New Phytologist

Research review

Complex pigment evolution in the Caryophyllales

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Received: *6 January 2011*
Accepted: *2 February 2011*

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New Phytologist (2011) **190**: 854–864
doi: 10.1111/j.1469-8137.2011.03687.x

Key words: anthocyanins, betalains, Caryophyllales, chemotaxonomy, pigmentation.

Summary

Carotenoids and flavonoids including anthocyanins are the predominant pigments in flowering plants, where they play important roles in pollination, seed dispersal, protection against stress and signalling. In certain families within the *Pentapetalae* order Caryophyllales, an unusual class of pigments, known as betalains, replaces the more common anthocyanins. This isolated occurrence of betalains in the Caryophyllales has stimulated over half a century of debate and experimentation. Numerous hypotheses have been suggested to explain the phylogenetically restricted occurrence of betalains and their apparent mutual exclusion with anthocyanins. In this review, we evaluate these hypotheses in the face of a changing interpretation of Caryophyllales phylogeny and new comparative genetic data. Phylogenetic analyses expose substantial gaps in our knowledge of the early evolution of pigments in the Caryophyllales and suggest pigmentation to be much more labile than previously recognized. Reconstructions of character evolution imply multiple switches from betalain to anthocyanin pigmentation, but also allow for possible multiple origins of betalains. Comparative genetic studies propose possible mechanisms underlying switches between pigment types and suggest that transcriptional down-regulation of late-acting enzymes is responsible for a loss of anthocyanins. Given these insights from molecular phylogenetics and comparative genetics, we discuss outstanding questions and define key goals for future research.

Introduction

Pigments perform critical roles in the biology of angiosperms, where they act as visible signals to attract fauna for pollination and seed dispersal, protect plants from light damage, phytopathogens and heavy metals, and are involved in internal signalling (reviewed by Koes *et al.*, 2005). Flavonoids, including anthocyanins, are widely distributed across angiosperms, where they are responsible for the colouration found in flowers, leaves, fruit and seeds (Tanaka *et al.*, 2008) (see Fig. 1). Betalains are pigments that are structurally and biosynthetically distinct from flavonoids and anthocyanins, and appear to have evolved at least twice: once within a fungal lineage – the Basidiomycetes; and once within a flowering plant order – the core Caryophyllales (Bischoff, 1876; Mabry, 1964) (see Fig. 1). The core Caryophyllales is a well-defined clade of eudicots comprising *c.* 29 families and *c.* 9000 species (APG, 2003). In betalain-producing families within the core Caryophyllales, anthocyanins have never been detected (Bate-Smith, 1962; Mabry, 1964), indicating that betalain pigmentation substitutes for the otherwise ubiquitous anthocyanins. However, in two

Fig. 1 Similar hues, different pigments. (a) Anthocyanin pigmentation in *Antirrhinum majus*. (b) Betalain pigmentation in *Mirabilis jalapa*.

families in the core Caryophyllales, the Molluginaceae and Caryophyllaceae, anthocyanins are produced instead of betalains, suggesting that anthocyanins and betalains are mutually exclusive pigment types (Mabry, 1964).

The novel origin of betalains within the core Caryophyllales and their mutual exclusion with anthocyanins have stimulated over half a century of debate in the fields of taxonomy and systematics, phytochemistry and evolutionary biology. In this article, we examine the scope and limitations of existing hypotheses that have been proposed to explain the complex patterns of pigment variation in the core Caryophyllales. We review the state of knowledge regarding the biosynthesis and regulation of anthocyanin and betalain pigmentation, review insights from recent studies that take a comparative genetic approach to betalain and anthocyanin synthesis, assess the impact of recent molecular phylogenetic analyses on the reconstruction of pigment evolution and identify key questions and hypotheses for future research.

The scope and limitations of existing hypotheses

Several hypotheses have been proposed to explain the evolution of betalain pigmentation; however, explanations of pigment evolution in the core Caryophyllales must be able to account for the following three phenomena: (1) the unique derived origin of betalain pigmentation; (2) the presence of both anthocyanin-pigmented and betalain-pigmented lineages in the core Caryophyllales; and (3) the mutual exclusivity of the two pigment types. As discussed below, many of the hypotheses that have been advanced to date are not mutually exclusive, but none alone adequately explains these three phenomena.

The unique origin of betalains

Ehrendorfer (1976) argued that the unique presence of betalains in Caryophyllales was the consequence of an unusual evolutionary history, in which the ancestor to Caryophyllales evolved in arid to semi-arid conditions before the radiation of major pollinator lineages. In this open, pollinator-deprived environment, wind pollination may have prevailed, and anthocyanin pigmentation was lost as there was no need to attract pollinators. Subsequently, following the radiation of pollinator lineages and the colonization of less marginal habitats, reversion to zoophily engendered a return to pigmentation in the form of betalains rather than anthocyanins (Ehrendorfer, 1976). This hypothesis is difficult to evaluate (Clement & Mabry, 1996) and the phylogenetic concept of Caryophyllales has changed considerably since Ehrendorfer (1976). Some morphological features of extant Caryophyllales are consistent with a wind-pollinated ancestor, including an ancestrally uniseriate undifferentiated flower (i.e. a perianth of one organ type rather than differentiated sepals and petals); however, several newly placed lineages (*Rhabdodendron*, *Asteropeia*, *Macarthuria*) suggest that entomophily may be the ancestral state (Brockington *et al.*, 2009). Furthermore, because anthocyanins and betalains accumulate and function in both vegetative and reproductive tissues, it is unreasonable to explain the evolutionary changes in pigmentation as a result of the absence or presence of pollinators alone (reviewed in Whittall & Strauss, 2006). There are few competing explanations for the unique presence of betalains in the core Caryophyllales; however, Stafford (1994) suggested possible horizontal transfer of genes from betalain-pigmented Basidiomycetes – an intriguing if improbable scenario that cannot yet be ruled out.

The presence of both betalain and anthocyanin lineages

To explain the presence of both betalain and anthocyanin lineages, Clement & Mabry (1996) suggested that the two compounds may have co-occurred in an ancestor to the core Caryophyllales. The two pigments might have been selectively maintained in ancestors of extant Caryophyllales, with the subsequent loss of one or other of the pigments in extant lineages. Clement & Mabry (1996) did not identify the nature of the selection maintaining this co-occurrence, or its loss, but betalains may have evolved as a result of their fungicidal properties (Mabry, 1980; Piatelli, 1981), rather than pigmentation; thus, the two pigments could have co-existed as a result of the complementary pigmentation and antifungal properties. The most significant objection to this hypothesis is that no taxon has been identified that contains both anthocyanins and betalains, which is surprising if they are complementary in function. It remains possible that the two pigment types co-exist in unexplored taxa; however, the maintenance of both pigments in a common ancestor does not provide mechanisms for their ultimate mutual exclusion. Clement & Mabry (1996) referred to the 'stochastic loss' of one or other pigment in extant lineages, but did not provide specific mechanisms to explain the loss of a pigment.

