New Phytologist

Research

Widespread flower color convergence in Solanaceae via alternate biochemical pathways

Julienne Ng and Stacey D. Smith

Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO 80309, USA

Author for correspondence:
Julienne Ng
Tel: +1 303 492 9668
Email: julienne.ng@colorado.edu

Received: *5 January 2015*
Accepted: *18 June 2015*

New Phytologist (2015)
doi: 10.1111/nph.13576

Key words: ancestral state reconstruction, anthocyanins, carotenoids, flower color evolution, phenotypic convergence, phylogenetic signal, pigment pathway, Solanaceae.

Summary

- Phenotypic convergence is rampant throughout the tree of life. While recent studies have made significant progress in ascertaining the proximate mechanisms underlying convergent phenotypes, less is known about the frequency and predictability with which convergent phenotypes arise via the same or multiple pathways at the macroevolutionary scale.
- We investigated the proximate causes and evolutionary patterns of red flower color in the tomato family, Solanaceae, using large-scale data mining and new sequence data to reconstruct a megaphylogeny of 1341 species. We then combined spectral and anatomical data to assess how many times red flowers have evolved, the relative contribution of different pathways to independent origins of red, and whether the underlying pathway is predicted by phylogenetic relatedness.
- We estimated at least 30 relatively recent origins of red flowers using anthocyanins, carotenoids, or a dual production of both pigments, with significant phylogenetic signal in the use of anthocyanins and dual production, indicating that closely related red-flowered species tend to employ the same mechanism for coloration.
- Our study is the first to test whether developmental pathways exhibit phylogenetic signal and implies that historical contingency strongly influences the evolution of new phenotypes.

Introduction

Phenotypic convergence, whereby distantly related species evolve similar traits, provides a unique opportunity to study naturally occurring evolutionary replicates. Convergence in phenotype is often taken as evidence that natural selection is acting to drive traits to a particular optimum in response to the same selective regime, and can therefore be used to understand whether the evolutionary process is predictable under the same selective pressures (Harvey & Pagel, 1991; Schluter, 2000; but see discussion in Losos, 2011 for alternative explanations). For example, the evolution of convergent phenotypes across entire evolutionary radiations facing similar environmental pressures suggests that morphological diversification can be not only deterministic but repeatable as well (e.g. Gillespie, 2004; Melville *et al.*, 2006; Muschick *et al.*, 2012; Mahler *et al.*, 2013; Grundler & Rabosky, 2014). It remains less clear, however, whether the underlying developmental, biochemical and genetic changes are also predictable at the macroevolutionary (phylogenetic) scale.

Intrinsic factors, such as underlying developmental and biochemical pathways, have long been recognized to shape the trajectory of phenotypic evolution (Maynard Smith *et al.*, 1985; Wake, 1991; Schluter, 1996; Wake *et al.*, 2011). Moreover, lineage-specific historical contingency can also influence how traits evolve (Gould, 2002; Rosenblum *et al.*, 2014). The potential role of intrinsic factors and historical contingency in trait evolution is exemplified by instances of 'incomplete' trait convergence, whereby species have become more similar to each other than were their ancestors but have not, or only partially, reached phenotypic convergence (Herrel *et al.*, 2004), or 'imperfect' convergence, where traits evolve along a similarly oriented trajectory towards a new region in morphospace but exhibit greater variation than seen among other species under different selective regimes (Collar *et al.*, 2014). Identifying the intrinsic factors responsible for trait development is therefore integral to understanding the evolution of convergent phenotypes.

In some systems, convergent traits have been demonstrated to arise via the same pathway, suggesting that phenotypic convergence may be linked to developmental and molecular convergence. For example, the repeated evolution of melanic phenotypes across a range of vertebrates, including lizards, birds, mice and mammoths, traces back to the same pigmentation pathway, although the specific gene and mutation responsible can vary (Hoekstra, 2006; Manceau *et al.*, 2010). There are, however, other cases where convergent phenotypes have arisen via different pathways. For example, the convergent elongated body shape of fossorial salamanders is attributable to either an increased number of vertebrae or the elongation of individual vertebrae (Parra-Olea & Wake, 2001). Despite the detailed knowledge of the mechanisms of convergence in these model systems, less is known about the frequency with which the same pathways lead to convergent phenotypes at broad macroevolutionary scales (Shapiro

et al., 2006), and whether use of the same pathway for the convergent phenotype is more likely among closely related species (Hall, 2003) or not (Arendt & Reznick, 2008).

Angiosperm flower color presents an excellent opportunity to test the frequency and predictability with which convergent phenotypes arise via the same or multiple pathways at the macroevolutionary scale. First, angiosperms are known not only for their diversity of flower color and form, but also for the widespread convergence of independent lineages on similar floral phenotypes (e.g. Brown & Kodric-Brown, 1979; Johnson *et al.*, 1998; Papadopulos *et al.*, 2013). Second, the three major biosynthetic pathways that underlie floral pigmentation (carotenoid, anthocyanin and betalain biosynthetic pathways) have been well characterized and are readily identifiable (Grotewold, 2006; Tanaka *et al.*, 2008). Third, these different pigment pathways can produce the same flower coloration, allowing investigations into how different pathways contribute to phenotypic convergence across the phylogeny. Nonetheless, few studies have examined the relative importance of these distinct pathways to flower color variation on a macroevolutionary scale (but see Brockington *et al.*, 2011). Here, we investigated convergence in flower color and biochemical pathways in the Solanaceae family, with particular focus on the evolution of red flower coloration.

Solanaceae comprises *c.* 2700 species that vary widely not only in flower coloration, but also in floral morphology and life form (Knapp, 2002, 2010; Sarkinen *et al.*, 2013). Red-flowered species can be found in different Solanaceae clades, suggesting that red flowers have independently evolved multiple times. Red coloration can arise from all three of the major pigment pathways in angiosperms (Grotewold, 2006; Tanaka *et al.*, 2008), but only carotenoids and anthocyanins are found in Solanaceae (Eich, 2008). Furthermore, carotenoids and anthocyanins can co-occur, and the combined effect of the two pigments may also result in reds, oranges or even bronze or brown hues that neither pigment is capable of producing alone (Griesbach, 1984; Forkmann, 1991). In addition to the presence of these pigments, there are other factors that may affect the hue of a flower, such as a change in vacuolar pH and cell shape (Forkmann, 1991; Mol *et al.*, 1998). In this study, we focus on identifying which of the two pigment pathways (carotenoid or anthocyanin) underlies the convergent evolution of red flowers in Solanaceae. Given the importance of a resolved phylogeny to address questions about phenotypic convergence, we first reconstructed a new phylogenetic tree for Solanaceae through large-scale data mining and the generation of new sequence data for red-flowered species and their close relatives. Using this expanded phylogeny of *c.* 1300 species, we addressed three main questions: (i) Is red flower coloration a convergent trait in Solanaceae?; (ii) What is the frequency with which different biochemical pathways are involved in the evolution of red coloration of Solanaceae flowers?; (iii) Do more closely related species tend to use the same pathway to produce red flowers, that is, does pathway use show significant phylogenetic signal? This will form the first test in any system of the hypothesis that more closely related species are likely to achieve convergent phenotypes by repeated recruitment of the same developmental pathway.

Materials and Methods

Phylogenetic reconstruction of Solanaceae

By searching the literature and herbarium collections, we identified 36 species of Solanaceae described as having red flowers (Supporting Information Table S1). We included species that are polymorphic for red coloration and those whose corolla is partly red, because we were interested in the evolution of the ability to produce red pigmentation in the corolla. Although the phylogeny of the family has been the subject of many systematic studies (Olmstead *et al.*, 2008; Sarkinen *et al.*, 2013), many of the red-flowered species have not been sampled, and resolution within a number of clades containing red-flowered species is poor (Montero-Castro *et al.*, 2006; Fregonezi *et al.*, 2012). In order to obtain an accurate estimate of the total number of transitions to red flowers, we reconstructed a new Solanaceae phylogeny with increased sampling of species and genes in clades containing red flowers. We used two approaches to obtain sequence data: we accessed publically available sequences using the PHLAWD pipeline (Smith *et al.*, 2009), and we generated new sequence data for red-flowered species and closely related non-red congeners that did not have available sequences for the target genes (Table S2; GenBank accession numbers within).

