were imaged at the base, middle and tip of the spur due to the size of the spur; fig. 4A). Overall cell length and width was then calculated from the average base, middle and tip of the spur. Overall anisotropy was calculated as the ratio of overall length to cell width. To count cell number, multiple high-resolution images were taken along the length of the spur, and then merged in Adobe Photoshop so that cell number could be counted along the length of the spur (fig. 4B). A line was drawn along the length of the spur, and all cells dissected by this line were counted using the 'Cell Counter' ImageJ plug-in.

Statistical analysis

To determine whether there were differences in growth rate between the eight species of *Linaria* used to study the natural variation in spur length, a grouped linear regression was used. Given that the growth curves have the appearance of a sigmoidal curve, with an initial slower growth phase, followed by a steep increase in growth that levels off, it was necessary to determine where the steep increase in growth occurred in each species. For this goal, the 'segmented' function in R was used to find two breakpoints on averaged data for each species (Muggeo, 2008; Lemoine, 2012). This approach divided up each species into three segments, and provided a gradient for each slope. The second segment gave the time points for the main growth phase for each species, and these time points were used in the grouped linear

regression. Each species was compared with *L. becerrae*. An overall ANOVA was used to ascertain that this approach was acceptable, and a significant difference was found ($p<0.001$).

To determine whether there was a significant difference in initiation or end of the spur growth, start (when a spur is first observed) and end (when spur length no longer increases) of spur growth was recorded for each of the five individual replicates. Both the start and end of spur growth were compared using the non-parametric Kruskal-Wallis and *post hoc* Dunn test.

An ancestral state reconstruction of spur growth rate was conducted based on the phylogeny of Fernández-Mazuecos *et al*. (2017). We used the coalescent-based species tree topology obtained using the NJ_{st} method with branch lengths estimated by maximum likelihood (for details see Fernández-Mazuecos *et al*., 2017). The tree was made ultrametric in Mesquite (Maddison and Maddison 2011), and growth rate (averaged over 13 days) was mapped as a continuous character using the maximum likelihood method implemented by the contMap function of the R package *phytools* (Revell, 2012).

A non-parametric Kruskal-Wallis was used to test the influence of developmental stage on cell length and number in *L. clementei* and *L. becerrae*. This was also used to investigate how location on the spur influenced cell length in *L. becerrae* and *L. clementei* across all developmental stages. A non-parametric Mann-Whitney U test was used to compare cell number and cell length in the mature spurs of *L. clementei* and *L. becerrae*. The Kruskal-Wallis and Mann-Whitney U tests were used because the data were not normal and variances were not equal (Dytham, 2010). All statistical analyses were performed in R version 3.2.2.

RESULTS

Evolutionary variation in nectar spur length can largely be attributed to changes in growth rate rather than in developmental timeframe

Spurs of eight closely related *Linaria* species were measured over 13 days to determine whether there were differences in growth (fig. 2). We hypothesised that longer spurred species may start growth earlier than shorter spurred species. There is a significant difference in initiation (X^2 = 20.79; d.f. 7; p<0.001) and end of spur growth (X^2 = 25.1; d.f. 7; p<0.001) among the eight species (see table 2 and 3). However, a *post hoc* Dunn test revealed that although there are discrepancies, there is no significant difference in spur growth initiation or termination between the longest-spurred species, *L. algarviana,* and the species with the shortest spur, *L. clementei* (p>0.05). When comparing the sister species *L. becerrae* and *L. clementei*, there was a significant difference in timing of spur initiation (p<0.05), however there was no difference in when termination of spur growth occurred (p>0.05).

To test whether the growth rate within the growth period determined by the segmented function was different between species (table 4), we used a grouped linear regression comparing species with *L. becerrae*. It determined that *L. clementei* (p<0.001), *L. onubensis* (p<0.01) and *L. salzmannii* (p<0.001) had a significantly different growth rate from *L. becerrae* (the other five species were not significantly different). There was in addition a significant interaction between species and time (p<0.001). As expected, there was also a significant difference between time and spur length (p<0.001). An overall ANOVA confirmed the above results.

To determine the direction of evolutionary change across the clade, particularly between *L. becerrae* and *L. clementei*, evolution of spur growth rate (averaged over 13 days) was

reconstructed and plotted on the phylogeny (fig. 3). The maximum likelihood value for the rate of the common ancestor of *L. becerrae* and *L. clementei* was intermediate between the rates of both species. Although error intervals were broad, there was a well-supported decrease in growth rate in *L. clementei* from that ancestor.

