Commentary

How to spot a flower

The great diversity of floral form has always fascinated biologists, and many studies have sought to explain this diversity by focussing on the interactions of pollinators with different floral morphologies in a wide range of species. By contrast, our understanding of the developmental genetic mechanisms through which floral diversity is generated is mostly limited to a handful of model species. *Antirrhinum majus* and *Petunia hybrida* have proved excellent models in which to explore the generation of floral symmetry (Luo *et al.*, 1996), flower colour (Quattrocchio *et al.*, 1998, 1999; Albert *et al.*, 2011), petal texture (Noda *et al.*, 1994; Perez-Rodriguez *et al.*, 2005) and venation patterning (Schwinn *et al.*, 2006). However, many other aspects of floral diversity merit closer examination, and advances in sequencing and gene expression analysis now allow the study of a wider range of traits in non-model organisms. For example, in this issue of *New Phytologist*, Martins *et al.* (pp 958–969) present an exciting analysis of petal spot development in *Clarkia gracilis* (Onagraceae), a native North American species.

'Petal spots are intimately linked to plant fitness through their influence on pollinator-mediated pollen transfer and resultant seed set.'

Petal spots are groups of pigmented cells that occur in a defined region or regions of the petal, and are sometimes associated with elaborated epidermal cell morphologies. They are widely distributed among many angiosperm families (Fig. 1), and their effects on pollinators have been studied in several systems, including two different *Clarkia* species (Jones, 1996; Moeller, 2005; Eckhart *et al.*, 2006). The petal spots of *Clarkia gracilis* were the subject of a classical genetic study in the 1980s, which demonstrated that the presence and position of the spot were controlled by two independent loci (Gottlieb & Ford, 1988). Martins *et al.* show that *Clarkia* petal spots develop through differential regulation of the enzymes involved in anthocyanin synthesis. Essentially, expression of the gene encoding F3′H (flavonoid 3′-hydroxylase) occurs throughout the petal early in development, but production of the resulting red/purple cyanidin/peonidin pigments is limited to the spot area by the spatially restricted expression of *DFR2* (encoding dihydroflavonol reductase) (Fig. 2). Martins *et al.* isolated an additional duplicate copy of *DFR*, *DFR1*, that is expressed later in petal development throughout the petal. This allows pigment to be produced everywhere in the petal, but the concurrent late expression of the gene encoding F3′5′H (flavonoid

Fig. 1 Examples of flowering plant families with species that exhibit petal spots. (a) *Nemophila maculata* (Boraginaceae); (b) *Gorteria diffusa 'Soeb'* (Asteraceae); (c) *Cistus cyprius* (Cistaceae); (d) *Digitalis purpurea* (Plantaginaceae); (e) *Pelargonium echinatum* (Geraniaceae); (f) *Sparaxis elegans* (Iridaceae); (g) *Linum grandiflorum* 'Bright eyes' (Linaceae); (h) *Dendrobium nobile* (Orchidaceae).

New Phytologist

3′,5′-hydroxylase) means that this pigment is pale pink malvidin. Spot formation is therefore, in part, the product of a duplication at the *DFR* locus. This was followed by the acquisition of a distinct spatio-temporal expression pattern for each of the duplicated gene copies, combined with temporal regulation of the genes encoding F3′H and F3′5′H.

Studies of the genus *Clarkia* in native habitats have demonstrated that insect pollinators can visually distinguish between spotted and non-spotted flowers. Populations of *Clarkia xantiana* ssp. *xantiana* are polymorphic for the presence and number of red petal spots and pollinated by the specialist bee species *Hesperapis regularis* (Melittidae), *Lasiglossum pullilabre* (Halictidae) and *Ceratina sequoia* (Apidae), which all show frequency dependent behaviour in selecting between spotted and non-spotted flowers (Moeller, 2005; Eckhart *et al.*, 2006). By contrast, the spotted form of *Clarkia gracilis* was shown to set up to 32% more seed than the non-spotted form when grown in a mixed plot (Jones, 1996). These data emphasize the importance of extrinsic factors such as pollinators in understanding the evolutionary processes leading to the formation of petal spots, in addition to understanding genetic regulation. Petal spots are intimately linked to plant fitness through their influence on pollinator-mediated pollen transfer and resultant seed set. Consequently these traits are the target of natural selection, and, by influencing pollinators, may play an important role in establishing species boundaries. Thus, petal spots offer an important opportunity to link natural genetic variation underlying diverse spot phenotypes with the fitness of this phenotypic variation for pollinator attraction. As work continues in diverse species it will become interesting to compare the findings from the *Clarkia* system with parallel experimental systems such as that of the South African daisy *Gorteria diffusa*. *Gorteria diffusa* has raised black petal spots on its ray florets, exists as several floral variants, and has been subjected to a detailed morphological analysis (Thomas *et al.*, 2009). The *G. diffusa* spot is surprisingly complex, consisting of different cell types and localized pigment deposition. As with *Clarkia*, pollination data suggest a complex interaction between these petal spots and their pollinator, in this case the small bee-fly *Megapalpus capensis* (Ellis & Johnson, 2010).

