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Transgressive phenotypes and generalist pollination in the floral evolution of *Nicotiana* polyploids

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Polyploidy is an important driving force in angiosperm evolution, and much research has focused on genetic, epigenetic and transcriptomic responses to allopolyploidy. *Nicotiana* is an excellent system in which to study allopolyploidy because half of the species are allotetraploids of different ages, allowing us to examine the trajectory of floral evolution over time. Here, we study the effects of allopolyploidy on floral morphology in *Nicotiana*, using corolla tube measurements and geometric morphometrics to quantify petal shape. We show that polyploid morphological divergence from the intermediate phenotype expected (based on progenitor morphology) increases with time for floral limb shape and tube length, and that most polyploids are distinct or transgressive in at least one trait. In addition, we show that polyploids tend to evolve shorter and wider corolla tubes, suggesting that allopolyploidy could provide an escape from specialist pollination via reversion to more generalist pollination strategies.

Polyploidy, or whole genome duplication (WGD), is an important driving force in the evolution of angiosperms. Ancient polyploid events are shared by all seed plants, all angiosperms and all core eudicots, and there is evidence of multiple independent ancient WGDs across the angiosperms^{1–5}. Polyploidy is associated with many genomic changes, including chromosomal rearrangements, retrotransposon activity, gene silencing and epigenetic changes, and it can also yield novel phenotypes, which may facilitate establishment of polyploids as species distinct from their progenitors¹. Potentially, such phenotypic changes in floral structures may affect pollination. Flowers attract pollinators with colour, scent, shape, size of floral display, nectar and pollen rewards, and aspects such as corolla tube or nectar spur dimensions can influence pollinator access to any reward offered. Pollinator-mediated selection on floral traits has been shown in many studies^{6–10} and can even occur in generalist pollination systems because of different selective pressures from varying pollinator assemblages¹¹.

Despite the importance of the changes following allopolyploidy, clear patterns of phenotypic evolution associated with allopolyploidy are hard to discern. In some cases, floral morphology in hybrids is intermediate between that of the parents, whereas in others phenotypes are transgressive (outside the range of the two parental species). For example, F₄ hybrids between *Nicotiana alata* and *N. forgetiana* are intermediate in 12 corolla tube and floral limb (the portion of the flower that opens at anthesis) measurements¹². However, other homoploid (diploid) hybrids display transgressive phenotypes, especially when alleles segregate in F₂ and subsequent generations¹³. F₁ hybrids between two species of *Petunia* with different corolla tube length and floral limb size have transgressively larger floral limbs, but intermediate corolla tube length; in the segregating F₂ population, floral limb size is transgressive in both directions, and tube length can be

transgressively larger¹⁴. In addition, autopolyploidy alone can yield floral changes even without the diversity generated by hybridization. For example, autotetraploids of *Heuchera grossularifolia* have larger, differently shaped flowers than diploids and attract different floral visitors; 6 of 15 common floral visitor species preferred either diploid or autotetraploid flowers¹⁵.

Here, we study the effects of allopolyploidy on floral morphology in the genus *Nicotiana*. *Nicotiana* is an excellent system in which to study allopolyploidy because about half of its species are allotetraploids of different ages, including polyploids formed approximately 0.2, 1, 4.5 and 10 Myr ago; recent (synthetic) polyploids are also available (Table 1)^{16–17}. This series of ever older polyploid lineages enables us to examine both the consequences of polyploidy at formation and how polyploids may have diverged over time from an expected intermediate floral morphology based on that of known diploid progenitors^{16,18–21}. Examples of floral morphology of *Nicotiana* polyploids and their diploid progenitors are found in Fig. 1. Our hypotheses are (1) polyploid floral divergence, from its predicted form at polyploid origin, will increase with age; (2) polyploid divergence in floral morphology will increase with greater progenitor morphological differentiation because there is the potential for greater floral variation on which selection/drift can act; and (3) polyploid species will diverge independently along lineage-specific trajectories.

