Original article

The effect of polyploidy and hybridisation on the evolution of floral colour in *Nicotiana* (Solanaceae)

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Running title: Floral colour evolution in *Nicotiana* polyploids

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Abstract

- *Background and Aims* We investigate whether changes in floral colour accompany polyploid and homoploid hybridisation, important processes in angiosperm evolution. Potentially, changes in floral colour can facilitate speciation through pollinator shifts.
- *Methods* We examined spectral reflectance of corolla tissue from 60 *Nicotiana* (Solanaceae) accessions (41 taxa) based on spectral shape (corresponding to pigmentation) as well as bee and hummingbird colour perception to assess patterns of floral colour evolution. We compared polyploid and homoploid hybrid spectra to those of their progenitors to evaluate whether hybridisation has resulted in floral colour shifts.
- *Key Results* Floral colour categories in *Nicotiana* seem to have arisen multiple times independently during the evolution of the genus. Polyploid and homoploid hybrids can display a floral colour: 1) intermediate between progenitors, 2) like one or other progenitor, or 3) a transgressive or divergent colour not present in either progenitor.
- *Conclusions* Floral colour evolution in *Nicotiana* is weakly constrained by phylogeny, but colour shifts occur and are sometimes associated with allopolyploid or homoploid speciation. Transgressive floral colour in *N. tabacum* has arisen by inheritance of anthocyanin pigmentation from its paternal progenitor while having a plastid phenotype like its maternal progenitor. Potentially, floral colour evolution has been driven by, or resulted in, pollinator shifts.

Key words: evolution, floral colour, hybridisation, *Nicotiana*, pollinator shifts, polyploidy, spectral reflectance, transgressive traits

Introduction

Polyploidy, or whole genome multiplication, has played an important role in the evolution of flowering plants (Soltis *et al.*, 2009; 2014). Allopolyploidy, arising from interspecific hybridisation and polyploidy, can cause 'genomic shock' (McClintock, 1984), that may trigger a suite of genetic changes, including (retro)transposition, differential gene expression, chromosome rearrangements, and epigenetic changes (Leitch and Leitch, 2008). These events and novel *cis-trans* interactions between progenitor genomes may generate variation, including transgressive phenotypes, and facilitate rapid divergence of both homoploid and allopolyploid hybrids (Wittkopp *et al.*, 2004; Chen, 2007; Gaeta *et al.*, 2007; Anssour *et al.*, 2009; Tirosh *et al.*, 2009; Clare *et al.*, 2013).

Speciation in angiosperms can be accompanied by, or perhaps be driven by, changes in floral colour that may influence pollinator preference and reproductive isolation. Many pollinators, such as bumblebees and hummingbirds, are generalists that visit a range of flower colours (Waser *et al.*, 1996). Several species of flower-naive bumblebees have innate colour preference for violet and blue shades, although preferences in experienced foragers are largely determined by learned associations between colours and rewards (Raine *et al.*, 2006). Hummingbirds appear to have no innate preferences for particular colours, but are likewise good at forming associations between flower visual displays and rewards (Goldsmith and Goldsmith, 1979; Chittka and Waser, 1997). Hummingbirds have specialised red receptors, whereas many insects do not. Consequently, red flowers are poorly detectable to bee pollinators, but conspicuous for hummingbirds. Therefore, hummingbird-visited flowers are often red, whereas those pollinated by bees typically have a range of other colours (Rodriguez-Girones and Santamaria, 2004; Shrestha *et al.*, 2013). Flowers visited by nocturnal pollinators are more often white than those pollinated in full daylight, probably to maximise their detectability in dim light conditions (Kevan *et al.*, 1996). Because of such

differences in affinities of various pollinator classes to certain flower colours, differences in flower colour can contribute to restricting gene flow between phenotypes, although they will rarely result in complete reproductive isolation; for this, differences in multiple traits are typically essential. In *Aquilegia* (Ranunculaceae), blue-, red- and white/yellow-flowered species are primarily pollinated by bees, hummingbirds and hawkmoths, respectively (Grant, 1952; Whittall and Hodges, 2007). In *Petunia axillaris* (Solanaceae), hawkmoths prefer white flowers to pink flowers transformed to express *ANTHOCYANIN2*, whereas bumblebees prefer pink *ANTHOCYANIN2* flowers to white flowers, demonstrating that expression of a single gene can cause differences in pollinator visitation (Hoballah *et al.*, 2007). Similarly, manipulation of a single locus controlling carotenoid production in *Mimulus* flowers (Phrymaceae) results in a pollinator shift, reaffirming the importance of floral colour in determining pollinator behaviour (Bradshaw and Schemske, 2003).

To analyse floral colour in the context of pollination, it is necessary to consider both colour theory and pollinator visual systems. There are several important differences between the colour vision systems of humans and those of various pollinator types. Humans and many insects are trichromatic, having three discrete photoreceptor types; however, humans possess red- (with peak sensitivity (λ_{max}) near 560nm), green- (λ_{max}=535nm) and blue-sensitive (λ_{max}=420nm) photoreceptors (Bowmaker and Dartnall, 1980), whereas many insects have ultraviolet- (UV-, λ_{max}=~350nm), blue- (λ_{max}=~440nm) and green-sensitive (λ_{max}=~530nm) receptors (Peitsch *et al.*, 1992; Briscoe and Chittka, 2001; Kelber *et al.*, 2003). Many birds (Bowmaker, 1998) and some butterflies (Kelber, 2001) have tetrachromatic colour vision. In birds, photoreceptors are sensitive to red, green, blue and either violet or UV wavelengths (Hart and Hunt, 2007). Hummingbirds have four single cone types with peak sensitivities in the UV (λ_{max}=370nm), blue (λ_{max}=440nm), blue-green (λ_{max}=508nm) and yellow (λ_{max}=560nm)— sensitivity of the last extends significantly into the

red (Herrera *et al.*, 2008). Hummingbirds can learn to distinguish near-UV light (370 nm) from darkness, confirming that they use their UV receptors for colour vision at a behavioural level (Goldsmith, 1980).

It is also important to consider the pigments responsible for giving flowers their colour and the placement of these pigments within floral cells. Lipid soluble pigments, like carotenoids and chlorophyll, are constrained to plastids, whereas water soluble pigments, like anthocyanins, are found in the vacuole. Cell size can affect concentration of vacuolar pigments and should also be taken into consideration. Spectral colour shifts can occur in anthocyanins due to hydroxylation and methylation, which result in different types of anthocyanins (Castaneda-Ovando *et al.*, 2009; Andersen and Jordheim, 2010), and differences in pH as well as copigmentation with other pigments, including carotenoids and colourless flavonoids, or metal ions can cause spectral shifts in the same anthocyanin compound (Grotewold, 2006; Andersen and Jordheim, 2010).