The mutual exclusion of anthocyanins and betalains

Additional hypotheses are needed to explain the mutual exclusion of these pigment types and/or the replacement of one pigment type with another. Clement & Mabry (1996) explored the relative metabolic cost of betalain and anthocyanin synthesis, but reached no clear conclusions, in part because the full physiological roles of anthocyanins and betalains are not thoroughly understood, and in part because true metabolic costs are not easily assessed. The relative effectiveness of the pigments has also been discussed in terms of molar absorptivity, with the suggestion that much smaller amounts of betacyanin are needed to absorb equivalent amounts of visible light relative to their anthocyanic counterparts, making betacyanins more 'cost-effective' (Clement & Mabry, 1996). Evaluating the cost-effectiveness on the basis of visible light alone is a limitation as UV light can be an important component of pollinator vision, for example UV light receptors in the honeybee *Apis mellifera* (Menzel & Blakers, 1975). Betalains are inefficient at absorbing in the UV spectrum, a property that may result in more UV reflectance and thus enhance a flower's appearance to prospective pollinators.

Stafford (1994) adopted a different line of reasoning, proposing that similar patterns in the accumulation of the two pigments in both vegetative and floral tissues are indicative of a common regulatory system. In this situation, should the ultimate step in anthocyanin synthesis be in some way inhibited by an end product of betalain synthesis (Stafford, 1994), anthocyanin accumulation might be repressed and replaced entirely by betalain pigmentation.

The biosynthesis of betalains and anthocyanins

An introduction to biosynthesis and the regulation of anthocyanin and betalain pigmentation is essential in evaluating these different hypotheses and assessing the impact of comparative genetic data. Here, we describe the two distinct biochemical pathways leading to anthocyanin and betalain pigmentation and illustrate their salient differences and occasional similarities (Figs 2, 3). It will become apparent that there is considerable disparity in our understanding of betalain and anthocyanin synthesis. This reflects, in part, the limited development of molecular tools within the betalain-pigmented Caryophyllales relative to well-established anthocyanic model organisms, for example *Arabidopsis*, *Antirrhinum*, *Petunia* and *Zea*.

Anthocyanin biosynthesis and regulation

Anthocyanin biosynthesis has been the subject of several comprehensive reviews, and we refer the reader to the following papers and citations within Dooner *et al.* (1991), Koes *et al.* (2005), Grotewold (2006) and Tanaka *et al.* (2008). Here, we provide a summary of the steps of this pathway, which is conserved among seed plants. Anthocyanins are derived from a branch of the flavonoid pathway, being synthesized from phenylalanine. Initially, phenylalanine is converted into *p*-coumaroyl-CoA via the intermediates cinnamic acid and *p*-coumaric acid, mediated by the enzymes phenylalanine ammonia lyase (Fig. 2: PAL), cinnamate-4-hydroxylase (Fig. 2: C4H) and 4-coumaroyl:CoA-ligase (Fig. 2: 4CL). Acting in the cytosol, chalcone synthase (Fig. 2: CHS) then catalyses the condensation of one molecule of *p*-coumaroyl-CoA with three molecules of malonyl-CoA to form the chalcone tetra-

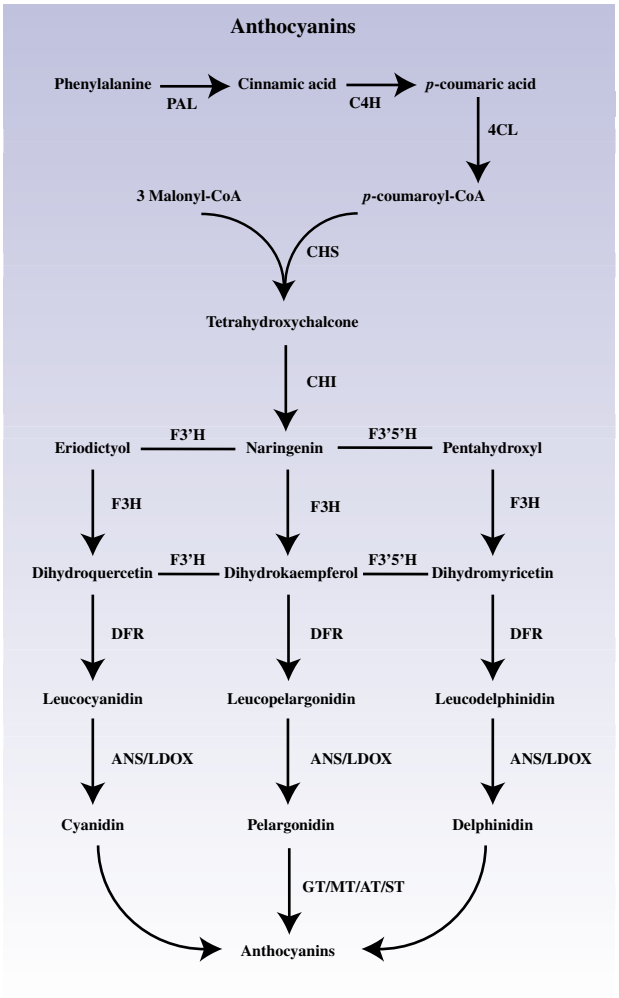

Fig. 2 Summary of the anthocyanin biosynthetic pathway showing the main intermediates and key enzymatic steps. Enzymes in the anthocyanin pathway: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3′H, flavanone 3′-hydoxylase; F3′5′H, flavanone 3′,5′-hydoxylase; F3H, flavanone-3-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS/LDOX, anthocyanidin synthase/leucoanthocyanidin dioxygenase; GT, glucosyl transferase; MT, methyl transferase; AT, acyl transferase; ST, sulphyl transferase.