We constructed a supermatrix for Solanaceae that comprised two nuclear regions (the granule-bound starch synthase (GBSSI or *waxy*) gene and the internal transcribed spacer (ITS) region), and three chloroplast regions (*mat*K, *ndh*F and *trnT-trnF*). We used the PHLAWD pipeline to retrieve homologous sequences from GenBank (accessed 10 February 2015; Smith *et al.*, 2009), which involved a BLAST search of user-supplied sequences against the GenBank nucleotide database. We specified that all varieties, forms, affinities and unknown species were to be excluded. This provided sequences for 1327 species, of which we removed 11 that were subspecies or suspect sequences that were placed in an unexpected clade in gene tree reconstructions. We sampled four of the outgroups used in Olmstead *et al.* (2008): three from the sister clade to Solanaceae, Convolvulaceae (*Convolvulus arvensis*, *Evolvulus glomeratus* and *Dinetus truncatus*), and one from a more distantly related group within Solanales (*Montinia caryophyllacea*).

To generate our own sequences, we used silica-dried leaf tissue from the field, samples from herbarium specimens, and fresh leaves from glasshouse-grown individuals from commercial sources. We also obtained tissue, already extracted DNA, or PCR products from L. Bohs, R. Olmstead, L. Freitas and Kew Gardens' DNA bank. Genomic DNA was extracted from leaf tissue using a modified 2× CTAB protocol (Doyle & Doyle, 1987). Protocols for amplification of the five targeted regions (GBSSI, ITS, *mat*K, *ndh*F and *trnT-trnF*) are provided in the supplementary material (Methods S1). We inspected sequence quality using GENEIOUS v5.1.4 (Drummond *et al.*, 2006) and used MAFFT v7.150b (Katoh & Standley, 2013) to add our sequences to the other GenBank-retrieved sequences that had been aligned via the PHLAWD pipeline. We conducted phylogenetic analyses on the concatenated matrix using partitioned maximum likelihood

(ML) searches in RAxML v7.2.8 (Stamatakis, 2006). We used a GTR model with gamma distributed rate variation for each gene partition with 1000 nonparametric bootstrap replicates. The resulting phylogeny was ultrametricized using semiparametric penalized likelihood with the chronopl function in the ape R package and a smoothing parameter of 1 (Sanderson, 2002; Paradis *et al.*, 2004). We assigned the root age as 49 million yr (Myr) following Sarkinen *et al.* (2013).

Quantifying reflectance for red flowers

In order to quantitatively characterize flower coloration, we obtained reflectance data for 24 putative red-flowered species using a JAZ spectrometer with a built-in pulsed xenon light source (Ocean Optics, Dunedin, FL, USA) (Table S1; spectra deposited in the Floral Reflectance Database; www.reflectance.co.uk). We measured reflectance from three corollas of each species freshly obtained either from plants in the field or from glasshouse-grown individuals. For polymorphic taxa (Table S1), we selected individuals of the red morph for these measurements (and for anatomical and HPLC analysis; see next section). We used an anodized aluminum probe holder which both eliminated ambient light and ensured measurements were taken at a 45° angle tip to prevent specular reflection (glare) (Endler, 1990). Each measurement was standardized using a Spectralon white standard (Labsphere, North Sutton, NH, USA). For each corolla, we measured the corolla lobe, the midpoint of the tube's exterior and, if there was variation along the tube exterior, the distal portion of the exterior. We used the pavo package in R (Maia *et al.*, 2013; R Core Team, 2014) to average corolla measurements across sampled individuals of a species and to smooth spectral curves to remove electrical noise introduced by the spectrometer.

We characterized flower color hue using the reflectance at the midpoint between the minimum and maximum reflectances ($\lambda_{R\text{mid}}$). As $\lambda_{R\text{mid}}$ is based on two reflectance measurements, it is a more reliable measure than solely using the wavelength at which reflectance is highest (Montgomerie, 2006), and is an established metric that has been used to characterize color in a range of taxa (e.g. Pryke *et al.*, 2001; Hofmann *et al.*, 2006; Cummings, 2007; Ng *et al.*, 2013). We calculated $\lambda_{R\text{mid}}$ from the reddest portion of the corolla of each species. To classify these spectra, we defined the $\lambda_{R\text{mid}}$ bounds by obtaining spectral data for Munsell color standards denoted to have a red hue ('*R*') (Munsell, 1976). We considered the flowers to be red if they fell within the range of these red Munsell spectra.

We were unable to obtain reflectance data for the remaining 12 species that had been described as having red flowers. We conducted all analyses with and without scoring these species as red and, as the results were qualitatively similar, we only report those in which the 12 species are included.

Characterizing pigment pathways

We conducted an anatomical study of petal tissue of 27 species to determine the presence of anthocyanin and carotenoid pigments in red corollas (Table S1). Cells colored by water-soluble anthocyanins have a uniform color across the cell as these pigments are stored within the vacuole, which comprises most of the plant cell volume (Tanaka *et al.*, 2008). Carotenoids, in contrast, are produced and stored in plastids (chromoplasts), and appear as discrete, colored intracellular compartments (Tanaka *et al.*, 2008). Cells that produce both classes of pigments thus have a colored vacuole as well as colored chromoplasts. We conducted petal peels to observe epidermal and parenchymatous petal cells with light microscopy and scored each species for the presence or absence of each class of pigment. When fresh tissue could not be obtained (in three species), we used silica-dried tissue or material from herbarium specimens. Conducting tests using available glasshouse material, we confirmed that the presence of both pigments could be accurately identified using a combination of examining dried tissue directly under the microscope as well as after rehydration with water. We verified our visual assessment by using high-performance liquid chromatography (HPLC) with five representative species to test for the presence or absence of anthocyanin pigments (Methods S2).

Reconstructing the evolutionary transitions to red flowers

We assessed the level of convergence for red flowers by reconstructing ancestral states across the family as either red-flowered or non-red-flowered. We mapped the evolution of red flowers onto the ML phylogeny using two different models: the Mk2 model, which allows for unequal transition rates (Pagel, 1994), and the binary state-dependent speciation and extinction (BiSSE) model, which additionally allows for the possibility of state-dependent diversification (Maddison *et al.*, 2007). We included the latter approach because reconstruction of a trait's history can be biased if the trait itself affects diversification (Maddison, 2006; Goldberg *et al.*, 2008; Ng & Smith, 2014). For the Mk2 model, we estimated ancestral states using ML with the asr.marginal function in the R package diversitree (FitzJohn, 2012). To test the robustness of our results, we repeated this analysis for all 1000 bootstrap trees. For the more complex BiSSE model, we first estimated posterior distributions for the model parameters using a Markov chain Monte Carlo (MCMC) chain of 10 000 steps and an exponential prior with rate $1/(2r)$, where r is the estimated diversification rate (following Johnson *et al.*, 2011). We accounted for missing taxa using the 'skeleton tree' approach, which assumes that unsampled species are randomly distributed (FitzJohn *et al.*, 2009). After visual inspection of the MCMC chain, we discarded the first 1107 steps as burn-in, and reconstructed ancestral states (again with the asr.marginal function) for each set of parameter values from the remaining 8893 steps. We then calculated the average probability of having red flowers and the 95% credibility interval at each node across these 8893 reconstructions. We considered nodes with red flower states as those with at least 95% posterior probability.

Testing for phylogenetic signal of pigment pathways

We tested whether phylogenetic relatedness predicts the pigment pathway used to make red flowers by estimating the phylogenetic