We investigate evolution of floral colour across *Nicotiana* (Solanaceae) in the context of polyploidy and hybridisation. *Nicotiana* is an excellent group in which to study the effects of hybridisation as nearly half of the 76 species are allotetraploids of different ages (Chase *et al.*, 2003; Clarkson *et al.*, 2004; Clarkson *et al.*, 2005; Leitch *et al.*, 2008; Kelly *et al.*, 2013), and several putative diploid homoploid hybrids have also been detected (Clarkson *et al.*, 2010; Kelly *et al.*, 2010), which add to the reticulate nature of the genus. Floral colours of *Nicotiana* polyploid and homoploid hybrids and the extant diploid species most closely related to the original parents, hereafter called 'progenitors,' are shown in Fig. 1. Because various animal groups have different sensitivities to colour, it is necessary to model colour perception of specific pollinator classes to understand the significance of floral colour signals. Here, we consider floral colours from both a bee perspective (Chittka, 1992), which can also be used to represent other trichromatic insects such as hawkmoths due to similarities

in photoreceptor sensitivities (Kelber *et al.*, 2003), and a hummingbird perspective (Herrera *et al.*, 2008; Restrepo, 2013) as hummingbirds and hawkmoths are known to visit *Nicotiana* species (Aigner and Scott, 2002; Kaczorowski *et al.*, 2005; Kessler and Baldwin, 2006; Nattero and Cocucci, 2007).

Our specific questions are as follows. 1) What types of spectral reflectance are found within *Nicotiana* and how do they appear to bee and hummingbird pollinators? 2) Do polyploid and homoploid hybrids have reflectance spectra that resemble one of their progenitors or are they divergent? 3) Is evolution of floral colour strongly constrained by phylogeny, or is there evidence that shifts in floral colour have been frequent in the evolution of the genus *Nicotiana*?

Materials and Methods

Spectral reflectance measurements

Spectral reflectance measurements were recorded for 60 *Nicotiana* accessions (41 taxa; Supplemental Table S1); three flowers from different plants, where possible, were used for each accession. Reflectance spectra from three *N. otophora* accessions were pooled because spectra were similar.

Spectral reflectance of flowers at anthesis was measured from 300-700 nm using an Avantes AvaSpec-2048 spectrophotometer with an Avantes AvaLight-DHS light source and calibrated with a barium sulphate white standard from labsphere®. *Nicotiana mutabilis* was also measured later as flowers change from white to pink when mature; pink flowers are less likely to have a nectar reward, but add to the attraction of the overall floral display, and therefore are still relevant to pollinators (R. Kaczorowski, University of Haifa, personal communication). Reflectance spectra contain the proportion of light reflected by the flower at any given wavelength. Spectra were visualised and exported in one nanometre increments

using the program AvaSoft version 7.0.3 Full (Avantes BV, Eerbeek, The Netherlands) and imported into Excel.

Spectra for each accession or colour morph were averaged and then smoothed three times, using a rolling average over nine nanometres. Spectra for all accessions were submitted to the Floral Reflectance Database (FReD; www.reflectance.co.uk; Arnold *et al.*, 2010).

Some spectra had a spike at ~656 nm, which corresponded to a narrow peak in the light source spectrum, suggesting that the spectra were saturated at ~656 nm; however, smoothing served to neutralise this spike. Several spectra (*N. arentsii*, *N. mutabilis*, *N. suaveolens* and *N. wigandioides*) included an anomalous reflectance minimum from 475-500 nm, which could not be explained by the light source spectrum. Remeasured spectra of *N. arentsii*, *N. suaveolens* and *N. wigandioides* lacked this minimum, but further material of *N. mutabilis* was unavailable, so these spectra were included despite the anomalies.

Calculation of colour loci in the bee colour hexagon

A reflectance spectrum can be represented as a single point in the bee colour hexagon space (a graphical representation of a bee's colour visual experience) based on the relative excitation of UV-, blue-, and green-sensitive photoreceptor types (Chittka, 1992). Vertices of this hexagon represent theoretical states where one or two photoreceptor types are at maximal excitation whereas at least one receptor type is at zero excitation (for example, the top vertex of the hexagon corresponds to maximal blue receptor excitation and zero signal from UV and green receptors, whereas the top right vertex corresponds to maximal signal in both blue and green receptors, but no signal in the UV receptor, and so forth; see Supplemental Fig. S1). The centre or origin of the hexagon is achromatic. Hue corresponds to angular position

around the origin, whereas spectral purity or saturation increases with distance from the origin.

Bee colour hexagon coordinates were calculated for all *Nicotiana* spectra. Illumination was assumed to be sunlight (D65; Wyszecki and Stiles, 1982); the background was represented by an average leaf spectrum (Gumbert *et al.*, 1999). Honeybee photoreceptor spectral sensitivity functions were used to determine bee colour hexagon coordinates; these are similar to bumblebee and hawkmoth photoreceptor sensitivity functions, so the bee colour hexagon can be used to approximate the colour vision of these insects as well (Menzel *et al.*, 1986; Peitsch *et al.*, 1992; Briscoe and Chittka, 2001; Kelber *et al.*, 2003 and references therein; Skorupski *et al.*, 2007). The equations used to determine colour hexagon coordinates are as follows, where E(G), E(B) and E(UV) represent the excitation of the green, blue and UV bee photoreceptors, respectively, elicited by a spectrum (Chittka, 1992):

$$x = \sqrt{3}/2\ (E(G) - E(UV))$$

$$y = E(B) - 0.5(E(UV) + E(G))$$

Because the colour loci of *Nicotiana* flowers were mostly close to the centre of the colour space, all colour hexagon displays presented are scaled so that only the central 40% is shown; the outline is therefore drawn as a dashed line. This results in a clearer spread of the colour loci to facilitate visual inspection. For a diagram explaining the colour hexagon, see Supplemental Information Fig. S1.

Calculation of colour loci in hummingbird colour space

For tetrachromatic hummingbirds, we chose to model flower colours in a 3-dimensional colour opponent space because *n*-1 colour opponent dimensions are necessary to code the information from *n* colour receptors (Chittka, 1996). The hummingbird colour

space can be displayed as a rhombic dodecahedron with 14 vertices (Restrepo, 2013). Like the bee colour hexagon, vertices of the space represent states where one, two or three photoreceptor types are at maximal excitation and at least one receptor type is at zero excitation.