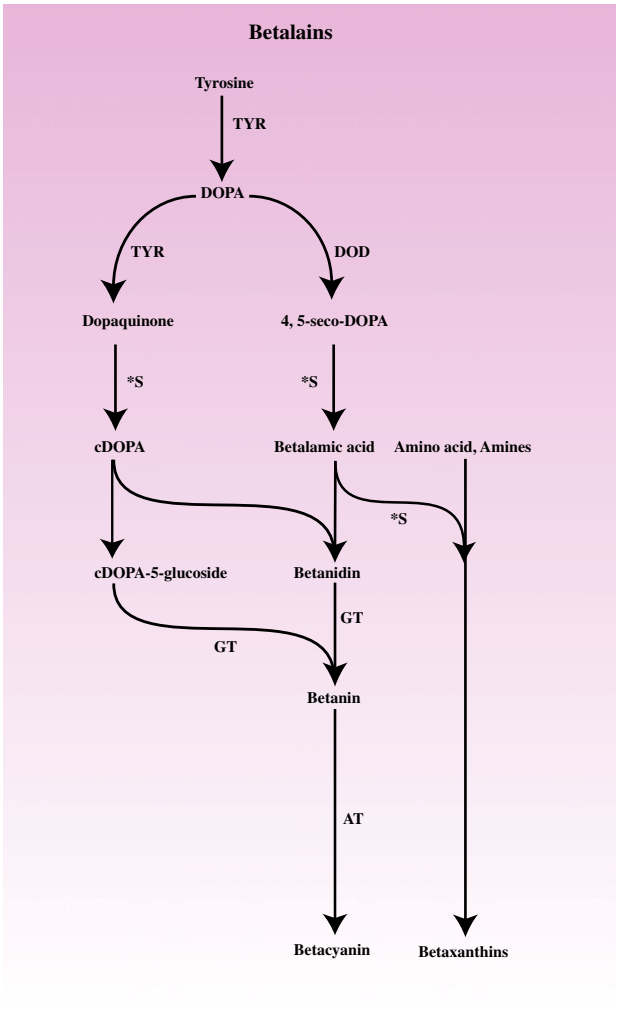

Fig. 3 Summary of the betalain biosynthetic pathway showing the main intermediates and key enzymatic steps. Enzymes in the betalain pathway: TYR, tyrosinase including tyrosinase hydroxylase and DOPA-oxygenase activity; DOD, DOPA 4,5-dioxygenase; *S, spontaneous steps; GT, glycosyl transferase; AT, acyltransferase. DOPA, 5,6-dihydroxy-phenylalanine.

hydroxychalcone. Isomerization of tetrahydroxychalcone to the flavone naringenin occurs through the enzymatic action of chalcone isomerase (Fig. 2: CHI), or spontaneously in some species. Naringenin can be converted to the additional colourless eriodictyol and pentahydroxyl flavanones by the enzymes flavanone 3′-hydroxylase (Fig. 2: F3′H) and flavanone 3′,5′-hydroxylase (Fig. 2: F3′5′H), respectively. Flavanone 3-hydroxylase (Fig. 2: F3H) hydroxylates the flavanones naringenin, eriodictyol and pentahydroxyl to the dihydroflavonols dihydrokaempferol, dihydroquercetin and dihydromyricetin, respectively. F3′H and F3′5′H can also catalyse the hydroxylation of dihydrokaempferol to the additional dihydroflavonols, dihydroquercetin and dihydromyricetin, respectively. Dihydroflavonol-4-reductase (Fig. 2: DFR) then catalyses the conversion of these dihydroflavonols to leucoanthocyanidins: for example, leucopelargonidin, leucocyanidin and leucodelphinidin. Leucoanthocyanidins are converted into coloured anthocyanidins (pelargonidin, cyanidin and delphinidin) through the enzymatic activity of anthocyanidin synthase/leucoanthocyanidin dioxygenase (Fig. 2: ANS/LDOX). Anthocyanidins may be modified by glycosylation, methylation, acetylation and sulfation reactions, generating a diversity of stable anthocyanin structures (Fig. 2: GT/MT/AT/ST). The addition of these different moieties varies taxonomically, contributing to species- and family-specific colouration. Stabilized anthocyanin pigments are then transported from the cytosol across the vacuolar membrane and stored in epidermal tissues.

Anthocyanin biosynthesis is one of the most studied regulatory systems in plants and is controlled by both external factors (e.g. light) and internal factors (e.g. the circadian clock) (Koes *et al.*, 2005). The structural genes of the pathway are largely regulated at the transcriptional level, controlling the spatial and temporal distribution of pigments in plant tissues (Mol *et al.*, 1998). Among the regulatory genes, common denominators include R2R3 MYB, basic-helix-loop-helix (bHLH) and WD40 repeat proteins, which form a complex regulating the structural genes of the pathway. Homologues of these genes have been isolated from many angiosperm species, where they are involved in the regulation of a range of epidermal processes, including anthocyanin pigmentation (Ramsay & Glover, 2005).

Betalain biosynthesis and regulation

Betalain synthesis is initiated through the hydroxylation of tyrosine, to give two precursor molecules of L-5,6-dihydroxy-phenylalanine (L-DOPA) (Piatelli, 1981). One molecule of L-DOPA is transformed into DOPA-quinone and spontaneously converted into *cyclo*-DOPA (Tanaka *et al.*, 2008). The second molecule of L-DOPA goes through a 4,5-extradiol oxidative cleavage to 4,5-*seco*-DOPA, which is recyclized to produce betalamic acid. Betalamic acid can condense with the imino group of *cyclo*-DOPA to give betanidin. Betanidin is then glycosylated to form betanin (Delgado-Vargas & Paredes-Lopez, 2002). Glycosylation can occur either on *cyclo*-DOPA before condensation with betalamic acid, or after the formation of betanidin. Subsequent to glycosylation, betanins may be acylated to form 1-*O*-acylglucosides (Delgado-Vargas & Paredes-Lopez, 2002). The alternative fate of betalamic acid is to condense with the imino or amino group of amino acids to give yellow betaxanthins. Together, these two classes, betacyanins and betaxanthins, make up the pigments known as betalains (Tanaka *et al.*, 2008).

Two enzymes are implicated in the production of the key molecules *cyclo*-DOPA and betalamic acid. The first enzyme in this pathway is a tyrosinase or phenol-oxidase complex that catalyses the conversion of tyrosine to L-DOPA and its

subsequent dehydrogenation to *O*-quinone (Delgado-Vargas & Paredes-Lopez, 2002) (Fig. 3). Tyrosinases involved in betalain synthesis were initially isolated from the basidiomycete *Amanita muscaria* (Mueller *et al.*, 1996) and subsequently from the Caryophyllid taxa *Portulaca grandiflora* (Portulacaceae) and *Beta vulgaris* (Amaranthaceae) (Steiner *et al.*, 1999). The isolated tyrosinases show the required monophenol monooxygenase and *O*-diphenol oxidase activity to produce *cyclo*-DOPA via the intermediate *O*-quinone (Delgado-Vargas & Paredes-Lopez, 2002). Two cDNA clones encoding polyphenol oxidase have also been isolated from *Phytolacca americana* (Phytolaccaceae), and transcript levels of these polyphenol oxidases have been shown to increase in the ripening betalain-pigmented fruit (Joy *et al.*, 1995).

The second enzyme DOPA-4,5-dioxygenase (DOD) converts L-DOPA to betalamic acid (Fig. 3: DOD). Purification of a DOD protein and cloning of the DOD gene were again first achieved in fungi, and the enzyme was shown to convert L-DOPA to betalamic acid and the fungal betalain muscaflavin (Mueller *et al.*, 1997). DOD was subsequently identified in *P. grandiflora* through cDNA subtraction. The activity of this *P. grandiflora* DOD was assayed in a white petal cultivar of *P. grandiflora*, where transient expression of DOD cDNA by particle bombardment generated red spots of betacyanin (Christinet *et al.*, 2004).