The paper by Martins *et al.* emphasizes the importance of two key themes in evolutionary biology – transcriptional regulation and gene duplication. Promoters are modular entities, comprising a composite assembly of distinct regulatory elements, which allow transcription of a single gene to be activated at different times and places to exert different functions. For example, in the common pea *Pisum sativum*, the single copy gene *UNIFOLIATA* is expressed at the margin of growing leaves, where it controls the formation of characteristic compound leaves, but is also later activated in the floral meristem to trigger flower development (Hofer *et al.*, 1997). Gene duplication can also contribute to this process of functional differentiation through neo- or sub-functionalization. The coding sequences of paralogous copies can diverge and create proteins with distinct properties. Alternatively, the expression of the two copies, now piloted by distinct regulatory regions, can change so that each duplicate ends up with its own unique spatio-temporal expression pattern. While several studies, including that of Martins *et al.*, have revealed the existence of such diverging expression patterns

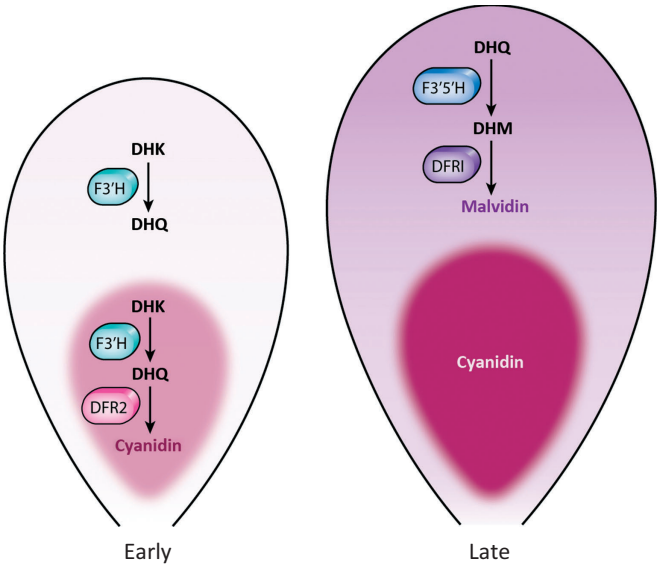

Fig. 2 Petal spot development in *Clarkia gracilis* is the result of spatially restricted expression of *DFR2* early in petal development, when *F3′H* expression leads to the production of cyanidin in the petal spot. Later expression of *DFR1* and *F3′5′H* throughout the petal leads to production of malvidin in the rest of the petal. DHK, dihydrokaempferol; DHQ, dihydroquercetin; DFR, dihydroflavonol reductase; DHM, dihydromyricetin; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′,5′-hydroxylase.

between duplicates, pinning down why and how the two copies are expressed differently is still challenging, even in a post-genomic era. Obtaining whole genome and transcriptome sequences for thousands of individuals has become relatively straightforward, yet it remains difficult to reconstruct transcriptional networks. While conserved protein sequences are easily identified, transcriptional regulatory modules are much more challenging to predict (Farnham, 2009). Thus, our ability to identify regulatory elements and, among them, those accounting for the acquisition of new expression patterns, is one of the next big challenges in evolutionary developmental biology.

By contrast, uncovering the transcriptional regulation responsible for differential expression patterns of pigmentation seems entirely feasible given our detailed understanding of anthocyanin synthetic pathways, and the availability of next-generation sequencing technologies. Anthocyanin synthesis is regulated by a complex comprising R2R3 MYB, bHLH (basic-helix-loop-helix) and WD40 proteins, which are a source of phenotypic variation and have been an essential component in generating plant epidermal cell diversity. While the bHLH and WD40 proteins controlling pigment synthesis are also involved in the production of compounds fulfilling various roles in vegetative tissues, the expression of R2R3 MYB transcription factors is, by contrast, highly tissue- or even cell-type specific: in *Petunia hybrida*, four R2R3 MYB genes have been shown to regulate different aspects of anthocyanin expression in the flower (Quattrocchio *et al.*, 1998, 1999; Albert *et al.*, 2011). Thus, R2R3 MYB regulators appear to be promising candidates to explain the diversity of spatio-temporal expression patterns of anthocyanin biosynthetic genes observed in *Clarkia*. In addition to these candidate gene approaches, next

generation sequencing technologies now allow the retrieval of differentially expressed regulators on a much greater scale. These technologies can be used to generate transcriptional profiles from tissue-specific developmental stages, to identify genes that regulate part of a biosynthetic pathway or development of a morphological trait, and to reveal the existence of duplicated genes, like those found by Martins *et al.*