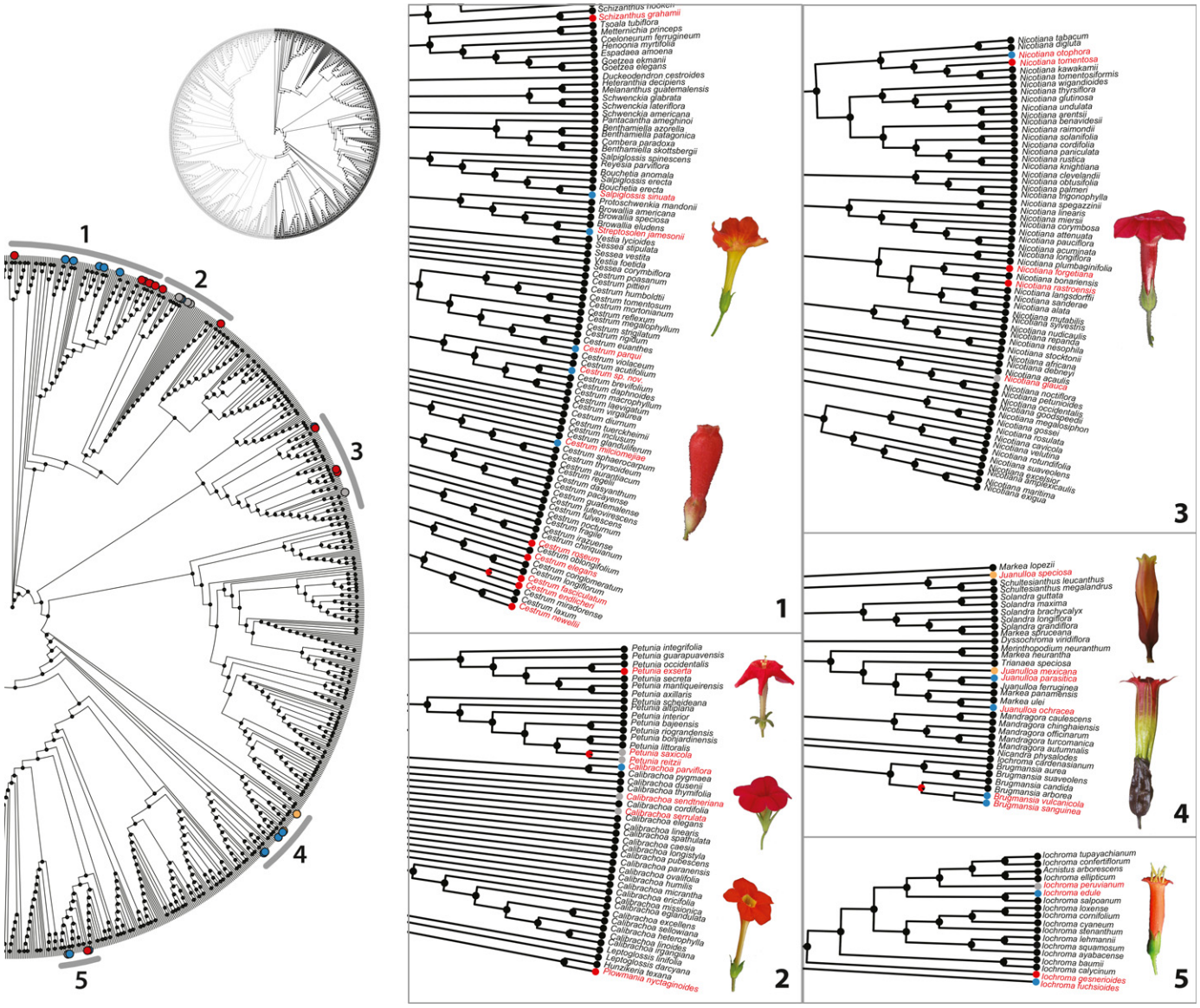

Fig. 1 Reconstruction of red flower evolution in Solanaceae. Topology from five-gene maximum likelihood analysis of 1341 species is shown. Only the portions of the tree (numbered 1–5) with red-flowered species (names in red) are shown in detail. The color of the circles for each red-flowered tip represents the pigment pathway used to produce red coloration: red circles, anthocyanin pathway; orange circles, carotenoids; blue circles, dual production of both anthocyanins and carotenoids. Gray circles represent red-flowered states of which the pigment pathway is unknown. Circles at nodes indicate the posterior probabilities from Bayesian ancestral state reconstruction, with red and black representing red- and non-red-flowered states, respectively. Flower images show red-flowered representatives of the respective groups (not to scale).

signal for each pathway following Maddison & Slatkin (1991). This approach compares the most parsimonious number of state changes, or transitions to that pathway, required to explain the distribution of tip states on our reconstructed phylogeny with a null distribution of 1000 randomly reshuffled tip states and the number of taxa in each state held constant. If our observed data showed significantly fewer parsimonious state changes, we considered the pathway to have phylogenetic signal and therefore species using that pathway were more related than expected by chance.

Results

Phylogeny of Solanaceae

Our final supermatrix comprised 1341 Solanaceae taxa, which included 313 kbp of new sequence data for 104 species. The matrix was 10931 bp in length, of which 7553 were variable, with 30.9% missing data. This data set is therefore a significant improvement in data completeness compared with previous

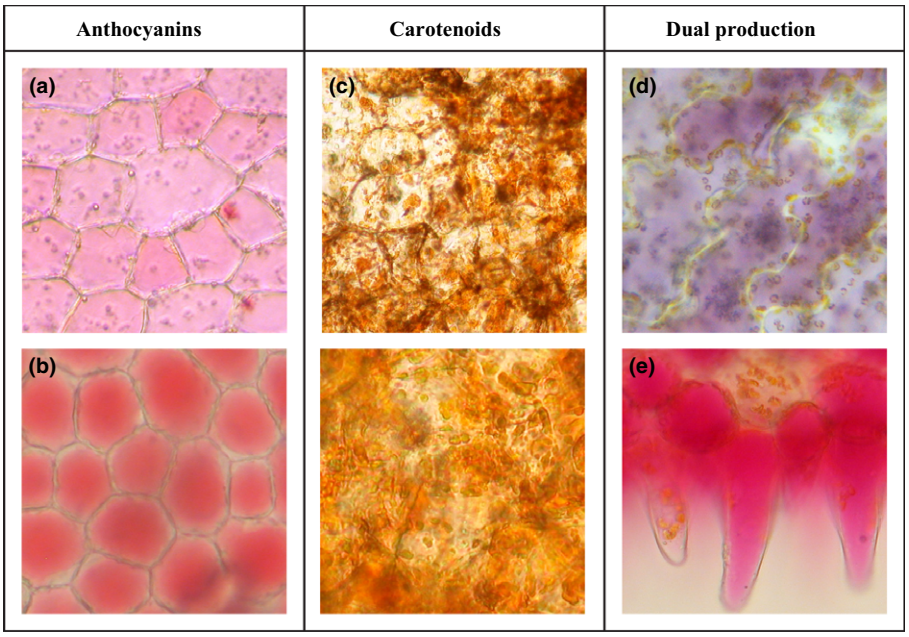

Fig. 2 Representative images of petal cells showing (a, b) anthocyanins, (c) carotenoids, and (d, e) dual production of both pigments. The uniform color across the red corolla lobe cells of (a) *Iochroma gesnerioides* and (b) *Petunia exserta* indicates the presence of anthocyanins. Clear leucoplasts are also present in the cells of *I. gesnerioides* (a) but no colored plastids are present. (c) Top and bottom images both show cells of the red part of *Juanulloa mexicana*'s corolla tube. Colored plastids and no uniform color in the cells indicate that carotenoids are responsible for the red coloration. Both colored plastids and a uniform color are seen in the cells of (d) *Cestrum parqui*'s red corolla tube and (e) *Calibrachoa parviflora*'s red lobe, indicating that dual production of both anthocyanins and carotenoids contributes to the red coloration.

Solanaceae data sets (e.g. 54.7% missing data, Sarkinen *et al.*, 2013). The topology of the major groups is consistent with Olmstead *et al.*'s (2008) phylogeny of the family (Figs 1, S1; TreeBase study ID S16617). We similarly found *Schizanthus* to be sister to the rest of Solanaceae (bootstrap support = 100%), and the tribe Goetzeoideae and *Duckeodendron* to be sister to the remaining Solanaceae. However, while *Reyesia* had previously been placed with Goetzeiodeae and *Duckeodendron* (Sarkinen *et al.*, 2013), we found *Reyesia* to be assigned to a moderately supported clade with *Salpiglossis* and *Bouchetia* (bootstrap support = 74%). *Reyesia* has previously been grouped with *Salpiglossis* based on morphology (Hunziker, 2001), but *Bouchetia* has previously been reported to be sister to *Plowmania* and *Hunzikeria* (Olmstead *et al.*, 2008; Sarkinen *et al.*, 2013). The 'X = 12' clade, which includes Solanoideae, the Australian endemic tribe Anthocercideae and *Nicotiana*, and is termed such because of a putative base chromosome number synapomorphy (Olmstead & Sweere, 1994), was recovered with moderate support (bootstrap support = 80%).

Our data set included 10 red-flowered species that had not previously been present in Solanaceae-wide phylogenies and, overall, included all but one of the 34 red-flowered species. Comparing our results with those of previous genus-level studies, we found that *Petunia reitzii* and *Petunia saxicola* were well-supported sister species with *Petunia exserta* more distantly related, as in previous analyses (Kulcheski *et al.*, 2006). *Cestrum* was also a well-supported clade (bootstrap support = 89%) but the relationships within the genus remained poorly resolved (Montero-Castro *et al.*, 2006). Within *Iochroma*, we recovered different relationships from those that had previously been found (Smith & Baum, 2006). For example, some previously well-supported clades, such as the 'A clade' that included the red-flowered *Iochroma edule* and *Iochroma peruvianum*, were no longer well supported, probably as a result of the addition of plastid sequences (D. Gates *et al.*, unpublished). Comparing our tree to previously reported relationships of *Nicotiana* (Clarkson *et al.*, 2004), we found that the red-flowered *Nicotiana* were placed in the same well-supported clades.