Several glucosylation enzymes have been characterized (Fig. 3: GT) which are homologous to glucosylation enzymes in the anthocyanin pathway: UDP-glucose:betanidin 5-O-GT (B5GT) and UDP-glucose:betanidin 6-O-GT (B6GT) (Vogt *et al.*, 1997, 1999) and cDOPA5GT (Sasaki *et al.*, 2005). B5GT and B6GT catalyse the glycosylation of betanidin to betanin, whereas cDOPA5GT catalyses the glycosylation of cDOPA before its condensation with betalamic acid. Glycosylation can therefore occur either before or after the formation of betanidin. The enzymes that catalyse the acylation of betanins (Fig. 3: AT) are not known, but may be glucose-dependent acyltransferases belonging to the serine carboxypeptidase-like (SCPL) enzyme family, as in the case of anthocyanin acylation (Tanaka *et al.*, 2008).

Relative to the detailed understanding of anthocyanin regulation, almost nothing is known about the regulation of betalain synthesis. As with anthocyanins, betalains accumulate in vacuoles in epidermal and subepidermal tissue layers, in both vegetative and reproductive tissues. The regulatory genes controlling betalain synthesis have not been characterized; however, betalains accumulate in response to similar external stimuli to anthocyanins (reviewed in Stafford, 1994). UV and red light (via phytochrome) have been implicated in the control of betalain synthesis in seedlings, but betalain synthesis is not always dependent on light, as betalains can accumulate in the seedlings of some species that have been kept in the dark. The application of hormones, such as cytokinins and indoleacetic acid, to cell culture lines also stimulates betalain biosynthesis (Stafford, 1994).

Insights from comparative genetics

Recent studies have sought to understand the mutual exclusion of betalains and anthocyanins at the molecular level through comparative genetic study (Shimada *et al.*, 2004, 2005, 2007). Although anthocyanins have not been detected in betalain-pigmented families, flavonol glycosides and flavonols are present in the perianth of *Astrophytum* (Cactaceae), and proanthocyanidins are common in the seeds of several Caryophyllales taxa (Bittrich & Amaral, 1991; Shimada *et al.*, 2005). These findings suggest a fully functioning anthocyanin pathway, at least to the stage of DFR activity. On the basis of this observation, Shimada *et al.* (2004, 2005) examined the betalain-producing taxa, *Spinacia oleracea* and *Phytolacca americana*, for the activity of enzymes in the anthocyanin pathway (DFR, ANS and CHS). These experiments revealed that CHS is expressed broadly throughout all tissues; DFR is expressed at very low levels in most mature plant organs, but transiently expressed during seedling growth and also expressed in seeds; ANS transcripts are expressed in seeds. The presence of both ANS and DFR in seeds is consistent with the presence of proanthocyanidins in seeds. These data imply that anthocyanin production in these betalain-pigmented taxa is lost through transcriptional level changes in the expression patterns of the late biosynthetic genes DFR and ANS. Importantly, the maintenance of ANS and DFR loci in betalain-pigmented taxa, together with their transcriptional down-regulation, provides a simple mechanism for reversals to the anthocyanic condition following the origin of betalain pigmentation. It would be interesting to conduct reciprocal experiments for structural genes from the betalain synthesis pathway to examine their expression and activity in anthocyanin-pigmented taxa. Such data might help to locate the hypothetical sites of repressive interaction between anthocyanin and betalain synthesis suggested by Stafford (1994).

A comparative genetics approach to understanding the evolution of betalain synthesis is challenging, as some of the major structural genes have not yet been isolated. Candidate tyrosinases from Caryophyllales taxa have only been characterized at the protein level and, although polyphenol oxidase genes have been cloned from *Phytolacca americana*, it is not certain whether they function in betalain synthesis (Joy *et al.*, 1995). DOD and glucosylation enzymes are better characterized at the genetic level. DOD has been cloned from *P. grandiflora* and *Mirabilis jalapa*, and three glucosylation enzymes have been cloned: B5GT and B6GT from *Doreanthus bellidiformis* (Vogt *et al.*, 1997, 1999) and cDOPA5GT from *M. jalapa* (Sasaki *et al.*, 2005). These data, although limited, provide some interest-

ing insights into the evolution of the betalain synthesis pathway. Homologues of DOD have been isolated from a range of anthocyanin-producing taxa across angiosperms (Christinet *et al.*, 2004). Some of these DOD homologues from anthocyanin-producing plants have been shown to have DOD catalytic activity *in vitro* (Tanaka *et al.*, 2008), although their *in vivo* function in these taxa is unknown. Similarly, B5GT and B6GT, which convert betanidin to betanin, are orthologues of genes that perform similar glucosylation functions in anthocyanin synthesis and can utilize flavonoids as well as betanidins as substrates (Vogt *et al.*, 1997, 1999). Together, these findings highlight that many of the key enzymes of the betalain synthesis pathway have orthologues in anthocyanic outgroups, and are probably present in anthocyanic lineages within the core Caryophyllales.

The impact of molecular phylogenetic analyses

Molecular phylogenetic analyses have a major impact on our understanding of pigment evolution in the Caryophyllales. Early taxonomic treatments, influenced by the mutual exclusion of anthocyanins and betalains, placed the anthocyanin-producing Molluginaceae and Caryophyllaceae together (Caryophyllinae), separated from the betalain-producing lineages (Chenopodiinae) (Mabry, 1976). In early classifications, therefore, betalains were synapomorphic for all the betalain-producing families. This hypothesis can now be rejected. Indeed, the Molluginaceae and Caryophyllaceae form disparate lineages within Caryophyllales (Rettig *et al.*, 1992; Manhart & Rettig, 1994), each allied to clades containing betalain-producing families (Cuenoud *et al.*, 2002; Brockington *et al.*, 2009). Molluginaceae is sister to a large clade of betalain-producing succulent plant families, including Cactaceae, whereas Caryophyllaceae is sister to Amaranthaceae, which produces betalains (Cuenoud *et al.*, 2002; Brockington *et al.*, 2009). Molluginaceae is therefore nested within betalain-producing lineages (Cuenoud *et al.*, 2002; Brockington *et al.*, 2009) (Fig. 4).

Recent studies have further shown that Molluginaceae is grossly polyphyletic, complicating the estimation of the number of anthocyanin lineages in Caryophyllales (Cuenoud *et al.*, 2002; Brockington *et al.*, 2009; Schafferhoff *et al.*, 2009; S. F. Brockington *et al.*, unpublished data). The anthocyanin-producing *Macarthuria* is clearly not a member of Molluginaceae, but actually constitutes a distinct lineage in the Caryophyllales branching after *Physena* and *Asteropeia* (S. F. Brockington *et al.*, unpublished data) (Fig. 4). The anthocyanin-producing *Hypertelis* appears to be firmly nested in an otherwise betalain-pigmented clade, thereby implying an additional anthocyanic lineage (Fig. 4) (Schafferhoff *et al.*, 2009; S. F. Brockington *et al.*, unpublished data). *Hypertelis salsaloides* and *Hypertelis bowkeriana* were initially determined to be anthocyanic by Beck *et al.* (1962), and Mabry (1977) reported *Hypertelis argaepequenae* to be anthocyanic (with no data). Clement *et al.* (1994) regarded the presence of anthocyanins in *Hypertelis* as 'doubtful', but Clement & Mabry (1996) later cited *Hypertelis* as anthocyanic. Given this confusion in the literature and the position of *Hypertelis* within a betalain-pigmented clade, the presence of anthocyanins in *Hypertelis* must be re-examined. Nonetheless, taking published findings at face value, a polyphyletic Molluginaceae results in two additional disparate anthocyanic lineages.