Hummingbird colour space coordinates were calculated for all *Nicotiana* spectra. Illumination was again assumed to be sunlight (D65; Wyszecki and Stiles, 1982) and the background an average leaf spectrum (Gumbert *et al.*, 1999) as was used for the bee colour hexagon. Photoreceptor spectral sensitivity functions from the green-backed firecrown hummingbird (*Sephanoides sephanoides*; Herrera *et al.*, 2008) were used to determine hummingbird colour space coordinates using the following equations (Restrepo, 2013):

$$x = \sqrt{3/4}\,E(B) - \sqrt{1/12}\,(E(UV) + E(G) + E(R))$$

$$y = \sqrt{2/3}\,E(G) - \sqrt{1/6}\,(E(UV) + E(R))$$

$$z = \sqrt{1/2}\,(E(UV) - E(R))$$

RStudio version 0.98.490 (http://www.rstudio.org/) was used to make 3D plots of the hummingbird colour space, and ImageJ version 1.48 (http://imagej.nih.gov/ij) was used to create an animation of the *Nicotiana* flower loci in the hummingbird colour space. Again, *Nicotiana* flower colour loci are close to the origin in the hummingbird colour space, so the graphs presented display only the central portion (either 25% or 50%) of the colour space for clarity. To further facilitate interpretation of these graphs, vertices representing individual excitation of the red, green, blue and UV photoreceptor types, as well as their excitation vectors from the origin, are shown in red, green, blue and black, respectively. Other vertices (representing excitation of two or three photoreceptor types) are shown in grey.

Clustering analyses

Clustering analyses were used to group spectra based on spectral shape (corresponding to pigmentation) and their position in both bee and hummingbird colour spaces. For spectral colour categories, spectra were normalised to the same integral under the curve in order to compare combinations of pigments, not the concentration of pigments. A distance matrix was calculated from the normalised spectral data in R version 3.0.2 (RCoreTeam, 2013; http://www.R-project.org/) using the dist() function. For the bee and hummingbird colour categories, the input data were the (x, y) or (x, y, z) coordinates of the spectra in the bee and hummingbird colour spaces, respectively.

Data were first imported into R. The function hclust() was used to perform agglomerative hierarchical clustering based on the average pairwise distances between groups. With this algorithm the observed points, which are initially all deemed to be distinct, are iteratively assigned to groups until eventually all points belong to the same group. At each step, the average distance between all groups is calculated (*i.e.* the mean distance from all points in group A to all points in group B - if either one of these is a single point then no averaging is needed), and the two groups with the minimum average distance are merged. The order in which groups are merged can be used to construct a dendrogram showing the spatial relationship between all data points. We can also look at the distribution of merge distances at each step in the algorithm and can use this distribution to estimate how many groups are present in the data. Points at which there is a steep increase in the average between-group distance ('elbow' points) highlight the spatial scale(s) at which there is clustering present in the data. By using one of these 'elbow' points as a cutoff in the algorithm, we can arrive at a distance grouping that captures the spatial clustering.

It should be noted that the determination of where to draw this threshold in a clustering analysis is, by definition, arbitrary. The number of categories (or clusters) determined obviously depends on where the threshold is set—if the threshold is set to define

only a very small area around every point in an *n*-dimensional space (*e.g.* a distance of 1 in Fig. 3A), the number of categories can be close to the actual number of data points. If, on the other hand, the threshold is set to a very high value (*e.g.* a distance of 7 in Fig. 3A), there will be only a few categories (two in this case). However, these two examples represent the extremes and illustrate why it is important to choose a threshold within the 'elbow' region of the between-group distance graph, as mentioned previously. The threshold values in our analyses were chosen from this 'elbow' region and determination of the specific point to be used was further informed by visual inspection of reflectance spectra, as well as distributions of colour loci in the perceptual colour spaces. For consistency, the same step in the algorithm, step 51, was chosen as the threshold for both bee and hummingbird groups, corresponding to a distance of ~0.7 and ~0.8 for bee and hummingbird groups, respectively (Fig. 3B,C).

Petal cell area measurements

To assess whether an increase in ploidy results in larger petal cells, cell area was measured from a subset of polyploids and their progenitors. The accessions used for the cell area measurements are the same as for spectral reflectance measurements, except for *N. tabacum*. For *N. tabacum* 'Samson', *N. sylvestris* A04750326, *N. rustica* var. *asiatica*, *N. rustica* var. *pavonii*, *N. paniculata*, *N. undulata* and *N. nudicaulis*, mature flowers were taken from plants and the adaxial petal surface was imprinted in Elite HD vinylpolysiloxane impression material (dental wax, supplied by Zhermack, Harrogate, UK). The wax was left to set, and then used as a mould for making epoxy petal casts. Devcon high-strength epoxy was mixed according to manufacturer's instructions, poured into the mould and allowed to set for 12 hours. The epoxy relief was removed and coated with gold using a Quorum K756X sputter coater. The samples were then imaged using a FEI Philips XL_{30} FEGSEM scanning electron

microscope. For *N. tomentosiformis*, *N. obtusifolia* var. *obtusifolia* TW143, *N. repanda* and *N. stocktonii*, only fixed material was available; whole mature flowers were fixed in formalin-acetic acid-alcohol (FAA) (60% ethanol; 6% formaldehyde; 5% acetic acid) for 72 hours before being transferred to a 70% ethanol (EtOH) wash for 24 hours. The samples were then dehydrated through an ethanol series of 2 hours each in 70%, 80%, 90% and two washes in 100% EtOH. The samples were dissected and then dried in an Autosamdri 815B critical point dryer. These were sputter coated and imaged as described above. For all samples, images were taken mid petal from an angle perpendicular to the surface, to minimise parallax error. Cell size measurements were carried out in ImageJ. The circumference of the cell base was drawn freehand and area was calculated for *circa* 100-150 cells until the cumulative mean stabilised. One-way ANOVA and Tukey's Honest Significance Tests were performed in RStudio to compare cell area of polyploids to those of their progenitors, repeating the tests for each polyploid section.

Ancestral state reconstruction

To examine evolution of colour within a phylogenetic context, ancestral state reconstructions were performed on trees inferred from plastid sequence data. Only species for which floral character data are available were included in these analyses. Because polyploid and homoploid hybrids originate via reticulate evolutionary processes, and therefore lack a history of tree-like evolution, ancestral characters were reconstructed using only non-hybrid diploid species. The states observed in hybrid species were then compared with the ancestral state reconstructions. Since sections *Repandae* and *Suaveolentes* have diversified to form several species following polyploidisation, characters were reconstructed for these sections separately to examine colour evolution subsequent to their origin. For non-hybrid diploid species, individual gene trees yield some conflicting topologies; nevertheless,

key nodes for the purposes of interpreting character evolution in hybrids are recovered in multiple gene trees and are supported by supernetwork analyses (Kelly *et al.*, 2010). Therefore, plastid data are suitable for these analyses.

Previously published sequences (Clarkson *et al.*, 2004) from four plastid regions (*mat*K, *ndh*F, *trn*L-F and *trn*S-G) were aligned separately using PRANK+F (Löytynoja and Goldman, 2008) and then concatenated to create a combined plastid dataset before further optimisation by eye in Mesquite version 2.74 (Maddison and Maddison, 2008). For *N. attenuata*, we used GenBank accessions AB040009 and AY098697 for the *matK* and *trnL-F* regions, respectively (due to likely misidentification of *N. attenuata* material used in Clarkson *et al.*, 2004; see Clarkson *et al.*, 2010); the other two regions were scored as missing data. Phylogenetic reconstruction by Bayesian inference was performed as described in Kelly *et al.* (2013) with the exception that BayesTrees v.1.3 (www.evolution.reading.ac.uk/BayesTrees.html) was used to construct 95% majority rule consensus trees. For sections *Repandae* and *Suaveolentes*, sequences representing their putative maternal progenitors were included during Bayesian inference to allow rooting of trees but were pruned from the trees prior to ancestral state reconstruction.