Greater cell division, rather than cell expansion, explains difference in spur length between L. clementei *and* L. becerrae

To determine whether differences in cell elongation or cell division are responsible for contrasting spur lengths, cell number, length and width were measured in nectar spur epidermal cells of both *L. becerrae* and *L. clementei* at five different developmental stages (fig. 4A, B). Cell number was found to differ strikingly between the two species (fig. 4C). Cell number in the *L. becerrae* spur shows a large increase from approximately 60 in stage two to approximately 230 in stage three (representing approximately two rounds of cell division). However, there is little difference in cell length between stages one and two (fig. 4D). Thus, most cell expansion takes place between stage two and the mature spur. Although cell expansion follows the same trend in *L. clementei,* cell number increases more slowly, from 35 at stage two to 40 at stage three; moreover, it increases throughout development, unlike in *L. becerrae*. There is a highly significant difference in cell number in the mature spur at the species level ($W = 73$; $p<0.001$) and at the level of developmental stage ($X^2 = 21.99$; d.f. 4; $p<0.001$) (fig. 4C, D).

The average overall length of a cell at the base of the mature nectar spur of *L. clementei* was 50 μm, and in *L. becerrae* it was 70 μm. These lengths reflected a fairly steady growth rate in both species, from 14 μm in *L. clementei* at stage 1 and 21 μm in *L. becerrae* at stage 1, maximum increase in length occurring between stage four and five for both *L. clementei* and

L. becerrae. Cell length in the mature spur was found to be significantly different between the species (W = 2949; p<0.001) and highly significantly different at contrasting developmental stages (X^2 = 658.95; d.f. 4; p<0.001) (fig. 4D).

Anisotropy does not explain the difference in spur length between L. clementei *and* L. becerrae

In both species there is a trend of cell length and cell width decreasing from the base to the tip of the spur (fig. 5). This differs from cells in *Aquilegia* which become larger towards the tip of the spur (see supplementary fig. 1). Cell length increases steadily in *L. clementei* at the base, middle and tip of the spur (fig. 5A). Cell length in *L. becerrae* shows a different trend; cell length decreasing at the base and middle of the spur from stage one to two indicates that cell division is taking place (fig. 5B). Cell length steadily increases until stage four, and there is then a large increase in cell length from stage four to five. Examination of cell width data in *L. clementei* reveals that mean cell width remains at approximately 14 μm across the base, middle and tip of the spur from stage one to stage four (perhaps as the epidermal cells of *L. clementei* divide through most of the developmental period), and then expansion of cell width occurs from stage four to stage five. *Linaria becerrae* shows a decrease in cell width at the base and middle of the spur, from stage one to stage two, which is again indicative of cell division. Steady growth then occurs across the base, middle and tip of the spur; a large increase in cell width occurs at stage five, which is more marked at the base of the spur. There was no significant difference between cell length and location on the spur (base, middle or tip of the spur) in *L. becerrae* (X^2 = 3.11; d.f. 2; p<0.05), in contrast with *L. clementei* (X^2 = 236; d.f. 2; p<0.001).

Overall cell anisotropy (measured at the base, middle and tip of the spur) at the five different developmental stages was calculated (fig. 6). Cells with equal length and width have an anisotropic value of 1, and therefore even at stage one both *L. becerrae* and *L. clementei* have longitudinally elongated epidermal cells, although the cells of *L. becerrae* are more elongated with an anisotropic value of 2, compared with *L. clementei* which has an anisotropic value of 1.5. The cells of *L. becerrae* maintain the anisotropic value of approximately 2 until stage four and five, when directed cell expansion begins to take place. This contrasts with the data from *L. clementei*, where a slow and steady increase in anisotropy occurs throughout the five developmental stages. Anisotropy in the mature cells was not significantly different between *L. becerrae* and *L. clementei* ($W=3$, $p>0.05$). Therefore anisotropy cannot explain the differences in spur length between the two species. The overall cell length of mature spurs of *L. becerrae* is 1.3 times the length of cells in *L. clementei*. Conversely, cell number is 3 times higher in *L. becerrae* compared with *L. clementei*.