Results

Floral morphological variation in *Nicotiana*. To examine the extent of variation present in floral limb shape, a geometric morphometric analysis of floral limb shape (Fig. 2a,b) was performed. The first two principal components of the resulting morphospace accounted for 58.18% and 20.33% of the variation present in the data, respectively. The morphospace of these two

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Table 1 | Polyploid and homoploid hybrid origins.

| Hybrid | Section | Maternal progenitor | Paternal progenitor | Age (millions of years) |
|---|--|---|---------------------------|---|
| <i>N. tabacum</i> | <i>Nicotiana</i> | <i>N. sylvestris</i> | <i>N. tomentosiformis</i> | <0.2 (ref. 16) |
| Synthetic <i>N. tabacum</i> QM | Synthetic <i>Nicotiana</i> | <i>N. sylvestris</i> | <i>N. tomentosiformis</i> | 0 (cross by K. Y. Lim, QMUL, UK) |
| Synthetic <i>N. tabacum</i> TH37 | Synthetic <i>Nicotiana</i> | <i>N. sylvestris</i> | <i>N. tomentosiformis</i> | 0 (ref. 56) |
| TH32 | Synthetic <i>Sylvestres-Tomentosae</i> | <i>N. sylvestris</i> | <i>N. otophora</i> | 0 (United States <i>Nicotiana</i> Germplasm Collection) (ref. 57) |
| <i>N. rustica</i> | <i>Rusticae</i> | <i>N. paniculata</i> | <i>N. undulata</i> | <0.2 (refs 17,58) |
| Synthetic U×P | Synthetic <i>Paniculatae-Undulatae</i> | <i>N. undulata</i> | <i>N. paniculata</i> | 0 (diploid cross, A. Kovařík) |
| Synthetic PUE1 F ₁ | Synthetic <i>Paniculatae-Undulatae</i> | <i>N. paniculata</i> | <i>N. undulata</i> | 0 (diploid cross, A. Kovařík) |
| Synthetic <i>N. rustica</i> PUE1-R10 S ₀ | Synthetic <i>Rusticae</i> | <i>N. paniculata</i> | <i>N. undulata</i> | 0 (synthetic PUE1 F ₁ doubled, C. Mhiri) |
| Synthetic <i>N. rustica</i> PUE1-R1 S ₁ | Synthetic <i>Rusticae</i> | <i>N. paniculata</i> | <i>N. undulata</i> | 0 (putative S ₁ from doubled PUE1 F ₁) |
| <i>N. arensii</i> | <i>Undulatae</i> | <i>N. undulata</i> | <i>N. wigandoides</i> | <0.2 (refs 17,58) |
| <i>N. clevelandii</i> | <i>Polydiciae</i> | <i>N. obtusifolia</i> | <i>N. attenuata</i> | ~1 (refs 17,58) |
| <i>N. quadrivalvis</i> | <i>Polydiciae</i> | <i>N. obtusifolia</i> | <i>N. attenuata</i> | ~1 (refs 17,58) |
| <i>N. × obtusiata</i> lines 1, 2 and 5 | Synthetic <i>Polydiciae</i> | <i>N. obtusifolia</i> | <i>N. attenuata</i> | 0 (ref. 59) |
| | | 'Baldwin' | 'Baldwin' | |
| <i>N. repanda</i> | <i>Repandae</i> | <i>N. sylvestris</i> | <i>N. obtusifolia</i> | ~4.5 (ref. 16) |
| <i>N. nesophila</i> | <i>Repandae</i> | <i>N. sylvestris</i> | <i>N. obtusifolia</i> | ~4.5 (ref. 16) |
| <i>N. stocktonii</i> | <i>Repandae</i> | <i>N. sylvestris</i> | <i>N. obtusifolia</i> | ~4.5 (ref. 16) |
| <i>N. nudicaulis</i> | <i>Repandae</i> | <i>N. sylvestris</i> | <i>N. obtusifolia</i> | ~4.5 (ref. 16) |
| <i>N. suaveolens</i> | <i>Suaevolentes</i> | Progenitors: sections <i>Noctiflorae</i> , <i>Petunioides</i> and <i>Sylvestres</i> | | ~10 (refs 17,21,58) |
| <i>N. glauca</i> * | <i>Noctiflorae-Petunioides</i> | Progenitors: sections <i>Noctiflorae</i> and <i>Petunioides</i> | | N/A |
| <i>N. linearis</i> * | <i>Noctiflorae-Petunioides</i> | Progenitors: sections <i>Noctiflorae</i> and <i>Petunioides</i> | | N/A |
| <i>N. glutinosa</i> * | <i>Tomentosae-Undulatae</i> | Progenitors: sections <i>Tomentosae</i> and <i>Undulatae</i> | | N/A |

*Homoploid hybrid evolution is difficult to detect and the age of origin of these hybrids have not been determined. QMUL, Queen Mary University of London.