Ancestral states for spectral reflectance colour categories and presence/absence of chloroplasts in petals (data in Table S2) were reconstructed using the parsimony reconstruction method in Mesquite version 2.74, under the unordered states assumption. To account for topological uncertainty, character states were reconstructed over all 36,000 post burn-in trees using the 'Trace Character Over Trees' option and summarised on the 95% majority rule consensus tree from the Bayesian analysis. Ancestral states were not calculated for bee or hummingbird colour categories because these are perceptual systems and the same colour category can result from different combinations of pigments; thus, a single category does not necessarily have a shared evolutionary history.

Phylogenetic signal in floral traits

In order to statistically test for phylogenetic signal in the phenotypic trait data (spectral reflectance, bee and hummingbird colour perception), we used Mantel tests to examine the correlation between phylogenetic distance and each of the respective continuous multidimensional traits (*e.g.* Cubo *et al.*, 2005; Muchhala *et al.*, 2014). Analyses were restricted to diploid species only, excluding homoploid and polyploid hybrids. Trees were edited in Newick format to include additional tips with zero branch lengths for taxa that are multiple in the trait datasets, either due to colour polymorphism (*N. otophora*) or multiple accessions (*N. sylvestris* and *N. obtusifolia* var. *obtusifolia*).

Statistical analyses were performed in R version 3.1.0. Phenotypic distance matrices were first calculated for the three trait datasets using Euclidean distance, and phylogenetic distance matrices were calculated (i) as genetic distance from the plastid alignment and (ii) for each of 36,000 post-burnin Bayesian trees using cophenetic.phylo(), part of the ape package version 3.1-2 (Paradis *et al.*, 2004). The second Bayesian set of tests was performed in order to account for evolutionary processes such as saturation and to estimate how phylogenetic uncertainty affects the correlation. Mantel tests were performed using Pearson's product-moment correlation coefficient, with 10,000 permutations of each distance matrix to test for significance; the mean p-value and its standard deviation were calculated for each set of 36,000 Mantel tests from the Bayesian trees, along with the percentage of trees that gave significant correlations. The function mantel() from the vegan package was used (Oksanen *et al.*, 2013).

Results

Petal cell area

Petal cell area was measured to determine whether an increase in ploidy results in larger floral cells. Polyploid petal cell area is significantly larger than both progenitors in both *N. tabacum* (ANOVA: F=376.3, df=2, $p<2x10^{-16}$) and *N. rustica* (ANOVA: F=371, df=3, $p<2x10^{-16}$) accessions, but is intermediate between progenitors in section *Repandae* polyploids (ANOVA: F=249.2, df=4, $p<2x10^{-16}$; Fig. 2). Tukey's Honest Significance Tests were performed to determine whether the average cell area between polyploids and their progenitors were significantly different, and the results can be found in Supplemental Information Table S3. Significantly different accessions (within polyploid sections and their progenitors) are represented by different letters above the bars in Fig. 2.

Clustering analyses

Nicotiana reflectance spectra were grouped based on spectral shape and position in the bee and hummingbird colour spaces using clustering analyses. The analysis based on spectral shape yielded eight colour categories, which roughly corresponded to flowers perceived by human observers as magenta, red, pink, UV-white, white, yellow, green, and dark green (Fig. 3A). *Nicotiana* spectra are displayed by spectral colour category in Fig. 4A,B, S2. The bee colour hexagon clustering resulted in eleven colour categories, which fell into the following areas of bee colour space: saturated green, UV-blue, high UV, UV-green, green, light green, blue-green, dark green, saturated UV-blue, saturated UV-green, and blue (the last four categories are each represented by only a single accession; Fig. 3B). These groups are shown in the bee colour hexagon (Fig. 4C). The hummingbird colour space clustering analysis also produced eleven colour categories: saturated green, green, UV-white, UV-green, pink, white, UV-pink, dark green, light pink, red, and saturated UV-pink (again the last four categories include only a single accession; Fig. 3C). These groups are shown in

the hummingbird colour space (Fig. 4D) and as an animation to better display the 3D nature of the colour space (Fig. S3).

Evolution of spectral reflectance in polyploids and homoploid hybrids

To assess evolution of polyploid floral colour, polyploid spectra were compared to those of their progenitors. The diploid progenitors and approximate age of polyploids and homoploid hybrids are found in Table 1. Most polyploids and homoploid hybrids are similar to at least one progenitor in spectral shape and in the bee and hummingbird colour spaces (Fig. 5, S4, S5). However, *N. tabacum* and TH32 spectra display shapes that are different from both progenitor spectra (Fig. 5A, S4A). The polyploid and homoploid hybrids that are classified into divergent colour groups from their progenitors are as follows: in spectral reflectance curve shape, *N. tabacum* 095-55 and *N. glauca*; in bee colour, *N. tabacum* 095-55, synthetic *N. tabacum* QM, *N. rustica* var. *asiatica*, synthetic U×P, synthetic F1, synthetic *N. rustica* S0, synthetic *N. rustica* S1 and *N. glauca* (Fig. 3, 5C,I, S4F); in hummingbird colour, *N. tabacum* 095-55, synthetic *N. tabacum* QM, synthetic U×P, *N. arentsii*, *N. clevelandii* and *N. glauca* (Fig. 3, 5B,H, S4E,H, S5B). *Nicotiana clevelandii* also lacks the reflectance minimum at 675 nm, which corresponds to the absorbance of chlorophyll *in vivo* (Haardt and Maske, 1987), unlike both progenitors (Fig. S5A).

Evolution of colour characters in a phylogenetic context

Reconstructed character states are shown for spectral reflectance colour categories (Fig. 6A) and the presence/absence of chloroplasts in petals (Fig. S6). Bee and hummingbird colour categories are also shown for extant species on the plastid tree (Fig. 6B,C). Although the deepest nodes are largely equivocal, evolution of spectral reflectance colour in *Nicotiana* seems to be dynamic (Fig. 6A). Green flowers likely have three independent origins: 1) in

sections *Paniculatae* and *Undulatae*, 2) in *N. langsdorffii* and 3) in the homoploid hybrid *N. glauca*. UV-white flowers also seem to have arisen three times independently: 1) in section *Trigonophyllae*, 2) in *N. pauciflora* and 3) in the homoploid hybrid *N. linearis*. Most polyploid and homoploid hybrid species exhibit a floral colour present in at least one of their progenitors. However, *N. tabacum* 095-55 is red and *N. glauca* is yellow and green, unlike their progenitors. UV-white flowers seem to have evolved *de novo* in *N. linearis*. UV-white flowers are also found in one of its progenitor sections, but the evolution of this state in *N. pauciflora* seems to have occurred subsequent to the formation of *N. linearis*, suggesting the two events are independent. It is unclear whether UV-white flowers also evolved *de novo* in *N. nudicaulis* because the ancestral node of section *Repandae* is equivocal.