Our study also provides the first targeted molecular phylogeny for *Juanulloa*. Evolutionary studies of *Juanulloa* had previously been restricted to morphological analyses (Knapp *et al.*, 1997) and our phylogeny (with new sequences for six *Juanulloa* species) supports previous results showing *Juanulloa* as a nonmonophyletic group. Similar to the morphological tree, the two red-flowered *Juanulloa* species, *Juanulloa mexicana* and *Juanulloa parasitica*, were placed in the same clade (although with low bootstrap support of 64%), and the other red-flowered *Juanulloa* species, *Juanulloa speciosa* and *Juanulloa ochracea*, were instead grouped with *Schultesianthus* and *Markea*, respectively (bootstrap support of 100 and 96%, respectively). Given that *Juanulloa* is nonmonophyletic with strong bootstrap support, we suggest that further work is now needed to define new generic boundaries in the Juanulloeae tribe (A. Orejuela, unpublished).

Quantifying reflectance for red flowers

Based on the $\lambda_{R\text{mid}}$ calculated from the spectral data for Munsell color standards denoted '*R*' (Munsell, 1976), we considered flowers to be red if $\lambda_{R\text{mid}}$ was between 575 and 665 nm. Two Cuban species, *Brunfelsia cestroides* and *Brunfelsia purpurea*, did not fit this criterion (Table S1). These species have been described as having purple flowers (e.g. León & Alain, 1974), or sometimes red or purplish red (Plowman, 1998; Knapp, 2010). However, we were only able to find specimens with purple flowers in Cuban herbaria and living collections (Herbario del Jardín Botánico Nacional and Herbario de la Academia de Ciencias)

and therefore removed these species from our analyses. This left a total of 22 of the measured species characterized as producing red flowers (Table S1).

Characterizing pigment pathways

We found that red-flowered species used different pathways to make red (Fig. 1; Table S1). Petal peels showed either the sole presence of anthocyanins without any visible colored plastids (12 species), only visible colored plastids without evidence of anthocyanins (two species), or the presence of both pigments (herein called dual production) (13 species) (Figs 2, S2). These results were verified using HPLC on five representative species (Fig. S3). Our finding of the presence of anthocyanins in some red-flowered Solanaceae confirms previous reports of the six red-flowered species whose pigments have been characterized (Table S1; Robinson & Robinson, 1934; Beale *et al.*, 1941; Miller *et al.*, 1967; Griesbach *et al.*, 1999; Ando *et al.*, 2000; Waterworth & Griesbach, 2001; Smith & Rausher, 2011). However, the additional contribution of carotenoids to red coloration in some of these same species has only been previously reported for *Calibrachoa* (Murakami, 2004). Our study also reveals that carotenoids alone can contribute to red-flowered Solanaceae.

Reconstructing the evolutionary transitions to red flowers

Our ancestral state reconstructions under the Mk2 model revealed 30 independent origins of red flowers. This was also supported by analyses performed on each of the 1000 bootstrap trees (30 ± 0.065 SE). When accounting for the possibility of state-dependent diversification, ancestral state reconstruction predicted 30–33 independent origins (taking into consideration the credibility intervals). The number of origins of red flowers may be an underestimation given that we are missing one red-flowered species in the phylogeny. Of the 31 transitions to red flowers, the utilization of anthocyanins to make red flowers is inferred to have arisen at least 11 times, utilization of carotenoids twice and the dual use of anthocyanins and carotenoids at least 12 times. Based on the inferred ages of both ancestral nodes and extant red-flowered species, we estimate that red flowers arose as early as 11 million yr ago (Ma) using anthocyanins. The earliest nodes reconstructed as having red coloration via dual production and solely carotenoids are more recent (145 000 yr ago and 72 000 yr ago, respectively; Fig. 3). Based on the confidence intervals for node age estimation in the most recent molecular dating analysis of Solanaceae, these estimated node ages may vary ± 4.5 Myr (Sarkinen *et al.*, 2013).

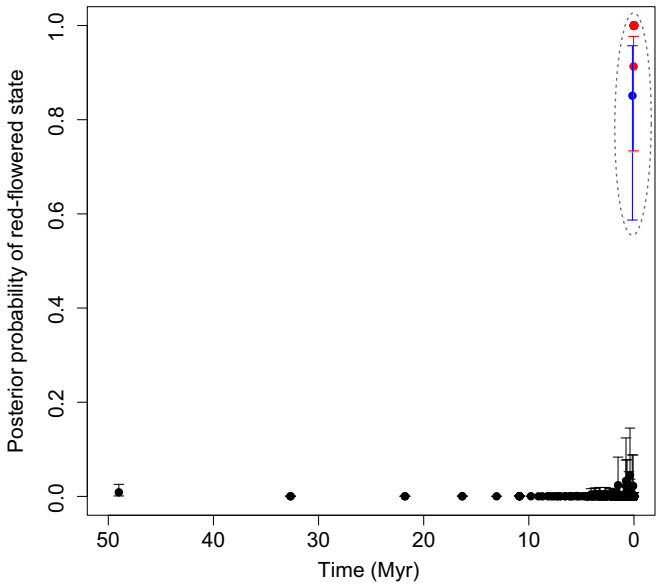

Fig. 3 Time plot showing posterior probabilities of nodes from Bayesian ancestral state reconstruction. Black circles indicate non-red flowers while colored circles within the dotted line represent red flowers. Colors of circles are the same as in Fig. 1. There are 36 colored circles, but many are superimposed (e.g. orange circles are hidden) as they are clustered towards the present (0 Myr). Bars represent 95% credibility intervals around the posterior probability of the node having a red-flowered state across the 8893 MCMC steps.

Testing for phylogenetic signal of pigment pathways

We found that, overall, species with red flowers were more closely related than expected by chance ($P < 0.05$). When testing individual pigment classes, we found that both anthocyanins and dual production of both anthocyanins and carotenoids exhibited phylogenetic signal, suggesting that red-flowered species using these pathways to produce red are more closely related than expected by chance ($P < 0.05$). In contrast, carotenoids alone did not exhibit phylogenetic signal, although this result may be a consequence of a small sample size of two.

Discussion

Convergent evolution of the red flower phenotype has occurred frequently across angiosperms (Cronk & Ojeda, 2008; Rausher, 2008). Our study found that, just within the Solanaceae, there have been at least 30 independent origins of red flowers and all relatively recently (within the last 11 Myr at most). These origins have occurred via alternate biochemical pathways, with most red-flowered species using the anthocyanin pathway, or in combination with the production of carotenoids at approximately the same frequency. We furthermore found that each of these two methods to produce red flowers tend to be used by more closely related species than expected by chance, which demonstrates that even highly convergent traits are impacted by historical contingency.

Drivers of red flower evolution

Evolutionary shifts in flower color are commonly thought to be driven by pollinator-mediated selection (Fenster *et al.*, 2004; Rodríguez-Gironés & Santamaría, 2004). One classic hypothesis is that flower color evolution is tied to shifts between different functional groups of pollinators, such as birds, moths, and bees

(Thomson & Wilson, 2008; van der Niet & Johnson, 2012). Red flowers in particular have long been associated with bird pollination (Faegri & van der Pijl, 1979) and, together with other traits, such as copious nectar, lack of scent and tubular corollas, form the bird pollination syndrome (Cronk & Ojeda, 2008). Shifts to bird pollination coincide with the evolution of red coloration in many taxa (Bradshaw & Schemske, 2003; Streisfeld & Kohn, 2007; Whittall & Hodges, 2007; Rosas-Guerrero *et al.*, 2014), including some Solanaceae (Ippolito *et al.*, 2004; Pérez *et al.*, 2006). However, this association is less clear in many floral radiations, suggesting that pollinator shifts alone may not explain color differences (Cooley *et al.*, 2008; Smith *et al.*, 2008; Knapp, 2010). Another possible pollinator-mediated explanation for flower color divergence, including shifts to red, is reproductive character displacement, where selection favors flower color divergence to minimize pollinator competition between co-occurring species (Brown & Wilson, 1956; Pfennig & Pfennig, 2012). This has been shown in *Iochroma* (Muchhala *et al.*, 2014) but remains to be tested in other Solanaceae clades with red-flowered species.