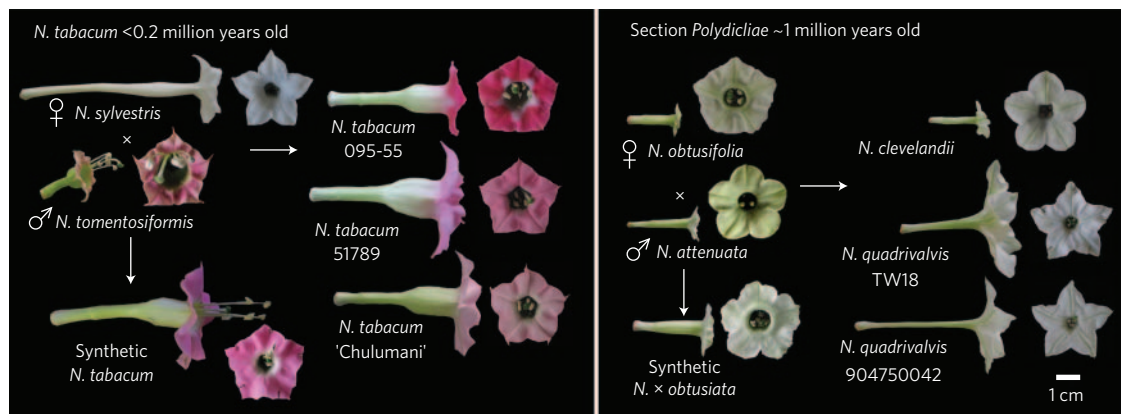


Figure 1 | Side and front floral morphology of *N. tabacum* and section *Polydiciae* polyploids and their diploid progenitors. Side photographs to scale (scale bar, 1 cm, lower right-hand corner), front photographs scaled to the same size. ♀ and ♂ symbols represent maternal and paternal progenitors, respectively.

1 principal components was defined by two characters on diagonal
2 axes; on one axis, the floral outline changed from round to
3 stellate, and, on the other, relative size of the corolla tube opening
4 ranged from small to large. The top right-hand corner of the
5 morphospace consisted of impossible shapes in which the internal
6 landmarks that mark the tube opening cross, creating negative
7 tube width (marked in grey in all figures). Figure 2b shows the
8 extent of the variation present in floral limb shape. Flowers from
9 the same accession clustered in the first two principal components
10 (Fig. 2b and Supplementary Fig. 1), but no clustering was seen in
11 subsequent principal components (for example, PC3 and PC4,
12 Supplementary Fig. 1).

Corolla tube length and width were measured to determine the
range of floral size. The accessions examined here clustered in the
scatter plot of tube width versus length (Fig. 2c). Tube length
ranged from 1.13 to 9.36 cm, and tube width ranged from 0.16
to 1.65 cm.

Polyploid floral morphology. An intermediate phenotype between
those of the diploid progenitors is the simplest null hypothesis
for estimating polyploid phenotype immediately following
polyploidization. The extent of the evolution of polyploid floral
morphology was assessed by comparing each polyploid mean to
its progenitor midpoint (the mean of progenitor averages) and