DISCUSSION

The developmental timeframe of spur growth in Linaria *does not vary with spur length*

We hypothesised that the longer-spurred *Linaria* species examined by us would have a longer developmental timeframe. However, we found that although there were some differences in timing of initiation and end of spur growth, the difference was not between the longest- and shortest-spurred species; rather, it was between species with intermediate sized spurs. Although there was a difference in spur initiation time for *L. becerrae* and *L. clementei*, termination of spur growth was not significantly different. In general, it is evident that both initiation and conclusion of spur growth are loosely synchronised among the clade of *Linaria*

species that we studied, including the two sister species *L. becerrae* and *L. clementei*, and that differences in spur length across species are mainly the result of changes in spur growth rate. This outcome contrasts with data from *Aquilegia*. Puzey *et al*. (2012) compared the growth period of four different *Aquilegia* species and found that growth duration differed between the shortest- and longest-spurred species, spur development in the longest spurred species taking six days longer than in the shorter-spurred species. This observation may indicate that *Aquilegia* and *Linaria* spur growth is fundamentally different. Although the *Linaria* data presented here show eight closely related species of varying spur length in the same clade, all pollinated by bees, the *Aquilegia* data show four species that were chosen to represent different pollination syndromes; for example, *Aquilegia vulgaris* is bee pollinated but *A. longissima* is hawkmoth pollinated. It would therefore be interesting to investigate duration of growth in other clades of *Linaria* and other spurred genera within the tribe Antirrhineae (*Kickxia*, *Chaenorhinum*, *Cymbalaria*), to determine whether the same trend is conserved across the tribe.

Cell number is a major factor in evolution of Linaria *spur length*

Spur development can only consist of cell division and/or anisotropic cell elongation (Box *et al.* 2011). Detected interspecific differences in spur growth rate generating length variation could be due to: (1) variation in initial cell divisions and cell number (resulting in faster or slower growth at the same rate of cell elongation); (2) variation in the rate of anisotropic elongation and in final cell size (resulting in faster or slower growth from the same number of cells); or (3) a combination of both. At a micromorphological scale, we observed that although cell length was significantly different between the mature spurs of *L. becerrae* and *L. clementei*, overall cell anisotropy was not significantly different. In addition, there were three times more cells in *L. becerrae* compared with *L. clementei*, whereas cell length was only 1.3 times the length of cells in *L. clementei.* Therefore, the major evolutionary change

explaining the difference in spur growth rate and length between these species (6.71 times longer in *L. becerrae* than in *L. clementei*) appears to be the decreased cell number (and therefore decreased cell division) in *L. clementei* in comparison with *L. becerrae*. This contrasts with observations on *Aquilegia*, in which cell number was only found to vary by 30±21% between the longest and shortest spurs. Puzey *et al*. (2012) found that increases in *Aquilegia* spur length were largely due to anisotropic cell expansion, which increases from the base to the tip of the spur. Mack and Davies (2015) also concluded that anisotropy was largely responsible for spur outgrowth in *Centranthus ruber*, but argued that anisotropic growth occurred equally across the spur. It should be noted that in this study we only measured and counted epidermal cells, and therefore cannot exclude the possibility that the subepidermal cell layers behave differently. Overall, nectar spur outgrowth is a good system for investigating novel organ outgrowth, and the use of modelling may help to give even greater insight into the initial outgrowth of the spur in *Linaria* (Coen and Rebocho 2016; Rebocho *et al.* 2017).

Mechanisms of nectar spur growth may vary in different plant systems

It is important to note that, in addition to the obvious phylogenetic differences, there are differences between the various systems in which nectar spur growth has been studied. *Centranthus* and *Linaria* both possess a single spur per flower, and while a trichomatous nectary within the spur is responsible for nectar secretion in *C. ruber*, in *Linaria* the nectary is situated above a single spur. In *Aquilegia* species, which possess five spurs per pentamerous flower, the nectary is situated within the spur, which may act as an organiser during spur initiation. Therefore, differences such as cell length in *Aquilegia* increasing from the base of the spur to the tip of the spur, while decreasing in *Linaria* from the base to the tip of the spur, may not be surprising.