Alternatively, the evolution of red flowers may not be attributable to any mode of pollinator-mediated selection. For example, red flower color may have arisen because it is genetically linked to another trait under selection or as a pleiotropic effect. Indeed, anthocyanins may be produced in floral tissue as a byproduct of the up-regulation of protective or defensive flavonoids in vegetative tissue (Armbruster, 2002; Strauss & Whittall, 2006; Rausher, 2008). Another possibility unrelated to pollinators is that convergent red flower color simply arose by chance (Rausher, 2008; Stayton, 2008). However, while a number of studies suggest that natural selection plays an important role (e.g. Waser & Price, 1983; Schemske & Bierzychudek, 2001; Streisfeld & Kohn, 2005; Schemske *et al.*, 2007), no study to date has supported the role of genetic drift in flower color variation. Further work is clearly needed, particularly at the microevolutionary scale, to understand when red flower coloration is adaptive, and if a common agent of selection or a combination of processes can explain the evolution of red-flowered species across Solanaceae.

Pathways to red flower coloration

Across angiosperms, red flower color has evolved via the betalain, carotenoid or anthocyanin pathway (Grotewold, 2006; Tanaka *et al.*, 2008; Brockington *et al.*, 2011). Among these, the anthocyanin pathway is the best understood in terms of its structure and regulation (Mol *et al.*, 1998; Koes *et al.*, 2005), and it is responsible for red flower color in many angiosperm clades (e.g. Pecket, 1960; Streisfeld & Rausher, 2009; Cooley *et al.*, 2011). Work on the contribution of carotenoids to red-colored flowers has primarily been in cultivars or model organisms (Zhu *et al.*, 2010; Ohmiya, 2011) and therefore their overall importance in the evolution of red flowers remains largely unknown. Our results, however, are likely to be representative of other angiosperm lineages, as previous studies have also shown that anthocyanins, carotenoids and the dual production of both occur in other groups (e.g. Yokoi & Saitô, 1973; Tatsuzawa *et al.*, 2010).

Our characterization of the pathways underlying phenotypic convergence of red flowers provides the first step towards understanding convergence at the molecular level. The red flowers that we found to employ the same biochemical pathway may also involve similar changes in gene expression or gene function. Indeed, red flowers that evolve via the anthocyanin pathway appear to arise from regulatory or functional mutations in a small number of 'hotspot' loci (Martin & Orgogozo, 2013). Biochemically, red anthocyanin pigments require fewer enzymatic steps than blue anthocyanins, and thus the specific mutations often cause loss of function or expression. For example, inactivation or down-regulation of flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), the enzymes responsible for blue anthocyanins, have been involved in transitions from blue to red flowers in at least four different plant genera (Des Marais & Rausher, 2010; Hopkins & Rausher, 2011; Smith & Rausher, 2011; Wessinger & Rausher, 2014). These loci are therefore strong candidates for the evolution of red flowers in Solanaceae lineages producing anthocyanins.

In contrast, the identity of carotenoid genes potentially responsible for the evolution of red flowers is less clear. Carotenoids are synthesized and stored within chromoplasts, which arise from pre-existing plastids, such as proplastids, chloroplasts or amyloplasts (Egea *et al.*, 2010; Li & Yuan, 2013). While the structural genes involved in the carotenoid biosynthesis pathway have been identified, regulation of carotenoid biosynthesis remains poorly understood and there are a number of carotenoids whose biosynthesis has yet to be characterized (Fraser & Bramley, 2004; Tanaka *et al.*, 2008; Li & Yuan, 2013). Furthermore, the regulation of chromoplast differentiation from pre-existing plastids is still largely to be elucidated (Egea *et al.*, 2010). Carotenoid studies in Solanaceae crop species (e.g. tomato (*Solanum lycopersicum*) and pepper (*Capsicum* spp.)), however, may make future investigations of convergence at the level of a carotenoid gene or mutation tractable in the family (Galpaz *et al.*, 2006; Paran & van der Knaap, 2007). Furthermore, these studies may provide insight into why the sole use of carotenoids to produce red flowers appears relatively rare among angiosperms (Ohmiya, 2011), including only two Solanaceae species.

Dual production of both anthocyanins and carotenoids to produce red flowers represents another level of convergence, as it requires the gain of anthocyanins or carotenoids, assuming that the ancestor already exhibited carotenoid-pigmented or anthocyanin-pigmented flowers, respectively. It is also possible that dual production evolved by the activation of both pathways from a white-flowered ancestor. However, the present data set, focused on only red-flowered species, does not allow us to distinguish among these alternatives. Blue or purple (anthocyanin-colored), yellow (carotenoid-colored) and white flowers are all common in Solanaceae (Knapp, 2010), and thus represent plausible ancestral states. A more fine-scale data set with anatomical and biochemical data for entire clades (e.g. Petunieae or Cestreae) would make it possible to reconstruct the range of trajectories by which lineages move among flower colors via changes in pigment expression.

Historical signal in pathway use

It is often assumed that convergent phenotypic evolution among closely related species is attributable to the same underlying pathway because evolution would begin at similar starting points (Arendt & Reznick, 2008; Losos, 2011; Rosenblum *et al.*, 2014) and may have access to the same standing genetic variation (Barrett & Schluter, 2008; Jones *et al.*, 2012). As lineages diverge, genetic backgrounds diverge as well, lowering the probability of using the same mechanisms for phenotypic evolution (Arendt & Reznick, 2008; Losos, 2011; Rosenblum *et al.*, 2014). However, many studies examining phenotypic convergence at the molecular level have shown that different developmental pathways can be responsible for phenotypic convergence among closely related taxa, while distantly related taxa can converge in phenotype using the same pathway (reviewed in Arendt & Reznick, 2008). This pattern suggests that there may be no direct relationship between phylogenetic distance and the developmental basis for convergent phenotypes. No studies before this, however, had yet explicitly tested whether pathway recruitment exhibits phylogenetic signal.

The results of our study show that, despite the evolutionary lability of flower color (Rausher, 2008), more closely related species tend to use the same pathway to make red flowers. We showed that the evolution of red flowers appears to be restricted to particular clades (Fig. 1), with no red-flowered species in groups such as *Solanum*, the most speciose genus in Solanaceae. This suggests that the likelihood of evolving red flowers, and the biochemical pathway utilized to make red flowers, are highly influenced by phylogenetic history. This phylogenetic clustering of red flowers and pigment pathway is consistent with the possibility that closely related species evolve red flowers via changes in the same genes and possibly via the same types of mutations.

The paradox of red flowers

Despite multiple independent transitions to red flowers via different pathways, species with red flowers remain curiously rare in Solanaceae. Only 34 of *c.* 2700 species, or 1% of the family, can be characterized as red. Red flowers also appear to be rare in other plant groups (e.g. Ghebrehiwet *et al.*, 2000; Wilson *et al.*, 2006; Ojeda *et al.*, 2012), and red pigmentation has even been found to be rare in other taxa, such as songbirds, despite having arisen multiple times from different classes of carotenoid pigments (Friedman *et al.*, 2014). One possible explanation for this rarity is nonequilibrium dynamics, with selection for red flowers, or the ability to produce red pigments, arising only recently, leaving the number of taxa with this state far lower than expected. However, if red flowers have indeed arisen as a result of pollinator-mediated selection (whether via the pollinator shift or competition models), we expect these dynamics to be much older than 11 Myr, as radiations of pollinating animals, such as bees and hummingbirds, began at least 30 Ma (Brown *et al.*, 2008; Rasmussen & Cameron, 2010). Furthermore, the pigment pathways themselves are quite old, with carotenoids being present in all plants (Frommolt *et al.*, 2008) and red anthocyanins probably dating back to the origin of land plants (Campanella *et al.*, 2014).

An alternative explanation for the rarity of red flowers is that red coloration increases the likelihood of a lineage's extinction, that is, that the trait results in an evolutionary dead end, essentially pruning itself from the phylogeny (Stebbins, 1957; Ng & Smith, 2014). Such traits may initially be fixed because of a short-term advantage, which appears to be the case for self-compatibility and polyploidy (Goldberg *et al.*, 2010; Mayrose *et al.*, 2011). Red flowers could be fixed within a lineage by selection, but disfavored in the long term because of pleiotropic effects associated with the genetic changes involved in the transition. For example, anthocyanins are produced in stems and leaves in response to stress (Chalker-Scott, 1999), and the loss-of-function mutations commonly fixed during the evolution of red floral anthocyanins (Smith & Rausher, 2011; Wessinger & Rausher, 2014) could negatively affect this stress response. The growing knowledge of the biochemistry and function of plant pigment pathways provides a clear avenue for identifying the genetic changes responsible for red flower evolution across Solanaceae and for testing the pleiotropic consequences of these transitions.