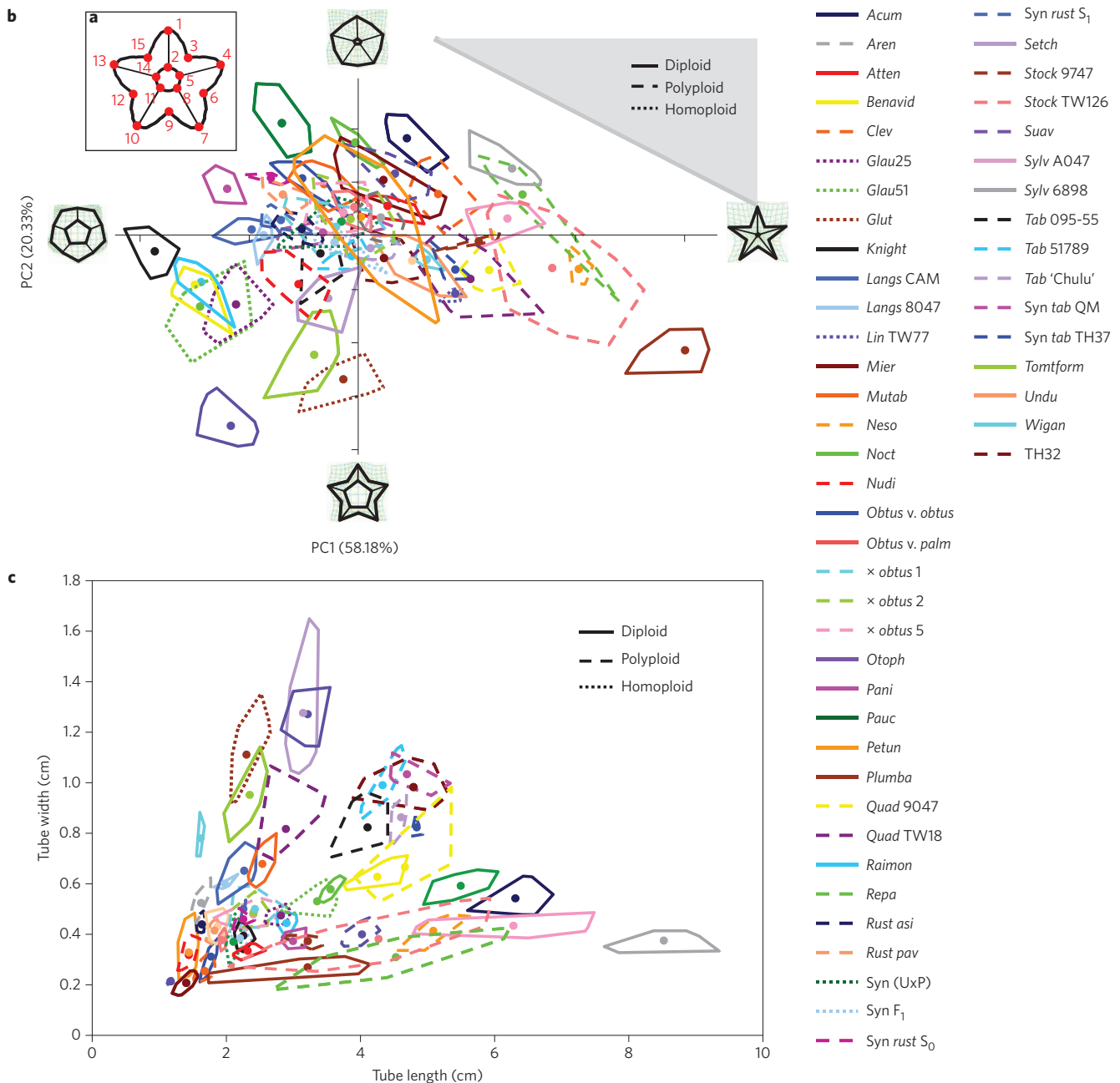


Figure 2 | Distribution of *Nicotiana* accessions in the floral limb shape morphospace and in tube length and width. a, Landmarks used to quantify floral limb shape via geometric morphometrics. **b**, First two components of the resulting morphospace from the geometric morphometric analysis, which consists of two morphological traits on diagonal axes: round to stellate floral outline on one and relatively small to large tube opening on the other. The images at the extremes of the axes show the relative warps of the landmarks at those points. The grey triangle in the right-hand corner represents impossible shapes. Convex polygons enclose the flower averages for each accession, representing the total variance of the accession; circles inside the polygons represent the mean. Diploid accessions have solid lines, polyploid accessions have dashed lines and homoploid or diploid hybrids have dotted lines. **c**, Corolla tube width is plotted against corolla tube length. Convex polygons and means are as described above. Accession abbreviations are found in the Methods.

1 progenitor midpoint range (estimated range of the progenitor
2 midpoint based on the range of progenitors; see Methods for
3 details). The oldest polyploid section, section *Suaveolentes*, was
4 likely to have been formed before diversification of several
5 *Nicotiana* sections²¹; therefore, a progenitor midpoint could not
6 be calculated for *N. suaveolens*. Instead, this polyploid was
7 compared with the morphology of all the species in its progenitor
8 sections (Supplementary Fig. 2). Owing to the difference in
9 comparison, this polyploid was left out of further calculations. In
10 floral limb shape, 50% of polyploids overlapped with their

progenitor midpoint range, 27% were distinct from the midpoint
11 range but within the combined ranges of their progenitors and
12 23% had a transgressive phenotype outside the combined ranges
13 of their progenitors (Table 2, Fig. 3 and Supplementary Fig. 2; see
14 Methods for further explanation of ‘overlapping’, ‘distinct’ and
15 ‘transgressive’). Evidence from *N. alata*, *N. forgetiana* and their
16 hybrids showed that control of tube length development is not
17 genetically linked to that of tube width^{12,22}. Therefore, we analysed
18 these characters independently. In tube length, 77% of polyploids
19 overlapped with their progenitor midpoint range, 9% were distinct
20