Heterochrony can help to explain the variation in spur length in different systems. Our reconstruction of the evolution of growth rate indicates that the common ancestor of *L. becerrae* and *L. clementei* was probably intermediate in growth rate, although we note that this is a statistical output based on the traits of the sister species, and that the rest of the clade contains species with long spurs. In any case, it is most likely that a decrease in growth rate occurred in the *L. clementei* lineage relative to its ancestor. Therefore, the shorter spur of *L. clementei* can be explained by neoteny, a category of paedomorphosis when there is no change in the timing of maturity but rather a decrease in the amount of development undergone before maturity is reached (Gould 1977; Box and Glover 2010). The data presented here indicate that neoteny in *L. clementei* is caused by a decrease in cell division, rather than a decrease in cell expansion. The molecular mechanisms behind both the outgrowth and variation in length of the spur are intriguing; they too may differ between the *Aquilegia* and *Linaria* systems (cf. Box *et al.* 2011; Yant *et al.* 2015).

CONCLUSIONS

This study used a comparative evo-devo approach to investigate nectar spur development at the micro and macro scale, aiming to discover how nectar spur development evolves in terms of tissue dynamics. We compared two sister species with dramatically different spur lengths to discover the basis of the variation in spur length. Our data indicate that spur length in *Linaria* is dependent on the number of cells, derived from initial cell divisions, which elongate at the same rate, resulting in different rates of spur elongation. Variation in cell division supports the idea that changes in the activity of cell cycle genes and their regulators may be involved in nectar spur evolution.

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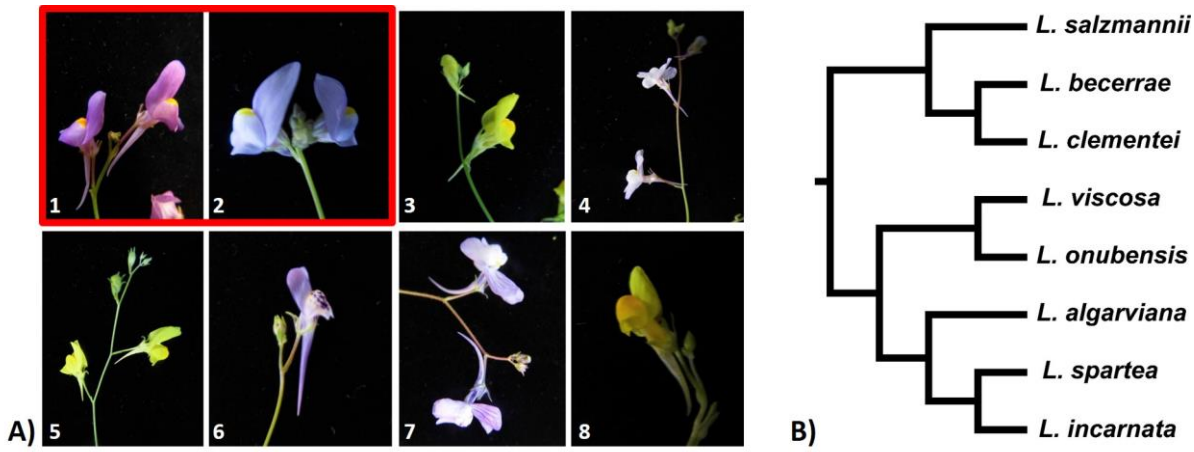

Figure 1. (A) The eight species of *Linaria* (Iberian clade of *Linaria* subsect. *Versicolores*) examined in this study. The sister species *L. clementei* and *L. becerrae*, which we focus on in this study, are highlighted in red. 1) *L. becerrae*, 2) *Linaria clementei*, 3) *Linaria spartea*, 4) *Linaria onubensis*, 5) *Linaria viscosa*, 6) *Linaria algarviana*, 7) *Linaria incarnata*, 8) *Linaria salzmannii*. (B) Phylogeny of the clade (Fernández-Mazuecos *et al.* 2017).