Acknowledgements

We thank D. Jackson, H. Biagini-Lee and L. Nalezny for their help in generating sequence data and L. Nalezny for help in assessing whether pigments could be accurately identified from dried tissue. We are also extremely grateful to A. Orejuela and R. Laport for help in the field and A. Berardi for help in obtaining HPLC data. We thank L. Bohs, R. Olmstead and L. Freitas for providing genomic DNA samples and PCR products, and three anonymous reviewers for constructive feedback on the manuscript. This work was supported by a National Science Foundation grant to SDS (NSF-DEB 1413855), and utilized the Janus supercomputer, which is supported by the National Science Foundation (award number CNS-0821794) and the University of Colorado Boulder. The Janus supercomputer is a joint effort of the University of Colorado Boulder, the University of Colorado Denver and the National Center for Atmospheric Research.

References

Ando T, Tatsuzawa F, Saito N, Takahashi M, Tsunashima Y, Numajiri H, Watanabe H, Kokubun H, Hara R, Seki H *et al.* 2000. Differences in the floral anthocyanin content of red petunias and *Petunia exserta*. *Phytochemistry* **54**: 495–501.

Arendt J, Reznick D. 2008. Convergence and parallelism reconsidered: what have we learned about the genetics of adaptation? *Trends in Ecology & Evolution* **23**: 26–32.

Armbruster WS. 2002. Can indirect selection and genetic context contribute to trait diversification? A transition-probability study of blossom-colour evolution in two genera. *Journal of Evolutionary Biology* **15**: 468–486.

Barrett RDH, Schluter D. 2008. Adaptation from standing genetic variation. *Trends in Ecology & Evolution* **23**: 38–44.

Beale GH, Price JR, Sturgess VC. 1941. A survey of anthocyanins. VII. The natural selection of flower colour. *Proceedings of the Royal Society of London. Series B - Biological Sciences* **130**: 113–126.

Bradshaw HD, Schemske DW. 2003. Allele substitution at a flower colour locus produces a pollinator shift in monkeyflowers. *Nature* **426**: 176–178.

Brockington SF, Walker RH, Glover BJ, Soltis PS, Soltis DE. 2011. Complex pigment evolution in the Caryophyllales. *New Phytologist* **190**: 854–864.

Brown JH, Kodric-Brown A. 1979. Convergence, competition, and mimicry in a temperate community of hummingbird-pollinated flowers. *Ecology* **60**: 1022–1035.

Brown JW, Rest JS, Garcia-Moreno J, Sorenson MD, Mindell DP. 2008. Strong mitochondrial DNA support for a Cretaceous origin of modern avian lineages. *BMC Biology* **6**: 6.

Brown WL Jr, Wilson EO. 1956. Character displacement. *Systematic Zoology* **5**: 49–64.

Campanella JJ, Smalley JV, Dempsey ME. 2014. A phylogenetic examination of the primary anthocyanin production pathway of the Plantae. *Botanical Studies* **55**: 10.

Chalker-Scott L. 1999. Environmental significance of anthocyanins in plant stress responses. *Photochemistry and Photobiology* **70**: 1–9.

Clarkson JJ, Knapp S, Garcia VF, Olmstead RG, Leitch AR, Chase MW. 2004. Phylogenetic relationships in *Nicotiana* (Solanaceae) inferred from multiple plastid DNA regions. *Molecular Phylogenetics and Evolution* **33**: 75–90.

Collar DC, Reece JS, Alfaro ME, Wainwright PC, Mehta RS. 2014. Imperfect morphological convergence: variable changes in cranial structures underlie transitions to durophagy in moray eels. *American Naturalist* **183**: E168–E184.

Cooley AM, Carvallo G, Willis JH. 2008. Is floral diversification associated with pollinator divergence? Flower shape, flower colour and pollinator preference in Chilean *Mimulus*. *Annals of Botany* **101**: 641–650.

Cooley AM, Modliszewski JL, Rommel ML, Willis JH. 2011. Gene duplication in *Mimulus* underlies parallel floral evolution via independent trans-regulatory changes. *Current Biology* **21**: 700–704.

Cronk Q, Ojeda I. 2008. Bird-pollinated flowers in an evolutionary and molecular context. *Journal of Experimental Botany* **59**: 715–727.

Cummings ME. 2007. Sensory trade-offs predict signal divergence in surfperch. *Evolution* **61**: 530–545.

Des Marais DL, Rausher MD. 2010. Parallel evolution at multiple levels in the origin of hummingbird pollinated flowers in *Ipomoea*. *Evolution* **64**: 2044–2054.

Doyle JJ, Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.

Drummond AJ, Kearse M, Heled J, Moir R, Thierer T, Ashton B, Wilson A, Stones-Havas S. 2006. *Geneious v4.6.1*. [WWW document] URL http://www.geneious.com/

Egea I, Barsan C, Bian W, Purgatto E, Latché A, Chervin C, Bouzayen M, Pech J-C. 2010. Chromoplast differentiation: current status and perspectives. *Plant and Cell Physiology* **51**: 1601–1611.

Eich E. 2008. *Solanaceae and Convolvulaceae: secondary metabolites*. Berlin, Germany: Springer-Verlag.

Endler JA. 1990. On the measurement and classification of color in studies of animal color patterns. *Biological Journal of the Linnean Society* **41**: 315–352.

Faegri K, van der Pijl L. 1979. *The principles of pollination ecology*. Oxford, UK: Pergamon Press.

Fenster CB, Armbruster WS, Wilson P, Dudash MR, Thomson JD. 2004. Pollination syndromes and floral specialization. *Annual Review of Ecology, Evolution, and Systematics* **35**: 375–403.

FitzJohn RG. 2012. Diversitree: comparative phylogenetic analyses of diversification in R. *Methods in Ecology and Evolution* **3**: 1084–1092.

FitzJohn RG, Maddison WP, Otto SP. 2009. Estimating trait-dependent speciation and extinction rates from incompletely resolved phylogenies. *Systematic Biology* **58**: 595–611.

Forkmann G. 1991. Flavonoids as flower pigments: the formation of the natural spectrum and its extension by genetic engineering. *Plant Breeding* **106**: 1–26.

Fraser PD, Bramley PM. 2004. The biosynthesis and nutritional uses of carotenoids. *Progress in Lipid Research* **43**: 228–265.

Fregonezi JN, Freitas LB, Bonatto SL, Semir J, Stehmann JR. 2012. Infrageneric classification of *Calibrachoa* (Solanaceae) based on morphological and molecular evidence. *Taxon* **61**: 120–130.

Friedman NR, McGraw KJ, Omland KE. 2014. Evolution of carotenoid pigmentation in caciques and meadowlarks (Icteridae): repeated gains of red plumage coloration by carotenoid C_4-oxygenation. *Evolution* **68**: 791–801.

Frommolt R, Werner S, Paulsen H, Goss R, Wilhelm C, Zauner S, Maier UG, Grossman AR, Bhattacharya D, Lohr M. 2008. Ancient recruitment by chromists of green algal genes encoding enzymes for carotenoid biosynthesis. *Molecular Biology and Evolution* **25**: 2653–2667.

Galpaz N, Ronen G, Khalfa Z, Zamir D, Hirschberg J. 2006. A chromoplast-specific carotenoid biosynthesis pathway is revealed by cloning of the tomato white-flower locus. *Plant Cell* **18**: 1947–1960.

Ghebrehiwet M, Bremer B, Thulin M. 2000. Phylogeny of the tribe Antirrhineae (Scrophulariaceae) based on morphological and *ndh*F sequence data. *Plant Systematics and Evolution* **220**: 223–239.

Gillespie R. 2004. Community assembly through adaptive radiation in Hawaiian spiders. *Science* **303**: 356–359.

Goldberg EE, Igić B, Posada D. 2008. On phylogenetic tests of irreversible evolution. *Evolution* **62**: 2727–2741.

Goldberg EE, Kohn JR, Lande R, Robertson KA, Smith SA, Igic B. 2010. Species selection maintains self-incompatibility. *Science* **330**: 493–495.

Gould SJ. 2002. *The structure of evolutionary theory*. Cambridge, MA, USA: The Belknap Press of Harvard University Press.

Griesbach RJ. 1984. Effects of carotenoid-anthocyanin combinations on flower color. *Journal of Heredity* **75**: 145–146.