Table 2 | Polyploid divergence from the progenitor midpoint.

| Species | Section | Ploidy | Age | Floral limb shape | Tube length | Tube width | At least one distinct (D) or transgressive (T)? |
|--|--|----------------|-----|-------------------|---------------|---------------|---|
| <i>N. tabacum</i> 095-55 | <i>Nicotiana</i> | Polyploid | 0.2 | Distinct | Overlapping | Overlapping | Yes, D |
| <i>N. tabacum</i> 51789 | <i>Nicotiana</i> | Polyploid | 0.2 | Distinct | Overlapping | Distinct | Yes, D |
| <i>N. tabacum</i> 'Chulumani' | <i>Nicotiana</i> | Polyploid | 0.2 | Overlapping | Overlapping | Overlapping | No |
| Synthetic <i>N. tabacum</i> QM | Synthetic <i>Nicotiana</i> | Polyploid | 0 | Overlapping | Overlapping | Distinct | Yes, D |
| Synthetic <i>N. tabacum</i> TH37 | Synthetic <i>Nicotiana</i> | Polyploid | 0 | Overlapping | Overlapping | Overlapping | No |
| TH32 | Synthetic <i>Sylvestres/Tomentosae</i> | Polyploid | 0 | Overlapping | Overlapping | Overlapping | No |
| <i>N. rustica</i> var. <i>asiatica</i> | <i>Rusticae</i> | Polyploid | 0.2 | Overlapping | Transgressive | Overlapping | Yes, T |
| <i>N. rustica</i> var. <i>pavonii</i> | <i>Rusticae</i> | Polyploid | 0.2 | Overlapping | Distinct | Overlapping | Yes, D |
| Synthetic <i>N. rustica</i> PUE-R10 | Synthetic <i>Rusticae</i> | Polyploid | 0 | Distinct | Overlapping | Overlapping | Yes, D |
| S ₀ | | | | | | | |
| Synthetic <i>N. rustica</i> PUE-R1 | Synthetic <i>Rusticae</i> | Polyploid | 0 | Overlapping | Overlapping | Overlapping | No |
| S ₁ | | | | | | | |
| <i>N. arentsii</i> | <i>Undulatae</i> | Polyploid | 0.2 | Overlapping | Overlapping | Overlapping | No |
| <i>N. clevelandii</i> | <i>Polydiciae</i> | Polyploid | 1 | Transgressive | Overlapping | Overlapping | Yes, T |
| <i>N. quadrivalvis</i> 904750042 | <i>Polydiciae</i> | Polyploid | 1 | Transgressive | Transgressive | Transgressive | Yes, T |
| <i>N. quadrivalvis</i> TW18 | <i>Polydiciae</i> | Polyploid | 1 | Transgressive | Distinct | Transgressive | Yes, T |
| <i>N. × obtusiata</i> line 1 | Synthetic <i>Polydiciae</i> | Polyploid | 0 | Overlapping | Overlapping | Transgressive | Yes, T |
| <i>N. × obtusiata</i> line 2 | Synthetic <i>Polydiciae</i> | Polyploid | 0 | Overlapping | Overlapping | Transgressive | Yes, T |
| <i>N. × obtusiata</i> line 5 | Synthetic <i>Polydiciae</i> | Polyploid | 0 | Overlapping | Overlapping | Transgressive | Yes, T |
| <i>N. nesophila</i> | <i>Repandae</i> | Polyploid | 4.5 | Transgressive | Overlapping | Overlapping | Yes, T |
| <i>N. nudicaulis</i> | <i>Repandae</i> | Polyploid | 4.5 | Transgressive | Transgressive | Overlapping | Yes, T |
| <i>N. repanda</i> | <i>Repandae</i> | Polyploid | 4.5 | Distinct | Overlapping | Overlapping | Yes, D |
| <i>N. stocktonii</i> 974750101 | <i>Repandae</i> | Polyploid | 4.5 | Distinct | Overlapping | Overlapping | Yes, D |
| <i>N. stocktonii</i> TW126 | <i>Repandae</i> | Polyploid | 4.5 | Distinct | Overlapping | Overlapping | Yes, D |
| <i>N. suaveolens</i> | <i>Suaveolentes</i> | Polyploid | 10 | Overlapping | Overlapping | Overlapping | No |
| <i>N. glauca</i> 51725 | <i>Noctiflorae/Petunioides</i> | Homoploid | – | Transgressive | Overlapping | Overlapping | Yes, T |
| <i>N. glauca</i> 51751 | <i>Noctiflorae/Petunioides</i> | Homoploid | – | Transgressive | Overlapping | Overlapping | Yes, T |
| <i>N. linearis</i> | <i>Noctiflorae/Petunioides</i> | Homoploid | – | Transgressive | Overlapping | Overlapping | Yes, T |
| <i>N. glutinosa</i> | <i>Tomentosae/Undulatae</i> | Homoploid | – | Overlapping | Overlapping | Overlapping | No |
| Synthetic U×P | Synthetic <i>Rusticae</i> | Diploid Hybrid | 0 | Overlapping | Distinct | Overlapping | Yes, D |
| Synthetic PUE F ₁ | Synthetic <i>Rusticae</i> | Diploid Hybrid | 0 | Overlapping | Overlapping | Overlapping | Yes, D |