Presence of chloroplasts in *Nicotiana* flowers is ancestral and has been lost three times in *N. sylvestris*, *N. noctiflora* and the most recent common ancestor of *N. acuminata* and *N. pauciflora* (Fig. S6). Whereas most polyploids and homoploid hybrids are similar to at least one progenitor, *N. clevelandii* has lost chlorophyll pigmentation.

Results from Mantel tests for phylogenetic signal for *Nicotiana* floral traits, for both genetic distance and the 36,000 post-burnin Bayesian trees, are shown in Table 2. All floral traits are significantly correlated with phylogenetic relationships for the Bayesian trees at a significance level of $p<0.05$. Only spectral reflectance is significant for the genetic distance tests whereas bee and hummingbird colour perception are just above the $p<0.05$ threshold. For the Bayesian trees, 90.1, 66.2 and 93.2 percent of trees are significantly correlated with the spectral reflectance, bee and hummingbird colour perception datasets, respectively. These results suggest that these floral traits are weakly constrained by phylogeny and that bee colour perception may be less constrained than spectral reflectance and hummingbird colour perception.

Discussion

Nicotiana is remarkable in its range of flower colours (white, UV-white, pink, magenta, red, yellow, green and dark green) and in the number and the variety of pollinators that visit the flowers (moth, bird, bee, bat; Knapp, 2010). Here, we describe a complex dynamic in the evolution of floral colour in *Nicotiana*. Spectral reflectance and bee and hummingbird colour perception are correlated with phylogeny, but multiple independent origins of various combinations of pigmentation suggest that the evolution of floral colour is not entirely phylogenetically constrained.

Known floral pigments in Nicotiana

Few studies have examined the specific pigments present in *Nicotiana* petals. Aharoni *et al.* (2001) confirm the presence of anthocyanin pigmentation in *N. tabacum*, which seems to be predominantly cyanidin derivatives. The yellow flower colour of *N. glauca* is due to carotenoid pigmentation (Zhu *et al.*, 2007). The reflectance minimum at 675nm seen in many of the *Nicotiana* spectra presented here (Fig. 4, S2) suggests the presence of chlorophyll in petals because chlorophyll absorbs at 675nm *in vivo* (Haardt and Maske, 1987).

Transgressive flower colour in N. tabacum *and the synthetic polyploid TH32*

Polyploids *N. tabacum* and synthetic TH32 are similar because they share a maternal progenitor, *N. sylvestris*, and their paternal progenitors, *N. tomentosiformis* and *N. otophora*, respectively, are both from section *Tomentosae* and have similar reflectance spectra: the paternal progenitors possess anthocyanin pigmentation as well as chlorophyll, whereas the maternal progenitor lacks both of these (Fig. S5G).

Genetic crosses in *Nicotiana* suggest that both green flower colour and the ability to produce floral anthocyanins are dominant and each may be determined by a single locus (Brieger, 1935). From this information, we can predict the expected floral phenotype for *N. tabacum* and TH32. The maternal progenitor, *N. sylvestris*, is recessive for green flower colour (it likely has colourless leucoplasts in its petals, like those found in *Arabidopsis* petals; Pyke and Page, 1998) and likely recessive for producing floral anthocyanins (pink flowers have never been recorded in *N. sylvestris*). The paternal progenitors, *N. tomentosiformis* and *N. otophora*, are dominant for green flower colour (they possess chlorophyll in their petals) and are dominant for anthocyanins (their flowers are pink, likely due to anthocynanin pigmentation). Therefore, *N. tabacum* and TH32 should be heterozygous, carrying two dominant and two recessive alleles for both green and pink flower colour, yielding a phenotype like that of their paternal progenitors: presence of both chlorophyll and anthocyanin pigments. However, this is not what is observed; *N. tabacum* accessions and TH32 possess anthocyanin pigmentation (two spectral peaks in the blue and red portions of the spectrum), but not chlorophyll (the lack of a reflectance minimum at 675nm; Fig. 4A, S4A). Therefore, *N. tabacum* and TH32 inherit anthocyanin floral pigmentation from their paternal progenitors, but a plastid phenotype (chlorophyll is only found in plastids) like that of their maternal progenitor; this floral phenotype is transgressive because it is unlike either progenitor and divergent from the expected phenotype. Intriguingly, both the *N. tomentosiformis* and *N. sylvestris* copies of the bHLH transcription factor involved in regulation of the anthocyanin biosynthetic pathway are expressed and functional in *N. tabacum* (Bai *et al.*, 2011), suggesting that a maternal gene has been co-opted into producing a paternal-type phenotype.

Polyploids typically inherit plastids from their maternal progenitor; it may be unsurprising, therefore, that *N. tabacum* and TH32 plastids have the maternal phenotype.

However, it is likely that the chloroplast-to-leucoplast transition in petal development is regulated by nuclear genes because most of the original plastid genome has been transferred to the nucleus, save those genes directly involved in photosynthesis (Puthiyaveetil and Allen, 2009). A study in *Arabidopsis* indicated that petal homeotic genes *APETALA3* and *PISTILLATA* down-regulate *BANQUO* genes, which are involved in accumulation of chlorophyll, suggesting that the breakdown of chloroplasts in petal development is linked to repression of genes involved in chlorophyll biosynthesis by nuclear encoded petal identity genes (Mara *et al.*, 2010). Furthermore, backcrosses of green-flowered F_1s to their non-green-flowered parent produced similar phenotypic ratios despite the direction of the cross (Brieger, 1935), suggesting that maternal plastid phenotype does not determine that of its offspring.

The polyploids *N. tabacum* and TH32 are heterozygous at the green-flowered locus, but it is unlikely that this non-green phenotype could arise via segregation in subsequent generations because these polyploids have fixed heterozygosity due to disomic inheritance (their progenitor genomes do not pair during meiosis). Also, synthetic *N. tabacum* QM is a first generation synthetic polyploid, suggesting that inheritance of the maternal-type leucoplast phenotype occurs immediately following polyploidisation. The *N. tabacum* and TH32 accessions examined here represent at least four independent origins (three synthetic and the natural accessions), and the same combination of pigments (the presence of anthocyanins, but the lack of chlorophyll) is observed in all of them, suggesting that the interplay between inheritance of plastid and vacuolar pigments yields a transgressive phenotype repeatedly in *N. tabacum* and TH32 polyploids.