Molluginaceae is widely cited as an anthocyanic family but, as several taxa have been removed through resolution of the polyphyletic condition, this claim is not supported by any published data. Of the nine genera that now constitute Molluginaceae *sensu stricto*, only three have been examined for the presence of pigments: *Adenogramma*, *Pharnaceum* and *Mollugo* (Fig. 4). Of these, the presence of anthocyanin in *Mollugo* was reported with no reference to experimental data (Mears, 1976), and anthocyanins in *Pharnaceum* have been reported without supporting data (Clement *et al.*, 1994). Pigments can fade with age and the absence of pigments reported in *Adenogramma* might be a result of the fact that only dried stems were analysed (Cuenoud *et al.*, 2002). The presence of pigments in vegetative tissue is also more likely to be dependent on environmental conditions, unlike floral tissue, which tends to be constitutively pigmented (Stafford, 1994). Clement *et al.* (1994) reported that most genera in Molluginaceae lack detectable pigments, but again with no reference to experimental data.

Finally, molecular phylogenetics impacts on our confidence in early character evolution in the core Caryophyllales. In the past decade, six previously obscure genera have been newly placed as lineages in the core Caryophyllales (Fig. 4): *Rhabdodendron* – originally associated with Rutaceae (Fay *et al.*, 1997); *Simmondsia* – formerly a member of the Buxaceae (Hoot *et al.*, 1999); *Asteropeia* – formerly placed within the Theales (Morton *et al.*, 1997); *Physena* – previously linked with Passifloraceae, Urticales or Sapindales (Morton *et al.*, 1997); *Macarthuria* – formerly a member of the Molluginaceae (Christin *et al.*, 2010; S. F. Brockington *et al.*, unpublished data); and *Microtea* – previously placed in Phytolaccaceae (Schafferhoff *et al.*, 2009). With the exception of *Macarthuria*, none of these taxa have been examined for their pigments, and it is therefore currently impossible to accurately reconstruct early pigment evolution in the core Caryophyllales.

The importance of character mapping

Reconstruction of character change over a well-resolved phylogeny is important in our understanding of trait evolution; yet, character mapping has hitherto played a minor role in directing research into Caryophyllid pigmentation.

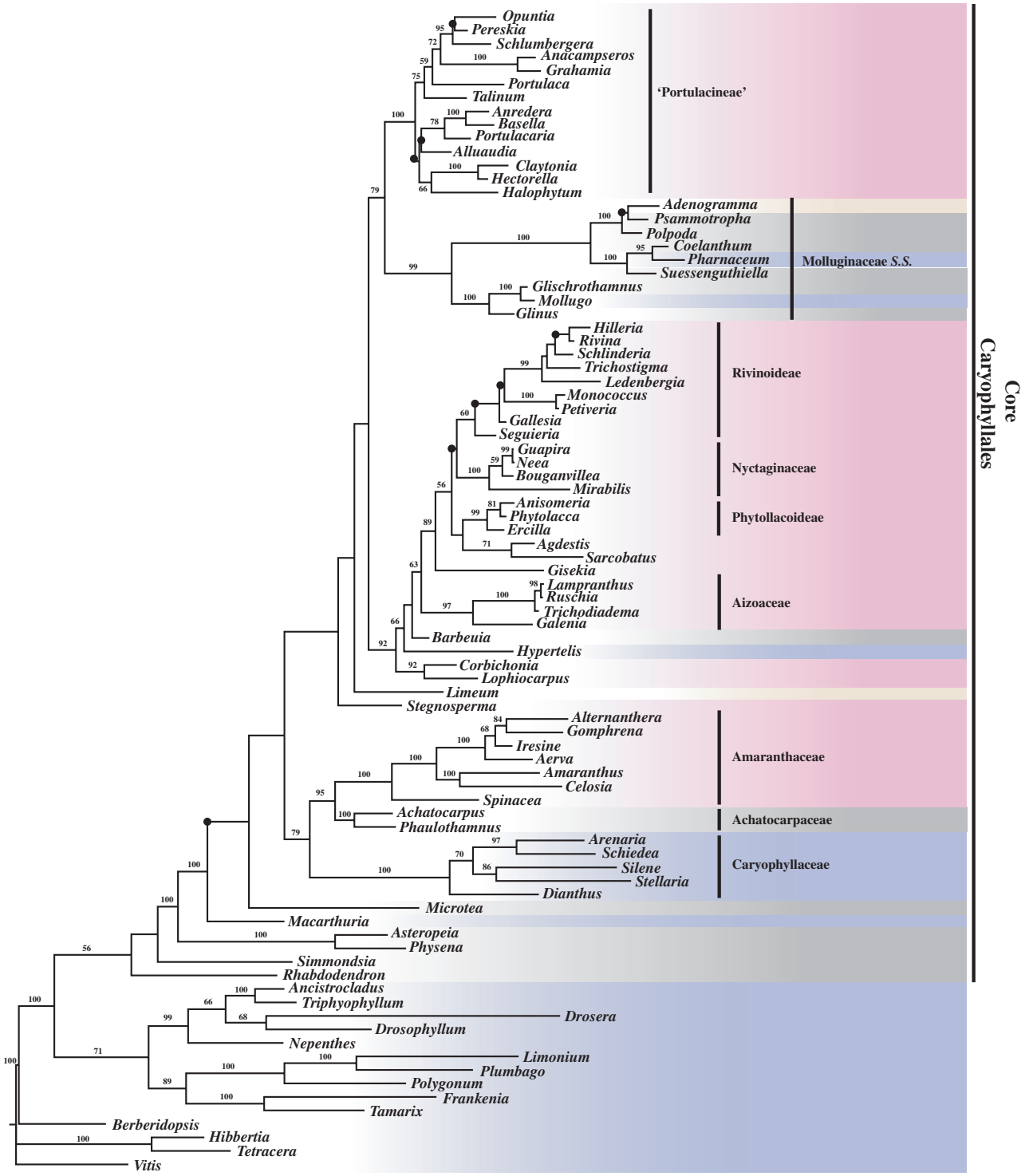

Fig. 4 Phylogram from parsimony analysis of combined *rbcL/matK* plastid genes from 82 Caryophyllid taxa and four outgroups. Circles indicate nodes that collapse in strict consensus. Numbers above the branches indicate bootstrap support. Pink blocks, betalain pigmentation; blue blocks, anthocyanin pigmentation; beige blocks, no pigments detected; grey blocks, no pigment data collected.