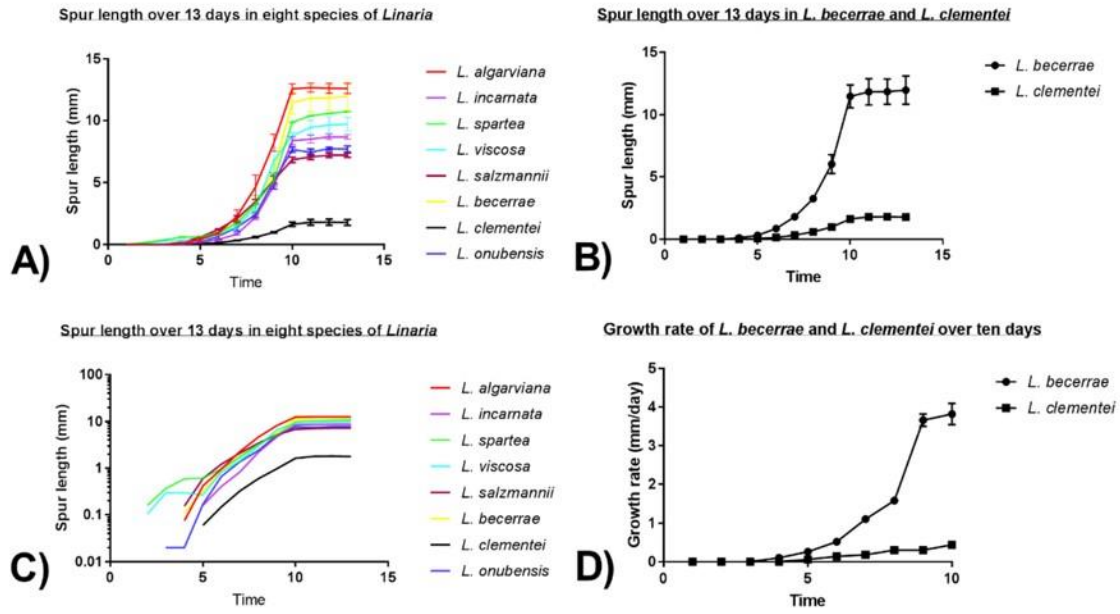

Figure 2. Spur length measured over 13 days in eight species of *Linaria*. Points represent the mean of five biological replicates. The flower opens at day 10. (A) Spur length over 13 days for eight species of *Linaria*, plotted on a linear scale ±SE. (B) Spur length over 13 days in *L.*

becerrae and *L. clementei* only, plotted on a linear scale ±SE. (C) Spur length over 13 days for eight species of *Linaria*, plotted on a logarithmic scale. (D) Growth rate of *L. becerrae* compared with *L. clementei*, calculated as increase in spur length/time per day until the flower opens.

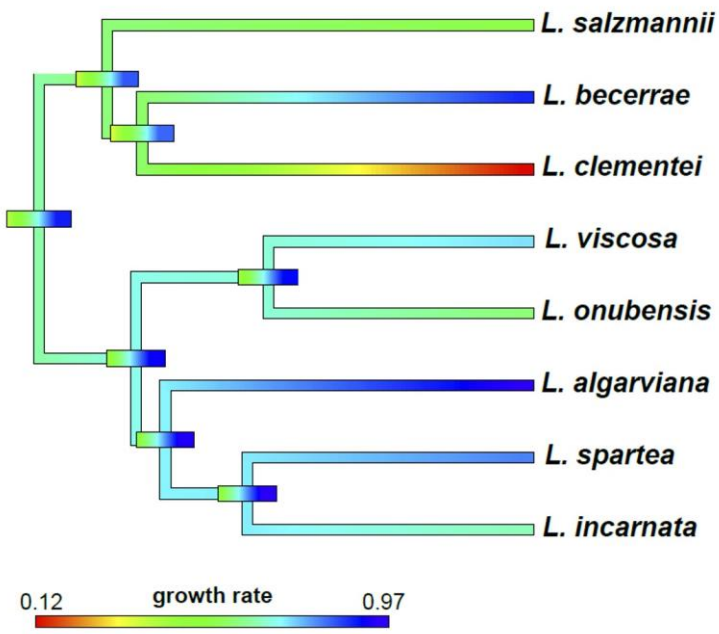

Figure 3. Evolution of spur growth rate (averaged over 13 days) plotted onto the phylogeny of the clade. The maximum likelihood reconstruction is represented as gradational colours along the branches; bars at nodes represent uncertainty (error range).