Griesbach RJ, Stehmann JR, Meyer F. 1999. Anthocyanins in the "red" flowers of *Petunia exserta*. *Phytochemistry* **51**: 525–528.

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

Grundler MC, Rabosky DL. 2014. Trophic divergence despite morphological convergence in a continental radiation of snakes. *Proceedings of the Royal Society B: Biological Sciences* **281**: doi:10.1098/rspb.2014.0413.

Hall BK. 2003. Descent with modification: the unity underlying homology and homoplasy as seen through an analysis of development and evolution. *Biological Reviews* **78**: 409–433.

Harvey PH, Pagel MD. 1991. *The comparative method in evolutionary biology*. Oxford, UK: Oxford University Press.

Herrel A, Vanhooydonck B, Van Damme R. 2004. Omnivory in lacertid lizards: adaptive evolution or constraint? *Journal of Evolutionary Biology* **17**: 974–984.

Hoekstra HE. 2006. Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity* **97**: 222–234.

Hofmann CM, Cronin TW, Omland KE. 2006. Using spectral data to reconstruct evolutionary changes in coloration: carotenoid color evolution in New World orioles. *Evolution* **60**: 1680–1691.

Hopkins R, Rausher MD. 2011. Identification of two genes causing reinforcement in the Texas wildflower *Phlox drummondii*. *Nature* **469**: 411–414.

Hunziker AT. 2001. *Genera Solanacearum: the genera of Solanaceae illustrated, arranged according to a new system*. Ruggell, Liechtenstein: A.R.G. Gantner Verlag Kommanditgesellschaft.

Ippolito A, Fernandes GW, Holtsford TP. 2004. Pollinator preferences for *Nicotiana alata*, *N. forgetiana* and their F_1 hybrid. *Evolution* **58**: 2634–2644.

Johnson SD, Linder HP, Steiner KE. 1998. Phylogeny and radiation of pollination systems in *Disa* (Orchidaceae). *American Journal of Botany* **85**: 402–411.

Johnson MTJ, FitzJohn RG, Smith SD, Rausher MD, Otto SP. 2011. Loss of sexual recombination and segregation is associated with increased diversification in evening primroses. *Evolution* **65**: 3230–3240.

Jones FC, Grabherr MG, Chan YF, Russell P, Mauceli E, Johnson J, Swofford R, Pirun M, Zody MC, White S *et al.* 2012. The genomic basis of adaptive evolution in threespine sticklebacks. *Nature* **484**: 55–61.

Katoh K, Standley DM. 2013. MAFFT Multiple sequence alignment software version 7: improvements in performance and usability. *Molecular Biology and Evolution* **30**: 772–780.

Knapp S. 2002. Floral diversity and evolution in the Solanaceae. In: Cronk QCB, Bateman RM, Hawkins JA, eds. *Developmental genetics and plant evolution*. London, UK: Taylor & Francis, 267–297.

Knapp S. 2010. On 'various contrivances': pollination, phylogeny and flower form in the Solanaceae. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 449–460.

Knapp S, Persson V, Blackmore S. 1997. A phylogenetic conspectus of the tribe Juanulloeae (Solanaceae). *Annals of the Missouri Botanical Garden* **84**: 67–89.

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.

Kulcheski F, Muschner V, Lorenz-Lemke A, Stehmann J, Bonatto S, Salzano F, Freitas L. 2006. Molecular phylogenetic analysis of *Petunia* Juss. (Solanaceae). *Genetica* **126**: 3–14.

León H, Alain H. 1974. *Flora de Cuba.* Koenigstein, Germany: Otto Koeltz Science Publishers.

Li L, Yuan H. 2013. Chromoplast biogenesis and carotenoid accumulation. *Archives of Biochemistry and Biophysics* **539**: 102–109.

Losos JB. 2011. Convergence, adaptation, and constraint. *Evolution* **65**: 1827–1840.

Maddison WP. 2006. Confounding asymmetries in evolutionary diversification and character change. *Evolution* **60**: 1743–1746.

Maddison WP, Midford PE, Otto SP. 2007. Estimating a binary character's effect on speciation and extinction. *Systematic Biology* **56**: 701–710.

Maddison WP, Slatkin M. 1991. Null models for the number of evolutionary steps in a character on a phylogenetic tree. *Evolution* **45**: 1184–1197.

Mahler DL, Ingram T, Revell LJ, Losos JB. 2013. Exceptional convergence on the macroevolutionary landscape in island lizard radiations. *Science* **341**: 292–295.

Maia R, Eliason CM, Bitton P-P, Doucet SM, Shawkey MD. 2013. pavo: an R package for the analysis, visualization and organization of spectral data. *Methods in Ecology and Evolution* **4**: 906–913.

Manceau M, Domingues VS, Linnen CR, Rosenblum EB, Hoekstra HE. 2010. Convergence in pigmentation at multiple levels: mutations, genes and function. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 2439–2450.

Martin A, Orgogozo V. 2013. The loci of repeated evolution: a catalog of genetic hotspots of phenotypic variation. *Evolution* **67**: 1235–1250.

Maynard Smith J, Burian R, Kauffman S, Alberch P, Campbell J, Goodwin B, Lande R, Raup D, Wolpert L. 1985. Developmental constraints and evolution: a perspective from the Mountain Lake conference on development and evolution. *The Quarterly Review of Biology* **60**: 265–287.

Mayrose I, Zhan SH, Rothfels CJ, Magnuson-Ford K, Barker MS, Rieseberg LH, Otto SP. 2011. Recently formed polyploid plants diversify at lower rates. *Science* **333**: 1257.

Melville J, Harmon LJ, Losos JB. 2006. Intercontinental community convergence of ecology and morphology in desert lizards. *Proceedings of the Royal Society B: Biological Sciences* **273**: 557–563.

Miller JH, Miller PM, Deal RH. 1967. Anthocyanin synthesis in *Salpiglossis sinuata*. *American Journal of Botany* **54**: 1163–1170.

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

Montero-Castro JC, Delgado-Salinas A, De Luna E, Eguiarte LE. 2006. Phylogenetic analysis of *Cestrum* section *Habrothamnus* (Solanaceae) based on plastid and nuclear DNA sequences. *Systematic Botany* **31**: 843–850.

Montgomerie R 2006. Analyzing colors. In: Hill GE, McGraw KJ, eds. *Bird coloration, Vol. 1, mechanisms and measurements*. Cambridge, MA, USA: Harvard University Press, 90–147.

Muchhala N, Johnsen S, Smith SD. 2014. Competition for hummingbird pollination shapes flower color variation in Andean Solanaceae. *Evolution* **68**: 2275–2286.

Munsell. 1976. *Munsell book of color, glossy finish collection (2 volumes)*. Baltimore, MD, USA: Munsell/Macbeth/Kollmorgen Corp.

Murakami Y. 2004. Floral coloration and pigmentation in *Calibrachoa* cultivars. *Journal of Horticultural Science & Biotechnology* **79**: 47–53.

Muschick M, Indermaur A, Salzburger W. 2012. Convergent evolution within an adaptive radiation of cichlid fishes. *Current Biology* **22**: 2362–2368.

Ng J, Landeen EL, Logsdon RM, Glor RE. 2013. Correlation between *Anolis* lizard dewlap phenotype and environmental variation indicates adaptive divergence of a signal important to sexual selection and species recognition. *Evolution* **67**: 573–582.

Ng J, Smith SD. 2014. How traits shape trees: new approaches for detecting character state-dependent lineage diversification. *Journal of Evolutionary Biology* **27**: 2035–2045.

van der Niet T, Johnson SD. 2012. Phylogenetic evidence for pollinator-driven diversification of angiosperms. *Trends in Ecology & Evolution* **27**: 353–361.

Ohmiya A. 2011. Diversity of carotenoid composition in flower petals. *Japan International Research Center for Agricultural Sciences* **45**: 163–171.

Ojeda I, Santos-Guerra A, Jaén-Molina R, Oliva-Tejera F, Caujapé-Castells J, Cronk Q. 2012. The origin of bird pollination in Macaronesian *Lotus* (Loteae, Leguminosae). *Molecular Phylogenetics and Evolution* **62**: 306–318.

Olmstead RG, Bohs L, Migid HA, Santiago-Valentin E, Garcia VF, Collier SM. 2008. A molecular phylogeny of the Solanaceae. *Taxon* **57**: 1159–1181.

Olmstead RG, Sweere JA. 1994. Combining data in phylogenetic systematics: an empirical approach using three molecular data sets in the Solanaceae. *Systematic Biology* **43**: 467–481.