The accessions of *N. tabacum* examined here vary in spectral shape and bee and hummingbird colour (Fig. 5A-C). Synthetic *N. tabacum* QM and *N. tabacum* 095-55 are distinct in both bee and hummingbird colour space, suggesting that these accessions will be

distinguishable from their progenitors by both bee (and likely hawkmoth, due to similarities in photoreceptor sensitivities) and hummingbird pollinators. The differences seen in the *N. tabacum* spectra may be due to the presence of different cyanidin derivatives, but vacuolar pH and the formation of heterodimers of anthocyanin and flavonol pigments can also cause shifts in spectral reflectance (Grotewold, 2006; Andersen and Jordheim, 2010). Cell size in *N. tabacum* is also significantly larger than the average cell size of its progenitors (Fig. 2), which likely affects the concentration of pigment found in petal cells. Synthetic *N. tabacum* TH37 and *N. tabacum* 'Chulumani' both have pale pink flowers (Fig. 1A), which may be at least partially explained by a decrease in the concentration of anthocyanin pigments due to an increase in cell size. Increased cell size may also explain the intermediate pigmentation concentration seen in *N. rustica* polyploids (Fig. 2, S4D). Duplicate pigment genes in polyploids are expected to result in an increase in the amount of pigment produced, and therefore an increased pigment concentration if cell size is similar to that of the progenitors. However, with an increase in cell size, the concentration should be intermediate between that of the progenitors, as is seen in *N. rustica* polyploids.

Polyploid divergence in floral colour

Many younger polyploids (<0.2 million years old) display divergent floral colours. As described above, *N. tabacum* and TH32 have a transgressive floral colour and some accessions are distinct from both progenitors in both bee and hummingbird colour space. Most *N. rustica* accessions are divergent from both progenitors in bee colour space, and *N. arentsii* is divergent in hummingbird colour space (Fig. 6). However, behavioural studies are still needed to determine whether the colour categories delineated here are actually distinct to insect and hummingbird pollinators. Most older polyploids (1-10 million years old) are similar in floral colour to at least one of their progenitors; *N. clevelandii* is the exception

because it is divergent in hummingbird colour space and lacks chlorophyll (Fig. 6, S6). However, as the age of a polyploid increases, there is an increased possibility that the most closely related extant diploid representatives of their progenitors differ in phenotype from those individuals actually involved in the polyploidisation event. Therefore, we cannot discount the possibility that change in these characters occurred in the diploid lineage and that *N. clevelandii* in fact resembles its true progenitor. Section *Repandae* polyploids seem to have evolved to be either like their maternal (*N. nesophila*, *N. repanda* and *N. stocktonii*) or paternal (*N. nudicaulis*) progenitor after diverging from the single original species formed via allopolyploidisation (Fig. 6). The maternal progenitor, *N. sylvestris*, is no longer sympatric with any of the section *Repandae* polyploids; therefore, *N. nesophila*, *N. repanda* and *N. stocktonii* can occupy the same pollination niche as their maternal progenitor without competition. Similarly, section *Suaveolentes* is native to Australasia, except for one species in Namibia, Africa, and is not sympatric with its progenitor sections in South America (Goodspeed, 1954); these polyploids and their diploid progenitors display similar floral colours, except *N. pauciflora*, which evolved spectrally UV-white flowers after the formation of section *Suaveolentes* (Fig. 6A). Sympatric taxa in the Iochrominae (Solanaceae) have a broader range of floral colours than allopatric taxa (Muchhala *et al.*, 2014), suggesting that competition for pollinators can drive floral colour diversification among closely related sympatric taxa.

Novel floral colour in homoploid hybrids

Homoploid hybrid *N. glauca* displays a novel floral colour in spectral, bee and hummingbird categories (Fig. 6). The combination of all floral traits displayed will determine pollinator behaviour, but this drastic change in floral colour may have played at least some role in the establishment of reproductive isolation between *N. glauca* and its

progenitors. Without reproductive isolation, homoploid hybrids often facilitate gene flow between their progenitors instead of becoming established as new species (Buerkle *et al.*, 2000; 2003). In experimental field plots of *Nicotiana alata* and *N. forgetiana*, pollinator fidelity decreased significantly in the presence of F_1 hybrids, increasing gene flow between the two progenitor species (Ippolito *et al.*, 2004).

Species of progenitor sections *Noctiflorae* and *Petunioides* mostly have vespertine flowers and many have long corolla tubes (Goodspeed, 1954), which suggest pollination by nocturnal hawkmoths. The only studies examining pollination in any of these species have confirmed that *N. attenuata* is pollinated by nocturnal hawkmoths but is also visited by hummingbirds (Aigner and Scott, 2002; Kessler and Baldwin, 2006). *Nicotiana glauca* is pollinated by hummingbirds in its native range (Nattero and Cocucci, 2007). Selection can still occur in the presence of generalist pollination based on differences in pollinator assemblage (Gomez *et al.*, 2009), so the floral colour shift in *N. glauca*, accompanied by a shift in the predominant pollinator, may have aided reproductive isolation and its establishment as a new species. Evolutionary shifts in characteristics known to affect pollinator preferences often occur together. Shift from insect to hummingbird pollination has occurred twice within *Mimulus* section *Erythranthe* (Phrymaceae), and red flowers, exserted stamens and pistils and reflexed upper petals (characters associated with hummingbird pollination) seem to have evolved at the same points on the phylogenetic tree as the shift in pollination (Beardsley *et al.*, 2003). In addition to a shift to yellow flowers, *N. glauca* has a reduced floral limb, the part of the corolla that opens, (associated with hummingbird pollination) compared with many species in its progenitor sections, suggesting the possibility of hummingbird-mediated selection on *N. glauca* floral traits.

Concluding remarks

Floral colour shifts in polyploid and homoploid hybrids may occur immediately after their formation, perhaps as a consequence of novel *cis-trans* interactions between progenitor genomes (Chen, 2007). Using genomic studies to examine plant-pollinator interactions will shed light on the complex interactions involved in successful pollination and pollinator-mediated evolution (Clare *et al.*, 2013). Transgressive and divergent floral colours may have aided hybrid speciation, but pollination studies of hybrids and their progenitors are needed to make these conclusions. Typically, synthetic and young polyploids (<0.2 million years old) have flowers that are divergent from their progenitors in the colour perception of at least one pollinator type. Older polyploids (1-10 million years old) tend to have a floral colour like at least one progenitor, perhaps due to the fact that the polyploids are no longer sympatric with one or both progenitors and/or because other floral traits were more important in the divergence from their progenitors.