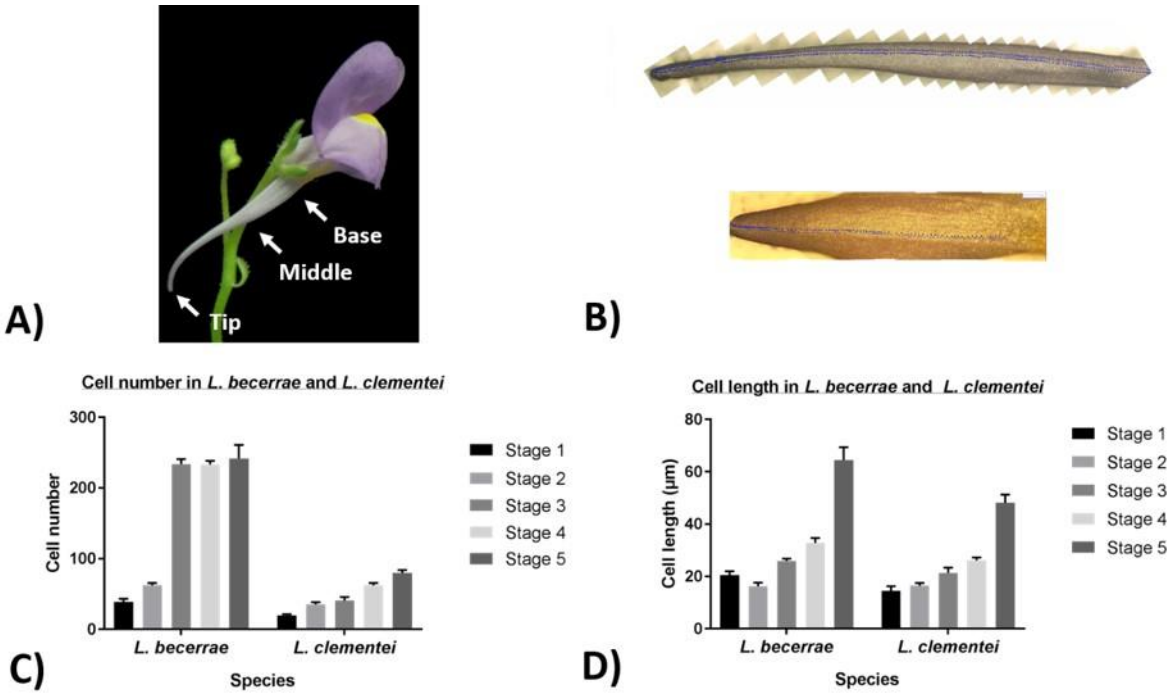

Figure 4. Micromorphological analysis of the spur. (A) Where the measurements at the base, middle and tip of the spur took place, illustrated with *L. becerrae*. (B) An example of a merged spur of *L. becerrae* at the top (spur length of approximately 12 mm), and a merged spur of *L. clementei* at the bottom (spur length of approximately 2 mm). The cells counted along the length of the spur are shown in blue. (C, D) A comparison of nectar spur cell number and cell length in *L. becerrae* and *L. clementei* is shown at five progressive developmental stages (table 1) mean ±SE is shown. Five biological replicates were taken. (C) Cell number in *L. becerrae* and *L. clementei*. (D) Overall cell length in *L. becerrae* and *L. clementei* (averaged data from the base, middle and tip of the spur). The data shown are the mean of 30 cell replicates at the base, middle and tip of the spur for five biological samples (apart from developmental stage one spurs, where only ten replicates were imaged at the base, middle and tip of the spur due to the small size of the spur).