In conclusion, Martins *et al.* have not only developed a promising system for future research into petal spot biology, but also present exciting insights that are broadly applicable to the development of many morphological traits in non-model systems. In particular, it will be interesting to determine the extent to which the genetic mechanisms identified here are able to explain sub-specific or inter-specific variation in spot morphology within the *Clarkia* genus as a whole. More broadly, it will also be important to compare genetic regulation of petal spot development in *Clarkia* with that found in the petal spots of diverse angiosperm lineages. The *Clarkia* system is an excellent example of the use of natural variation coupled with laboratory-based experimental techniques to explore the genetic regulation of morphological traits with quantifiable impacts on fitness. We hope to see many more such studies in coming years.

Beverley J. Glover*, Rachel H. Walker, Edwige Moyroud and Samuel F. Brockington

Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK
(*Author for correspondence: tel +44 1223 333938; email bjg26@cam.ac.uk)

References

Albert NW, Lewis DH, Zhang H, Schwinn KE, Jameson PE, Davies KM. 2011. Members of an R2R3-MYB transcription factor family in *Petunia* are developmentally and environmentally regulated to control complex floral and vegetative pigmentation patterning. *Plant Journal* **65**: 771–784.

Eckhart VM, Rushing NS, Hart GM, Hansen J. 2006. Frequency-dependent pollinator foraging in polymorphic *Clarkia xantiana* ssp. *xantiana* populations: implications for flower colour evolution and pollinator interactions. *Oikos* **112**: 412–421.

Ellis A, Johnson S. 2010. Floral mimicry enhances pollen export: the evolution of pollination by sexual deceit outside of Orchidaceae. *American Naturalist* **176**: E143–E151.

Farnham PJ. 2009. Insights from genomic profiling of transcription factors. *Nature Reviews Genetics* **10**: 605–616.

Gottlieb LD, Ford VS. 1988. Genetic studies of the pattern of floral pigmentation in *Clarkia gracilis*. *Heredity* **60**: 237–247.

Hofer J, Turner L, Hellens R, Ambrose M, Matthews P, Michael A, Ellis N. 1997. UNIFOLIATA regulates leaf and flower morphogenesis in pea. *Current Biology* **7**: 581–587.

Jones KN. 1996. Pollinator behaviour and postpollination reproductive success in alternative floral phenotypes of *Clarkia gracilis* (Onagraceae). *International Journal of Plant Sciences* **157**: 733–738.

Luo D, Carpenter C, Vincent C, Copsey L, Coen E. 1996. Origin of floral asymmetry in *Antirrhinum*. *Nature* **383**: 794–799.

Martins TR, Berg JJ, Blinka S, Rausher MD, Baum DA. 2012. Precise spatial-temporal regulation of the anthocyanin biosynthetic pathway leads to petal spot formation in *Clarkia gracilis* (Onagraceae). *New Phytologist* **197**: 958–969.

Moeller DA. 2005. Pollinator community structure and sources of spatial variation in plant–pollinator interactions in *Clarkia xantiana* ssp. *xantiana*. *Oecologia* **142**: 28–37.

Noda K, Glover BJ, Linstead P, Martin C. 1994. Flower colour intensity depends on specialized cell shape controlled by a Myb-related transcription factor. *Nature* **269**: 661–664.

Perez-Rodriguez M, Jaffe FW, Butelli E, Glover BJ, Martin C. 2005. Development of three difference cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of *Antirrhinum majus* flowers. *Development* **132**: 359–370.

Quattrocchio F, Wing JF, van der Woude K, Mol JNM, Koes R. 1998. Analysis of bHLH and MYB domain proteins: species-specific regulatory differences are caused by divergent evolution of target anthocyanin genes. *Plant Journal* **13**: 475–488.

Quattrocchio F, Wing J, van der Woude K, Souer E, de Vetten N, Mol J, Koes R. 1999. Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. *Plant Cell* **11**: 1433–1444.

Schwinn K, Venail J, Shang JY, Mackay S, Alm V, Butelli E, Oyama R, Bailey P, Davies K, Martin C. 2006. A small family of MYB-regulatory genes controls floral pigmentation intensity and patterning in the genus *Antirrhinum*. *Plant Cell* **18**: 831–851.

Thomas MM, Rudall PJ, Ellis AG, Savolainen V, Glover B. 2009. Development of a complex floral trait: the pollinator-attracting petal spots of the beetle daisy, *Gorteria diffusa* (Asteraceae). *American Journal of Botany* **96**: 2184–2196.

Key words: anthocyanin, differential regulation, gene duplication, petal spot, transcriptional control.