Supplementary Data

Supplementary data are available online and include the following. Table S1: *Nicotiana* accessions used in the spectral reflectance dataset and in petal cell area measurements. Table S2: Floral colour characters for all *Nicotiana* species examined. Table S3: Tukey's Honest Significance Test results for cell areas. Figure S1: Navigating the bee colour hexagon. Figure S2: *Nicotiana* reflectance spectra from 300-700 nm by spectral colour category. Figure S3: Animation of *Nicotiana* spectra in 3D hummingbird colour space. Figure S4: Reflectance spectra, bee colour hexagons and hummingbird colour space for TH32, *N. rustica* and *N. arentsii*. Figure S5: Reflectance spectra, bee colour hexagons and hummingbird colour space for section *Polydicliae*, section *Suaveolentes* and *N. glutinosa*. Figure S6: Ancestral state reconstruction of the presence/absence of chloroplasts in petals.

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Tables

Table 1 Polyploid and homoploid hybrid origins

Hybrid	Maternal Progenitor	Paternal Progenitor	Age (millions of years)
N. tabacum	*N. sylvestris*	*N. tomentosiformis*	<0.2 (Clarkson *et al.*, 2005)
synthetic *N. tabacum* QM	*N. sylvestris*	*N. tomentosiformis*	0 (cross by K. Y. Lim, QMUL, UK)
synthetic *N. tabacum* TH37	*N. sylvestris*	*N. tomentosiformis*	0 (Burk, 1973)
TH32	*N. sylvestris*	*N. otophora*	0 (United States *Nicotiana* Germplasm Collection; Moon *et al.*, 2008)
N. rustica	*N. paniculata*	*N. undulata*	<0.2 (Clarkson, 2006; Leitch *et al.*, 2008)
synthetic U×P	*N. undulata*	*N. paniculata*	0 (diploid cross, A. Kovařík)
synthetic PUE1 F_1	*N. paniculata*	*N. undulata*	0 (diploid cross, A. Kovařík)
synthetic *N. rustica* PUE1-R10 S_0	*N. paniculata*	*N. undulata*	0 (synthetic PUE1 F_1 doubled, C. Mhiri)
synthetic *N. rustica* PUE1-R1 S_1	*N. paniculata*	*N. undulata*	0 (putative S_1 from doubled PUE1 F_1)
N. arentsii	*N. undulata*	*N. wigandioides*	<0.2 (Clarkson, 2006; Leitch *et al.*, 2008)
N. clevelandii	*N. obtusifolia*	*N. attenuata*	~1 (Clarkson, 2006; Leitch *et al.*, 2008)
N. quadrivalvis	*N. obtusifolia*	*N. attenuata*	~1 (Clarkson, 2006; Leitch *et al.*, 2008)
N. × *obtusiata* lines 1, 2, and 5	*N. obtusifolia* 'Baldwin'	*N. attenuata* 'Baldwin'	0 (Anssour *et al.*, 2009)
N. repanda	*N. sylvestris*	*N. obtusifolia*	~4.5 (Clarkson *et al.*, 2005)
N. nesophila	*N. sylvestris*	*N. obtusifolia*	~4.5 (Clarkson *et al.*, 2005)
N. stocktonii	*N. sylvestris*	*N. obtusifolia*	~4.5 (Clarkson *et al.*, 2005)
N. nudicaulis	*N. sylvestris*	*N. obtusifolia*	~4.5 (Clarkson *et al.*, 2005)
N. benthamiana	sections *Noctiflorae* and *Petunioides*	*N. sylvestris*	~10 (Leitch et al., 2008)
N. forsteri	sections *Noctiflorae* and *Petunioides*	*N. sylvestris*	~10 (Leitch et al., 2008)
N. gossei	sections *Noctiflorae* and *Petunioides*	*N. sylvestris*	~10 (Leitch et al., 2008)
N. megalosiphon	sections *Noctiflorae* and *Petunioides*	*N. sylvestris*	~10 (Leitch et al., 2008)
N. occidentalis subsp. *hesperis*	sections *Noctiflorae* and *Petunioides*	*N. sylvestris*	~10 (Leitch et al., 2008)
N. suaveolens	sections *Noctiflorae* and *Petunioides*	*N. sylvestris*	~10 (Leitch et al., 2008)
N. glauca[*]	Progenitors: sections *Noctiflorae* and *Petunioides*		N/A
N. linearis[*]	Progenitors: sections *Noctiflorae* and *Petunioides*		N/A
N. glutinosa[*]	Progenitors: sections *Tomentosae* and *Undulatae*		N/A

[*]Homoploid hybrid evolution is more convoluted and difficult to detect; therefore, which progenitor was maternal or paternal, as well as the age of origin, has not been determined.

Table 2 Mantel test results

	Genetic Distance	Bayesian	
Trait	p-value	Mean p-value	% significant trees
Spectral reflectance	0.0229	0.0206±0.0215	90.1
Bee colour vision	0.0866	0.0410±0.0321	66.2
Hummingbird colour vision	0.0594	0.0198±0.0187	93.2

Figure legends

Fig. 1 Floral colour, as perceived by humans, of polyploid and homoploid hybrid *Nicotiana* and their diploid progenitors. Polyploid ages were estimated using a molecular clock calibrated with the geological age of volcanic islands with endemic *Nicotiana* species (Clarkson *et al.*, 2005). Absolute dates estimated by the clock should be treated with caution; however, relative ages between polyploid sections should reflect the true sequence of polyploidisation events. (A) Natural polyploids of *N. tabacum*, formed <0.2 million years ago (mya) via polyploidisation between maternal *N. sylvestris* and paternal *N. tomentosiformis* progenitors and synthetic polyploids of the same parentage. (B) Synthetic polyploid TH32 and maternal *N. sylvestris* and paternal *N. otophora* progenitors. (C) Natural *N. rustica* polyploids, which formed <0.2 mya from maternal *N. paniculata* and paternal *N. undulata* progenitors. Synthetic hybrids include a homoploid from a reciprocal cross (*N. undulata* as the maternal and *N. paniculata* as the paternal parent) and a polyploid series (F_1 homoploid, and S_0 and S_1 polyploids) of the same parentage as natural *N. rustica*. (D) *Nicotiana arentsii* was formed <0.2 mya from maternal *N. undulata* and paternal *N. wigandioides* progenitors. (E) Natural polyploids of section *Polydicliae*, *N. clevelandii* and *N. quadrivalvis*, speciated after a single polyploidisation event between maternal *N. obtusifolia* and paternal *N. attenuata* progenitors ~1 mya. Synthetic *N.* × *obtusiata* polyploid lines were made from a cross between the *N. obtusifolia* and *N. attenuata* accessions studied here. (F) Section *Repandae* includes four species, which speciated after a single polyploidisation event between maternal *N. sylvestris* and paternal *N. obtusifolia* progenitors ~4.5 mya. (G) Section *Suaveolentes* contains 26 polyploid species and *N. sylvestris* and sections

Noctiflorae and *Petunioides* appear to have played a role in its origin ~10 mya; *N. sylvestris* seems to be the paternal progenitor. Biogeographical analyses suggest that section *Suaveolentes* originated ~15 mya, before the aridification of Australia (Ladiges *et al.*, 2011), and this seems to be relatively congruent with the molecular clock results. (H) *Nicotiana glauca* and *N. linearis* are likely to be homoploid hybrids, which arose via hybridisation between sections *Noctiflorae* and *Petunioides*. (I) *Nicotiana glutinosa* seems to be a homoploid hybrid between sections *Tomentosae* and *Undulatae*. Photographs scaled to the same size.