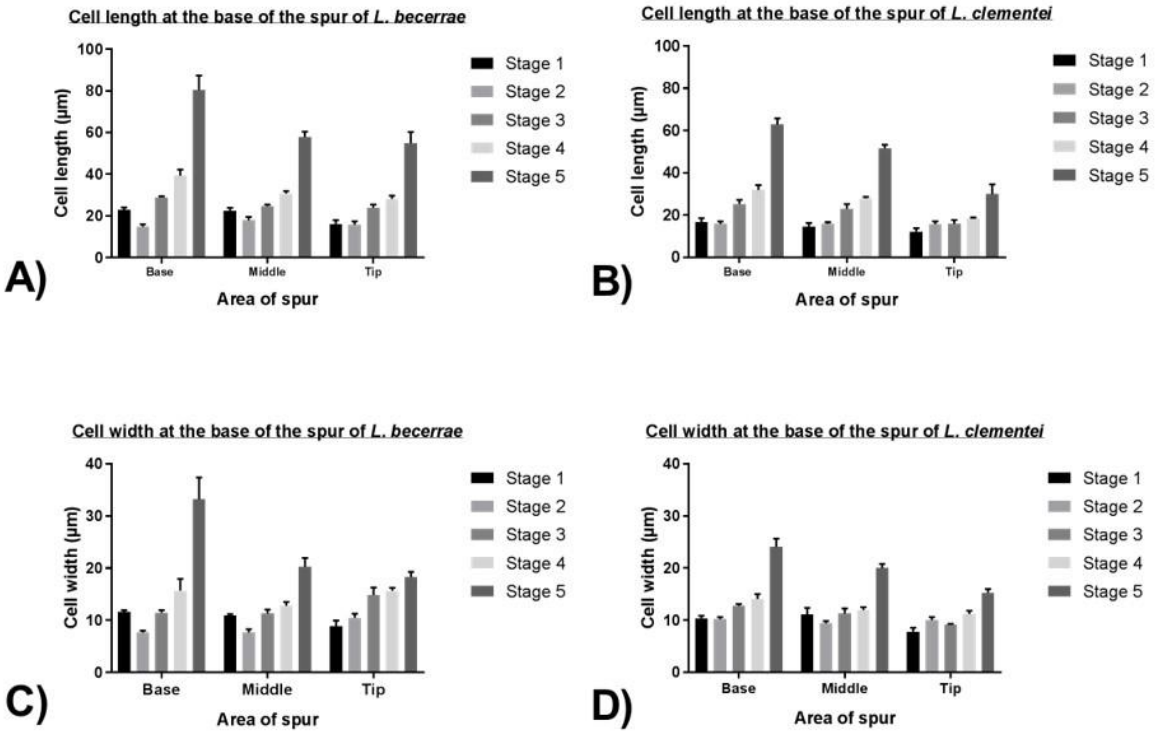

Figure 5. Cell length and width at five progressive developmental stages at the base, middle and tip of the spur in *L. becerrae* and *L. clementei*. Data shown are the mean of 30 replicates for each biological replicate, with five biological replicates ±SE. (A) Cell length along the spur of *L. clementei*. (B) Cell length along the spur of *L. becerrae*. (C) Cell width along the spur of *L. clementei*. (D) Cell width along the spur of *L. becerrae*.