Pagel M. 1994. Detecting correlated evolution on phylogenies: a general method for the comparative analysis of discrete characters. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **255**: 37–45.

Papadopulos AST, Powell MP, Pupulin F, Warner J, Hawkins JA, Salamin N, Chittka L, Williams NH, Whitten WM, Loader D *et al.* 2013. Convergent evolution of floral signals underlies the success of Neotropical orchids. *Proceedings of the Royal Society B: Biological Sciences* **280**: 20130960.

Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**: 289–290.

Paran I, van der Knaap E. 2007. Genetic and molecular regulation of fruit and plant domestication traits in tomato and pepper. *Journal of Experimental Botany* **58**: 3841–3852.

Parra-Olea G, Wake DB. 2001. Extreme morphological and ecological homoplasy in tropical salamanders. *Proceedings of the National Academy of Sciences, USA* **98**: 7888–7891.

Pecket RC. 1960. The nature of the variation in flower colour in the genus *Lathyrus*. *New Phytologist* **59**: 138–144.

Pérez F, Arroyo MTK, Medel R, Hershkovitz MA. 2006. Ancestral reconstruction of flower morphology and pollination systems in *Schizanthus* (Solanaceae). *American Journal of Botany* **93**: 1029–1038.

Pfennig D, Pfennig K. 2012. *Evolution's wedge: competition and the origins of diversity*. Berkeley, CA, USA: University of California Press.

Plowman T. 1998. *A revision of the South American species of Brunfelsia (Solanaceae)*. Chicago, IL, USA: Field Museum of Natural History.

Pryke SR, Lawes MJ, Andersson S. 2001. Agonistic carotenoid signalling in male red-collared widowbirds: aggression related to the colour signal of both the territory owner and model intruder. *Animal Behaviour* **62**: 695–704.

R Core Team. 2014. *R: a language and environment for statistical computing*. v.3.1.1. Vienna, Austria: R Foundation for Statistical Computing. URL http://www.R-project.org/

Rasmussen C, Cameron SA. 2010. Global stingless bee phylogeny supports ancient divergence, vicariance, and long distance dispersal. *Biological Journal of the Linnean Society* **99**: 206–232.

Rausher MD. 2008. Evolutionary transitions in floral color. *International Journal of Plant Sciences* **169**: 7–21.

Robinson GM, Robinson R. 1934. A survey of anthocyanins. IV. *Biochemical Journal* **28**: 1712–1720.

Rodríguez-Gironés MA, Santamaría L. 2004. Why are so many bird flowers red? *PLoS Biology* **2**: e350.

Rosas-Guerrero V, Aguilar R, Martén-Rodríguez S, Ashworth L, Lopezaraiza-Mikel M, Bastida JM, Quesada M. 2014. A quantitative review of pollination syndromes: do floral traits predict effective pollinators? *Ecology Letters* **17**: 388–400.

Rosenblum EB, Parent CE, Brandt EE. 2014. The molecular basis of phenotypic convergence. *Annual Review of Ecology, Evolution, and Systematics* **45**: 203–226.

Sanderson MJ. 2002. Estimating absolute rates of molecular evolution and divergence times: a penalized likelihood approach. *Molecular Biology and Evolution* **19**: 101–109.

Sarkinen T, Bohs L, Olmstead RG, Knapp S. 2013. A phylogenetic framework for evolutionary study of the nightshades (Solanaceae): a dated 1000-tip tree. *BMC Evolutionary Biology* **13**: 214.

Schemske DW, Bierzychudek P. 2001. Perspective: evolution of flower color in the desert annual *Linanthus parryae*: wright revisited. *Evolution* **55**: 1269–1282.

Schemske DW, Bierzychudek P, Schoen D. 2007. Spatial differentiation for flower color in the desert annual *Linanthus parryae*: was Wright right? *Evolution* **61**: 2528–2543.

Schluter D. 1996. Adaptive radiation along genetic lines of least resistance. *Evolution* **50**: 1766–1774.

Schluter D. 2000. *The ecology of adaptive radiation*. New York, NY, USA: Oxford University Press.

Shapiro MD, Bell MA, Kingsley DM. 2006. Parallel genetic origins of pelvic reduction in vertebrates. *Proceedings of the National Academy of Sciences, USA* **103**: 13753–13758.

Smith SA, Beaulieu JM, Donoghue MJ. 2009. Mega-phylogeny approach for comparative biology: an alternative to supertree and supermatrix approaches. *BMC Evolutionary Biology* **9**: 37.

Smith SD, Baum DA. 2006. Phylogenetics of the florally diverse Andean clade Iochrominae (Solanaceae). *American Journal of Botany* **93**: 1140–1153.

Smith SD, Hall SJ, Izquierdo PR, Baum DA. 2008. Comparative pollination biology of sympatric and allopatric Andean *Iochroma* (Solanaceae). *Annals of the Missouri Botanical Garden* **95**: 600–617.

Smith SD, Rausher MD. 2011. Gene loss and parallel evolution contribute to species difference in flower color. *Molecular Biology and Evolution* **28**: 2799–2810.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22**: 2688–2690.

Stayton CT. 2008. Is convergence surprising? An examination of the frequency of convergence in simulated datasets. *Journal of Theoretical Biology* **252**: 1–14.

Stebbins GL. 1957. Self fertilization and population variability in the higher plants. *American Naturalist* **91**: 337–354.

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

Streisfeld MA, Kohn JR. 2005. Contrasting patterns of floral and molecular variation across a cline in *Mimulus aurantiacus*. *Evolution* **59**: 2548–2559.

Streisfeld MA, Kohn JR. 2007. Environment and pollinator-mediated selection on parapatric floral races of *Mimulus aurantiacus*. *Journal of Evolutionary Biology* **20**: 122–132.

Streisfeld MA, Rausher MD. 2009. Genetic changes contributing to the parallel evolution of red floral pigmentation among *Ipomoea* species. *New Phytologist* **183**: 751–763.

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

Tatsuzawa F, Ichihara K, Shinoda K, Miyoshi K. 2010. Flower colours and pigments in *Disa* hybrid (Orchidaceae). *South African Journal of Botany* **76**: 49–53.

Thomson JD, Wilson P. 2008. Explaining evolutionary shifts between bee and hummingbird pollination: convergence, divergence, and directionality. *International Journal of Plant Sciences* **169**: 23–38.

Wake DB. 1991. Homoplasy: the result of natural selection, or evidence of design limitations? *American Naturalist* **138**: 543–567.

Wake DB, Wake MH, Specht CD. 2011. Homoplasy: from detecting pattern to determining process and mechanism of evolution. *Science* **331**: 1032–1035.

Waser NM, Price MV. 1983. Pollinator behaviour and natural selection for flower colour in *Delphinium nelsonii*. *Nature* **302**: 422–424.

Waterworth RA, Griesbach RJ. 2001. The biochemical basis for flower color in *Calibrachoa*. *HortScience* **36**: 1131–1132.

Wessinger CA, Rausher MD. 2014. Predictability and irreversibility of genetic changes associated with flower color evolution in *Penstemon barbatus*. *Evolution* **68**: 1058–1070.

Whittall JB, Hodges SA. 2007. Pollinator shifts drive increasingly long nectar spurs in columbine flowers. *Nature* **447**: 706–709.

Wilson P, Castellanos MC, Wolfe AD, Thomson JD. 2006. Shifts between bee and bird pollination among penstemons. In: Waser NM, Ollerton J, eds. *Plant pollinator interactions: from specialization to generalization*. Chicago, IL, USA: University of Chicago Press, 47–68.

Yokoi M, Saitô N. 1973. Light absorption patterns of intact *Rosa* flowers in relation to the flower colour. *Phytochemistry* **12**: 1783–1786.

Zhu C, Bai C, Sanahuja G, Yuan D, Farré G, Naqvi S, Shi L, Capell T, Christou P. 2010. The regulation of carotenoid pigmentation in flowers. *Archives of Biochemistry and Biophysics* **504**: 132–141.

Supporting Information

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

Fig. S1 Maximum likelihood phylogenetic reconstruction of the Solanaceae family.

Fig. S2 Example images of cells from a herbarium specimen flower sample (*Juanulloa parasitica*).

Fig. S3 Anthocyanidin pigments in flower tissue of red-flowered species separated using high-performance liquid chromatography.

Methods S1 PCR and thermocycling procedures.

Methods S2 High-performance liquid chromatography procedures.

Table S1 Information about Solanaceae species used in this study that have classically been described as having red flowers

Table S2 GenBank accession numbers for new sequence data generated for this study

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.