Fig. 2 Petal cell area from polyploids and their progenitors. Within each polyploid group, bars with different letters represent significantly different mean cell areas.

Fig. 3 Dendrograms based on distance clustering analyses for (A) spectral, (B) bee and (C) hummingbird colour categories. Coloured circles represent distinct colour categories as determined by the chosen threshold (dotted line).

Fig. 4 (A,B) *Nicotiana* reflectance spectra from 300-700 nm, which roughly correspond to colours perceived by human observers as pink (A) and green (B). See Supplemental Fig. S2 for other spectral colour categories. Solid lines are used for diploid taxa, dashed lines for polyploid taxa, and dotted lines for homoploid hybrid taxa. p=pink; syn=synthetic; g=green. (C) Colour hexagon displaying the distribution of *Nicotiana* colour loci in bee colour space. The hexagon has been scaled so that vertices represent 40% excitation of photoreceptors. UV=ultraviolet; UV-B=UV-blue; B=blue; B-G=blue-

green; G=green; UV-G=UV-green. Bee colour categories are delineated by coloured ovals; sat.=saturated. Species abbreviations are as follows: acum=*N. acuminata*; aren=*N. arentsii*; atten=*N. attenuata*; benavid=*N. benavidesii*; benth=*N. benthamiana*; clev=*N. clevelandii*; forst=*N. forsteri*; glau25=*N. glauca* 51725; glau51y=*N. glauca* 51751 yellow; glau51g=*N. glauca* 51751 green; glut=*N. glutinosa*; goss=*N. gossei*; knight=*N. knightiana*; langs=*N. langsdorffii*; lin9647=*N. linearis* 964750099; linTW77=*N. linearis* TW77; mega=*N. megalosiphon*; mier=*N. miersii*; mutab1w=*N. mutabilis* CPG12456 white; mutab1p=*N. mutabilis* CPG12456 pink; mutab3w=*N. mutabilis* CPG3 white; mutab3p=*N. mutabilis* CPG3 pink; neso=*N. nesophila*; noct=*N. noctiflora*; nudi=*N. nudicaulis*; ×obtus1=*N.* × *obtusiata* line 1; ×obtus2=*N.* × *obtusiata* line 2; ×obtus5=*N.* × *obtusiata* line 5; obtusB=*N. obtusifolia* var. *obtusifolia* 'Baldwin'; obtusTW=*N. obtusifolia* var. *obtusifolia* TW143; obtuspalm=*N. obtusifolia* var. *palmeri*; occhesp=*N. occidentalis* subsp. *hesperis*; otoph w=*N. otophora* white; otoph p=*N. otophora* pink; pani=*N. paniculata*; pauc=*N. pauciflora*; petun=*N. petunioides*; plumba=*N. plumbaginifolia*; quad9047=*N. quadrivalvis* 904750042; quadTW18=*N. quadrivalvis* TW18; raim=*N. raimondii*; repa=*N. repanda*; rustasi=*N. rustica* var. *asiatica*; rustpav=*N. rustica* var. *pavonii*; syn U×P=synthetic U×P; syn F1=synthetic PUE1 F_1; synrustS0=synthetic *N. rustica* PUE1-R10 S_0; synrustS1=synthetic *N. rustica* PUE1-R1 S_1; setch=*N. setchellii*; stock=*N. stocktonii*; suav=*N. suaveolens*; sylv6898=*N. sylvestris* 6898; sylvA047=*N. sylvestris* A04750326; tab09555=*N. tabacum* 095-55; tab51789=*N. tabacum* 51789; tabchulu=*N. tabacum* 'Chulumani;' syntabQM=synthetic *N. tabacum* QM; syntabTH37=synthetic *N. tabacum* TH37; tomtform=*N. tomentosiformis*; undu=*N. undulata*; wigan=*N. wigandioides*; TH32=TH32, synthetic *N. sylvestris* × *N. otophora*

polyploid. (D) The distribution of *Nicotiana* spectral loci in hummingbird colour space. The vertices of the hummingbird colour space represent 50% excitation of the photoreceptors; single photoreceptor type vertices (red, green, blue and UV) are coloured red, green, blue and black, respectively and all other vertices are grey. Red, green, blue and black arrows represent the vectors of these photoreceptors from the origin of the hummingbird colour space. *Nicotiana* loci are coloured according to hummingbird colour categories (see Fig. 3C), but are labelled with the accession name if the category includes only one taxon.

Fig. 5 (A,D,G) Reflectance spectra for polyploid or homoploid sections and their progenitors (A) *N. tabacum*, (D) section *Repandae* (G) *Noctiflorae-Petunioides* homoploid hybrids. Solid lines are used for diploid taxa, dashed lines for polyploid taxa, and dotted lines for homoploid hybrid taxa. (B,E,H) Hummingbird colour space for polyploid or homoploid sections and their progenitors: (B) *N. tabacum*, (E) section *Repandae*, (H) *Noctiflorae-Petunioides* homoploid hybrids. The vertices of the hummingbird colour space represent 25% (B,E) or 50% (H) excitation of the photoreceptors; single photoreceptor type vertices (red, green, blue and UV) are coloured red, green, blue and black, respectively and all other vertices are grey. Red, green, blue and black arrows represent the vectors of these photoreceptors from the origin of the hummingbird colour space. (C,F,I) Bee colour hexagons for polyploid or homoploid sections and their progenitors: (C) *N. tabacum*, (F) section *Repandae*, (I) *Noctiflorae-Petunioides* homoploid hybrids. Hexagons have been scaled so that vertices represent 40% excitation of photoreceptors. UV=ultraviolet; UV-B=UV-blue; B=blue; B-G=blue-

green; G=green; UV-G=UV-green. For information regarding how to interpret colour hexagons, see Supplemental Fig. S1. Female (♀) and male (♂) symbols mark maternal and paternal progenitors, respectively, in the hummingbird and bee colour spaces.

Fig. 6 (A) Results of the ancestral state reconstruction for spectral colour categories summarised on the 95% majority rule tree from the Bayesian analysis of plastid sequence data from non-hybrid diploids. Homoploid and polyploid hybrids are superimposed on the diploid tree; black and grey solid, dashed and dotted lines to the right of the tree represent hybridisation events. Orange branches were added to the tree where progenitors of the hybrid taxa are entire sections. Pie charts at internal nodes indicate character states inferred for that node during ancestral state reconstruction carried out on a set of 36,000 post burn-in trees from the Bayesian analyses. Pie charts at the tips of the branches indicate character states observed in extant species. (B) Bee and (C) hummingbird colour categories for extant species displayed on the plastid tree.