Phylogenetic character reconstruction can help with the following: (1) identify the context in which betalains arose; (2) estimate the number of times anthocyanin and betalain pigmentation have arisen; (3) determine the direction and temporal sequence of pigment evolution; and (4) specify which organisms should be compared in evaluating the causes of character change. With these four objectives in mind, we examine the evolutionary reconstruction of

pigmentation in the context of recent changes in our understanding of Caryophyllales phylogeny. We adopt both parsimony-based character reconstruction (implemented in Mesquite 4.05; Maddison & Maddison, 2008) and stochastic mapping (implemented in SIMMAP 1.0; Bollback, 2006). Stochastic mapping has several advantages over traditional parsimony-based reconstruction. First, it allows one to average over equally likely topologies, which is valuable because the positions of some taxa are poorly supported or poorly resolved. Second, it allows for character change along branches and is therefore a useful methodology for character reconstruction in the Caryophyllales, a clade in which long branches are common. Third, it provides a framework in which to test for correlated evolution and to assess whether the apparent mutual exclusivity of the two pigments is the result of chance alone.

In a pre-cladistic correlative approach to character evolution, Ehrendorfer (1976) associated the origin of betalain pigmentation with environmental variables, such as desertification, reduced pollinator abundance and wind pollination. It is difficult to have confidence in Ehrendorfer's (1976) hypothesis, as several newly placed lineages suggest an entomophilous ancestry. The pigment status of these newly placed lineages is unknown, but phylogenetic reconstructions indicate that, given current data, an ancestral anthocyanic condition is more likely (Fig. 5). An anthocyanic *Macarthuria* (Behnke *et al.*, 1983), together with the anthocyanic Caryophyllaceae and anthocyanic outgroups, raises the possibility that betalains may have arisen later than previously thought in the core Caryophyllales. Improved chemotaxonomic sampling is necessary to resolve the uncertainty surrounding early pigment evolution. This is an important undertaking, as it affects the inferred direction of changes in pigmentation in the core Caryophyllales and the number of inferred switches in pigmentation type.

Regardless of the inferred ancestral condition, reconstructions suggest multiple switches between pigment types (Fig. 5, Table 1). DELTRAN optimizations favouring parallel evolutionary events to explain homoplasy suggest

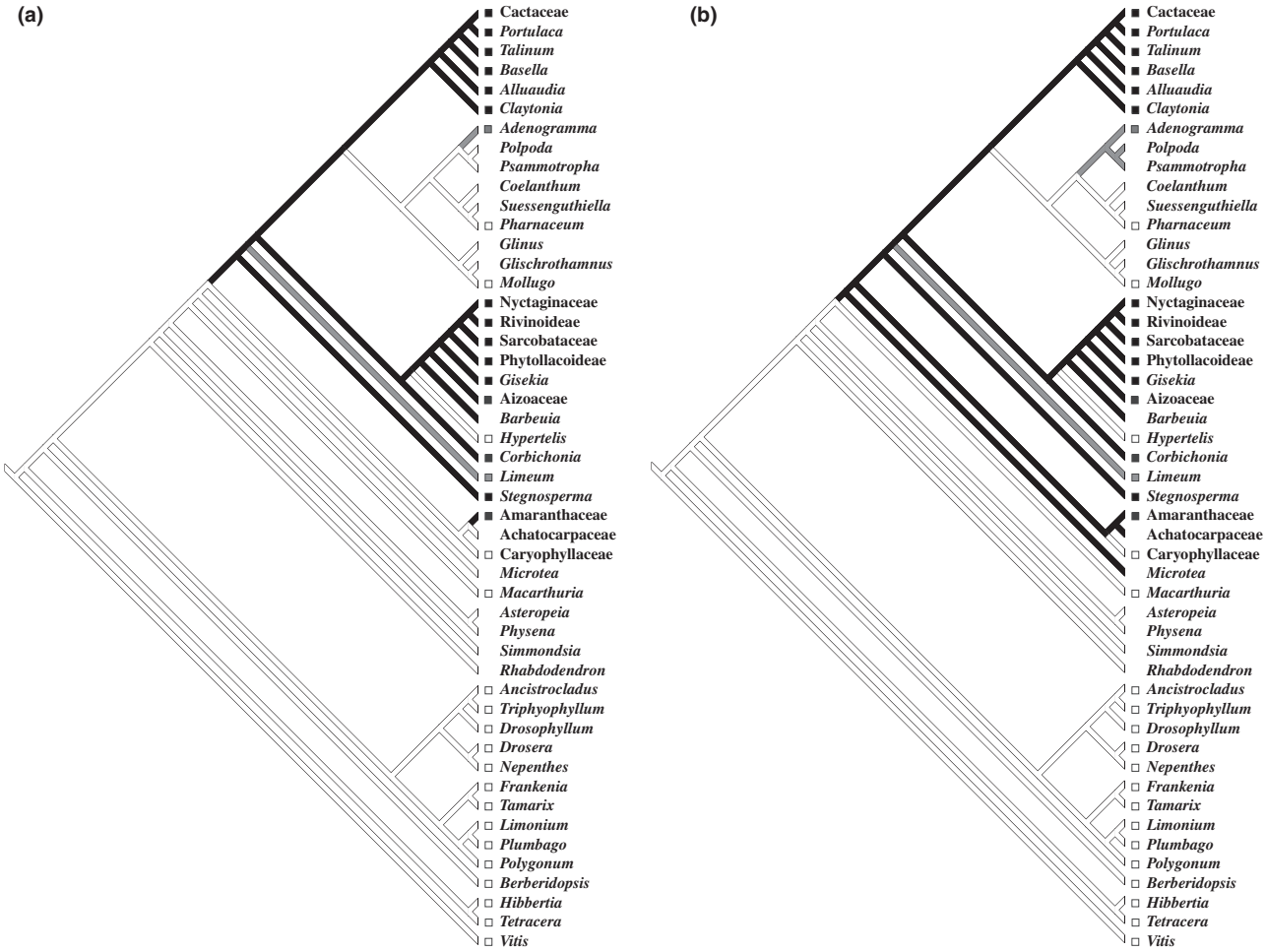

Fig. 5 Parsimony reconstruction of pigmentation across a simplified Caryophyllales tree. Black, betalain pigmentation; white, anthocyanin pigmentation; grey, no pigment detected. (a) DELTRAN optimization, (b) ACCTRAN optimization.