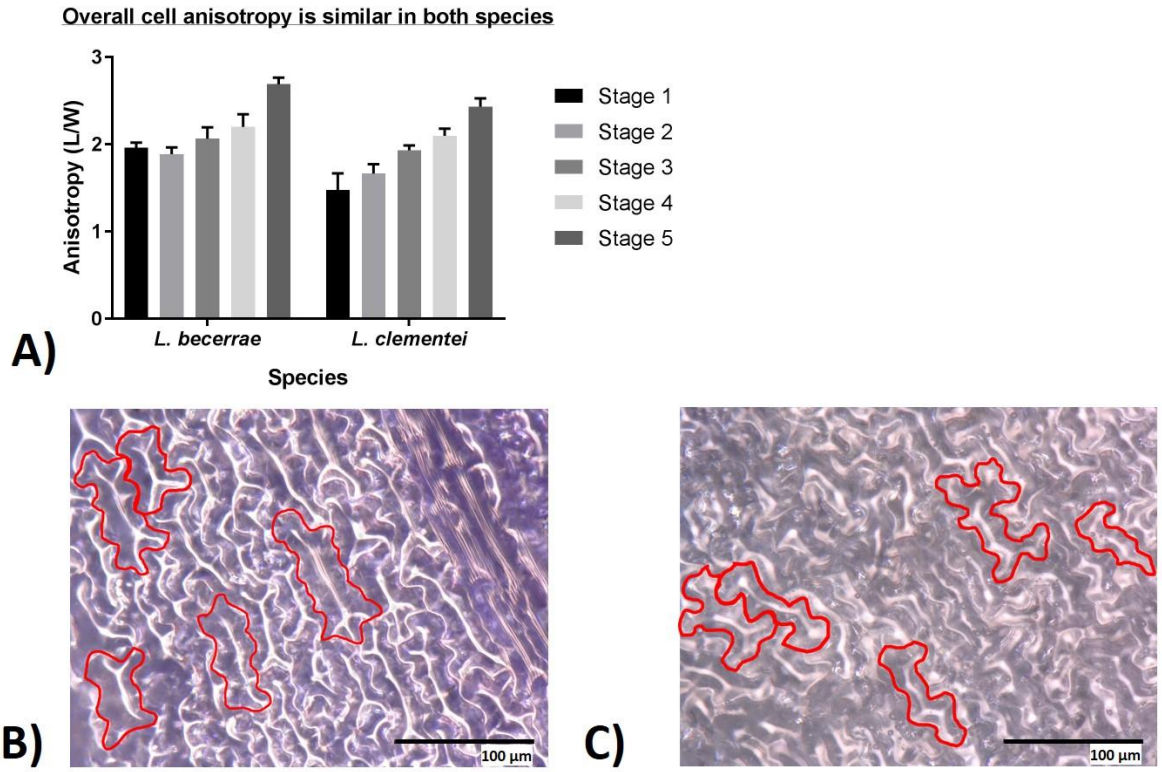

Figure 6. Overall cell anisotropy within the spur is similar in both *L. becerrae* and *L. clementei*. (A) Cell anisotropy within the spur of both *L. becerrae* and *L. clementei* was calculated by examining the ratio of cell length to cell width versus overall cell length and cell width in the mature spur. (B, C) Images of epidermal cells at the base of *L. becerrae* and *L. clementei* spurs. Five epidermal cells are outlined in red in each image as an example of cell boundaries. (B) *L. becerrae* spur. (C) *L. clementei* spur.

Stage	*L. becerrae* spur length (mm)	*L. clementei* spur length (mm)	Approximate number of days prior to anthesis
1	0.8	0.2	4
2	3.25	0.5	2
3	6	0.8	1
5	9	1.4	0.5
5	Open flower	Open flower	0

Table 1. Stages used for cell length and number measurements. These stages were selected as they represent five regularly interspaced stages of spur length for *L. becerrae*, and the equivalent stages for *L. clementei* were determined on the growth curves.

Species	*L. alga.*	*L. inc.*	*L. spa.*	*L. visc.*	*L. salz.*	*L. clem.*	*L. bec.*
L. inc.	ns						
L. spa.	*	***					
L. visc.	ns	*	ns				
L. salz.	ns	*	*	ns			
L. clem.	*	ns	***	**	*		
L. bec.	ns	*	*	ns	ns	*	
L. onu	ns	ns	**	ns	ns	ns	ns

Table 2. Results of post-hoc Dunn test when the initiation of spur growth of each individual species was compared to every other individual species studied. *L. alga, L. algarviana*; *L. onu, L. onubensis*; *L. spa, L. spartea*; *L. visc, L. viscosa*; *L. salz, L. salzmannii*; *L. clem, L. clementei*; *L. bec, L. becerrae*; *L. onu, L. onubensis*; ns, non-significant; *, p<0.05; **, p<0.01, ***, p<0.001.

Species	*L. alga.*	*L. inc.*	*L. spa.*	*L. visc.*	*L. salz.*	*L. clem.*	*L. bec.*
L. inc.	ns						
L. spa.	*	ns					
L. visc.	*	ns	ns				
L. salz.	*	ns	ns	ns			
L. clem.	ns	ns	*	*	*		
L. bec.	ns	ns	**	**	**	ns	
L. onu	ns	*	***	**	***	ns	ns

Table 3. Results of post-hoc Dunn test when the end of spur growth of each individual species was compared to every other individual species studied. *L. alga, L. algarviana*; *L. onu, L. onubensis*; *L. spa, L. spartea*; *L. visc, L. viscosa*; *L. salz, L. salzmannii*; *L. clem, L. clementei*; *L. bec, L. becerrae*; *L. onu, L. onubensis;* ns, non-significant; *, p<0.05; **, p<0.01, ***, p<0.001.

Species	Average initiation of spur (days)	Average end of spur growth (days)	Day segmented function identified	Average growth rate over 13 days (mm/day)
L. clementei	5.4	10.8	7-10	0.1
L. becerrae	4.4	10.4	8-10	0.9
L. onubensis	4.6	10.2	7-10	0.6
L. salzmannii	4.4	12.2	6-10	0.6
L. spartea	2.2	12.2	8-10	0.8
L. viscosa	3.4	12	8-10	0.7
L. algarviana	4.6	10.8	7-10	0.9
L. incarnata	5.4	11.4	8-10	0.7

Table 4. Dates of average initiation and end of spur growth (over 13 days) based on five replicates. The days that the segmented function identified as steep increases in growth rate predicted by the segmented package (which was used for the grouped linear regression) and the average growth rate (calculated as increase in spur length per day) over 13 days is shown.