Table 1 Comparison of the frequency of switches between pigment types as estimated by parsimony reconstruction and stochastic character mapping

Transformation type	Parsimony: ACCTRAN	Parsimony: DELTRAN	Stochastic mapping
Anthocyanin to nonpigmented	1	2	1.12 ± 0.01
Nonpigmented to anthocyanins	0	0	0.5 ± 0.01
Betalains to nonpigmented	2	1	1.16 ± 0.01
Nonpigmented to betalains	0	0	0.09 ± 0.01
Anthocyanins to betalains	1	2	1.67 ± 0.01
Betalains to anthocyanins	3	2	3.48 ± 0.01
Total number of transitions	7	7	7.67 ± 0.02

Stochastic character mapping was implemented in SIMMAP 1.0 with 100 realizations performed on 4500 post burn-in Bayesian trees (with branch lengths). The bias parameter was 0.33 and rate parameters were defined by an α value of 4.0 and a β value of 0.4 (see Supporting Information Methods S1).

two origins of betalains, two reversals to anthocyanin and two reversals to the unpigmented condition (Fig. 5a). DELTRAN optimizations therefore propose that the anthocyanic condition in Caryophyllaceae is a retention of a plesiomorphic condition, with an additional evolution of betalains in the Amaranthaceae. ACCTRAN optimizations that favour secondary reversals as an explanation of homoplasy suggest a single origin of betalains, with three reversals to anthocyanin pigmentation and two reversals to an unpigmented state (Fig. 5b). ACCTRAN optimizations therefore suggest that betalains evolved once early on, with a reversion to anthocyanin pigmentation in Caryophyllaceae.

Stochastic mapping analyses (SIMMAP 1.0; Bollback, 2006) also suggest a number of switches between pigment types with 1.76 (± 0.1) transitions to betalains and 3.98 (± 0.1) transitions to anthocyanins within the core Caryophyllales (Table 1). Although it is possible that the apparent mutual exclusion of pigments is the product of chance, a correlation analysis within the stochastic mapping framework suggests that we can reject a null hypothesis that the mutual exclusion of the two pigments is a result of chance ($P < 0.01$). It is therefore likely that we observe a significant macroevolutionary pattern.

Together, these analyses emphasize that pigmentation is a complex labile trait within the Caryophyllales. The phylogenetic position of anthocyanic Molluginaceae and *Hypertelis* implies that there have almost certainly been switches from betalain pigmentation back to anthocyanin pigmentation. These parallel switches from betalain to anthocyanin pigmentation and, possibly, from anthocyanins to betalains provide important opportunities for comparative genetics. As the betalain synthesis pathway becomes better characterized at the genetic level, it will be interesting to examine these derived switches to anthocyanin pigmentation. The study of separate instances of reversals to anthocyanin might help to elucidate whether the regulation of anthocyanin and betalain pathways intersect to cause mutual exclusion of the two pigment types. It would be valuable to determine whether parallel inhibition of betalain synthesis has occurred at the same enzymatic steps or through similar regulatory changes that may indicate constraints in biochemical evolution. The possibility of multiple origins of betalain pigmentation is also intriguing. Biochemical and molecular data from betalain-pigmented species have been treated collectively despite being derived from phylogenetically diverse lineages. Evaluating the possibility of multiple origins of betalain pigmentation may require careful consideration of taxonomic variation in betalain synthesis, for example the glycosylation of cDOPA vs betanidin (Vogt *et al.*, 1997, 1999; Sasaki *et al.*, 2005). Ultimately, however, the discovery that many of the enzymes involved in betalain synthesis have homologues in anthocyanic lineages (Christinet *et al.*, 2004), or can utilize flavonoid substrates (Vogt *et al.*, 1997, 1999), encourages speculation that betalain pigmentation could have arisen more than once within the core Caryophyllales.

Future perspectives

Pigmentation in the Caryophyllales has a long history of research, but there is much work to be done. Focused chemotaxonomic studies are required to identify pigment types in both phylogenetically critical taxa and in taxa whose pigment status is uncertain (e.g. Molluginaceae *sensu stricto* and *Hypertelis*). We need to develop further molecular tools in betalain-pigmented taxa in order to characterize better the structural components of the betalain synthesis pathway and their regulation. Characterization of new structural and regulatory genes, in conjunction with improved molecular-based phylogenies, will help to unravel the intersection of betalain and anthocyanin pathways. Phylogenetic analysis of these structural and regulatory genes will reveal the evolutionary origins of the genes involved in betalain synthesis, and comparative analysis of the genetic changes underlying switches between pigment types will provide an insight into the mutual exclusion of anthocyanins and betalains. Finally, we know very little about the roles of betalains vs anthocyanins. What are the functions of betalains relative to anthocyanins in vegetative tissues? What are the visual properties of betalains and how do pollinators perceive them? Computational modelling of betalain pigments in pollinator visual space, together with analyses of pollinator behaviour in response to betalains, may yield answers to some of these questions. Moreover, with second-generation sequencing, the integration of molecular phylogenetics, comparative genetics and molecular biology is likely to generate substantial advances in our understanding of pigmentation biology in the Caryophyllales.

Acknowledgements

We thank Khidir Hilu, Sunny Crawley and Michael Moore for reading earlier versions of the manuscript. This research was supported by a DDIG grant (DEB-0808342) to D. E. S. and S. F. B., and an EU Marie Curie IRG Fellowship to S. F. B. and B. J. G.

References

APG II. 2003. An update of the angiosperm phylogeny group classification for the orders and families of flowering plants: APG II. *Botanical Journal of the Linnean Society* **141**: 399–436.

Bate-Smith E. 1962. The phenolic constituents of plants and their taxonomic significance. *Botanical Journal of the Linnean Society* **58**: 95–173.

Beck E, Merxmuller H, Wagner H. 1962. Uber die art der anthocyane bei Plumbaginaceen, Alsinoideen und Molluginaceen. *Planta* **58**: 220–224.

Behnke HD, Mabry TJ, Neumann P, Barthlott W. 1983. Ultrastructural, micromorphological and phytochemical evidence for a ''central position'' of *Macarthuria* (Molluginaeae) within the Caryophyllales. *Plant Systematics and Evolution* **143**: 151–161.

Bischoff H. 1876. Das Caryophyllinenroth. *Inaugural Dissertation*. Tubingen, Germany: University of Tubingen.

Bittrich B, Amaral M. 1991. Proanthocyanidins in the testa of centrospermous seeds. *Biochemical Systematics and Ecology* **19**: 319–321.

Bollback JP. 2006. SIMMAP: Stochastic character mapping of discrete traits on phylogenies. *BMC Bioinformatics* **7**: 88.

Brockington S, Alexandre R, Ramdial J, Moore M, Crawley S, Dhingra A, Hilu K, Soltis P, Soltis D. 2009. Phylogeny of the Caryophyllales *sensu lato*: revisiting hypotheses on pollination biology and perianth differentiation in the core Caryophyllales. *International Journal of Plant Science* **170**: 627–643.

Christinet L, Burdet FRX, Zaiko M, Hinz U, Zryd JP. 2004. Characterization and functional identification of a novel plant 4,5-extradiol dioxygenase involved in betalain pigment biosynthesis in *Portulaca grandiflora*. *Plant Physiology* **134**: 265–274.

Clement JS, Mabry TJ. 1996. Pigment evolution in the Caryophyllales: a systematic overview. *Botanica Acta* **109**: 360–367.

Clement J, Mabry T, Wyler H, Dreiding A. 1994. Chemical review and evolutionary significance of the betalains. In: Behnke H-D, Mabry T, eds. *Caryophyllales: evolution and systematics*. Berlin, Germany: Springer, 247–261.

Cuenoud P, Savolainen V, Chatrou LW, Powell M, Grayer RJ, Chase MW. 2002. Molecular phylogenetics of Caryophyllales based on nuclear 18S rDNA and plastid *rbcL*, *atpB*, and *matK* DNA sequences. *American Journal of Botany* **89**: 132–144.

Delgado-Vargas F, Paredes-Lopez O. 2002. Anthocyanins and betalains. *Natural colorants for food and nutraceutical uses*. Boca Raton, FL, USA: CRC Press, 167–219.

Dooner HK, Robbins TP, Jorgensen RA. 1991. Genetic and developmental control of anthocyanin biosynthesis. *Annual Review of Genetics* **25**: 173–199.

Ehrendorfer F. 1976. Closing remarks: systematics and evolution of centrospermous families. *Plant Systematics and Evolution* **126**: 99–106.

Fay MF, Cameron KM, Prance GT, Lledo MD, Chase MW. 1997. Familial relationships of *Rhabdodendron* (Rhabdodendraceae) plastid *rbcL* sequences indicate a Caryophyllid placement. *Kew Bulletin* **52**: 923–932.

Grotewold, E. 2006. The genetics and biochemistry of floral pigments. *Annual Reviews of Plant Biology* **57**: 761–780.

Hoot SB, Magallon S, Crane PR. 1999. Phylogeny of basal eudicots based on three molecular data sets: *atpB*, *rbcL*, and 18S nuclear ribosomal DNA sequences. *Annals of the Missouri Botanical Garden* **86**: 1–32.

Joy RW, Sugiyama M, Fukuda H, Komamine A. 1995. Cloning and characterization of polyphenol oxidase cDNAs of *Phytolacca americana*. *Plant Physiology* **107**: 1083–1089.

Koes R, Verweij W, Quattrocchio F. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends in Plant Science* **10**: 236–242.

Mabry T. 1964. *The betacyanins, a new class of red violet pigments, and their phylogenetic significance*. New York, NY, USA: Roland Press.

Mabry T. 1976. Pigment dichotomy and DNA–RNA hybridization data for centrospermous families. *Plant Systematics and Evolution* **126**: 79–94.

Mabry TJ. 1977. The order Centrospermae. *Annals of the Missouri Botanic Gardens* **64**: 210–220.

Mabry T. 1980. Betalains. In: Bell E, Charwood B, eds. *Encyclopedia of plant physiology, secondary plant products*. Berlin, Germany: Springer-Verlag, 513–533.

Maddison W, Maddison D. 2008. *Mesquite: a modular system for evolutionary analysis, version 2.5*. [WWW document]. URL http://mesquiteproject.org [accessed 12 December 2010].

Manhart J, Rettig JH 1994. Gene sequence data. In: Behnke H-D, Mabry T, eds. *Caryophyllales: evolution and systematics*. Berlin, Germany: Springer, 235–246.

Mears J. 1976. Guide to research in plant taxonomy. *Chemical Plant Taxonomy Newsletter* **25**: 11–14.

Menzel R, Blakers M. 1975. Colour receptors in the bee eye – morphology and spectral sensitivity. *Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology* **108**: 11–13.

Mol J, Grotewold E, Koes R. 1998. How genes paint flowers and seeds. *Trends in Plant Science* **3**: 212–217.

Morton CM, Karol KG, Chase MW. 1997. Taxonomic affinities of *Physena* (Physenaceae) and *Asteropeia* (Theaceae). *Botanical Review* **63**: 231–239.

Mueller LA, Hinz U, Uze M, Sautter C, Zryd JP. 1997. Biochemical complementation of the betalain biosynthetic pathway in *Portulaca grandiflora* by a fungal 3,4-dihydroxyphenylalanine dioxygenase. *Planta* **203**: 260–263.

Mueller LA, Hinz U, Zryd JP. 1996. Characterization of a tyrosinase from *Amanita muscari*a involved in betalain biosynthesis. *Phytochemistry* **42**: 1511–1515.

Piatelli M. 1981. The betalains: structure, biosynthesis, and chemical taxonomy. In: Conn EE, ed. *The biochemistry of plants: a comprehensive treatise*. New York, NY, USA: Academic Press, 557–575.

Ramsay NA, Glover BJ. 2005. MYB–bHLH–WD40 protein complex and the evolution of cellular diversity. *Trends in Plant Science* **10**: 63–70.

Rettig JH, Wilson HD, Manhart JR. 1992. Phylogeny of the Caryophyllales – gene sequence data. *Taxon* **41**: 201–209.

Sasaki N, Wada K, Koda T, Kasahara K, Adachi T. 2005. Isolation and characterization of cDNAs encoding an enzyme with glucosyltransferase activity for cyclo-DOPA from Four O'clocks and Feather Cockscombs. *Plant and Cell Physiology* **46**: 666–670.

Schafferhoff B, Muller K, Borsch T. 2009. Caryophyllales phylogenetics: disentangling Phytolaccaceae and Molluginaceae and the description of Microteaceae as a new isolated family. *Willdenowia* **39**: 209–228.

Shimada S, Inoue YT, Sakuta M. 2005. Anthocyanidin synthase in non-anthocyanin-producing *Caryophyllales* species. *Plant Journal* **58**: 950–959.

Shimada S, Otsuki H, Sakuta M. 2007. Transcriptional control of anthocyanin biosynthetic genes in the Caryophyllales. *Journal of Experimental Botany* **58**: 957–967.

Shimada S, Takahashi K, Sato Y, Sakuta M. 2004. Dihydroflavonol 4-reductase cDNA from non-anthocyanin producing species in the Caryophyllales. *Plant and Cell Physiology* **45**: 1290–1298.

Stafford HA. 1994. Anthocyanins and betalains: evolution of the mutually exclusive pathways. *Plant Science* **101**: 91–98.

Steiner U, Schliemann W, Bohm H, Strack D. 1999. Tyrosinase involved in betalain biosynthesis of higher plants. *Planta* **208**: 114–124.

Tanaka Y, Sasaki N, Ohmiya A. 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids. *Plant Journal* **54**: 733–749.

Vogt T, Grimm R, Strack D. 1999. Cloning and expression of a cDNA encoding betanidin 5-O-glucosyltransferase, a betanidin- and flavonoid-specific enzyme with high homology to inducible glucosyltransferases from the Solanaceae. *Plant Journal* **19**: 509–519.

Vogt T, Zimmermann E, Grimm R, Meyer M, Strack D. 1997. Are the characteristics of betanidin glucosyltransferases from cell-suspension cultures of *Dorotheanthus bellidiformis* indicative of their phylogenetic relationship with flavonoid glucosyltransferases? *Planta* **203**: 349–361.

Whittall JB, Strauss S. 2006. Non-pollinator agents of selection on floral traits. In: Harder L, Barrett S, eds. *Ecology and evolution of flowers*. Oxford, UK: Oxford University Press, 120–138.

Supporting Information

Additional supporting information may be found in the online version of this article.

Methods S1 Methodology for Bayesian analyses, stochastic mapping and correlation analyses.

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