Overlapping, overlaps with progenitor midpoint range. Distinct, does not overlap with progenitor midpoint range. Transgressive, falls outside range of progenitors.

1 and 14% were transgressive (Table 2, Fig. 3 and Supplementary Fig. 2). In tube width, 68% of polyploids were overlapping, 9% are distinct and 23% are transgressive (Table 2, Fig. 3 and Supplementary Fig. 2). In examining all three traits together, most polyploids were either distinct or transgressive in at least one trait, whereas only 23% of polyploids overlapped with their progenitor midpoint range in all traits (Table 2).

Direction and degree of polyploid floral divergence from expectation. Directional distances of each polyploid mean from its progenitor midpoint were plotted with the origin as the progenitor midpoint (Fig. 4). Using this figure, the direction of change in morphology from predicted for each polyploid could be determined (based on the quadrant in which the polyploid falls). In floral limb shape, 70% of polyploids fell along the round to stellate floral axis, whereas 30% of polyploids diverged along the small to large tube opening axis (Fig. 4a). In tube length and width, 25% of polyploids had a longer and wider tube than expected, 50% had a shorter and wider tube and 25% had a shorter and narrower tube. No polyploid had a longer and narrower tube than expected (Fig. 4b). This distribution was significantly different from equal numbers of polyploids in each quadrant ($\chi^2 = 12$, d.f. = 3, $P = 0.0074$), and polyploids tend to have shorter and wider tubes than expected.

Older polyploids tend to be more divergent from the progenitor midpoint. To test whether younger polyploids had an intermediate floral morphology between those of their diploid progenitors whereas older polyploids had distinct or transgressive phenotypes, we plotted the distance of each polyploid from its progenitor midpoint against estimated polyploid age (Fig. 5).

In floral limb shape, distance from the midpoint increased with polyploid age. An asymptotic curve was fitted to the data ($y = 0.0953451 - 5.97903 \times 10^{-16}/x$; Fig. 5a). The shape of this curve implies that divergence from the progenitor midpoint occurs early in polyploid evolution. A Mood's median test on polyploid distance from the midpoint revealed that the median distances of the synthetic and young polyploids (0.2 million years old (myo)) were significantly smaller than those of older polyploids (1 and 4.5 myo; $\chi^2 = 12.27$, d.f. = 3, $P < 0.007$). However, although young and older polyploid groups were significantly different in distance, their ranges overlapped such that the most divergent synthetic and young polyploids were as divergent as the least divergent polyploids of 1 and 4.5 myo (Fig. 5a).

In tube length, regression analysis showed that polyploid distance from the midpoint and polyploid age were positively associated ($F = 4.63$; d.f. = 1, $P = 0.043$); however, older polyploids showed high variance in distance from the midpoint (Fig. 5b). This relationship also confirms our hypothesis that older polyploids tend to be

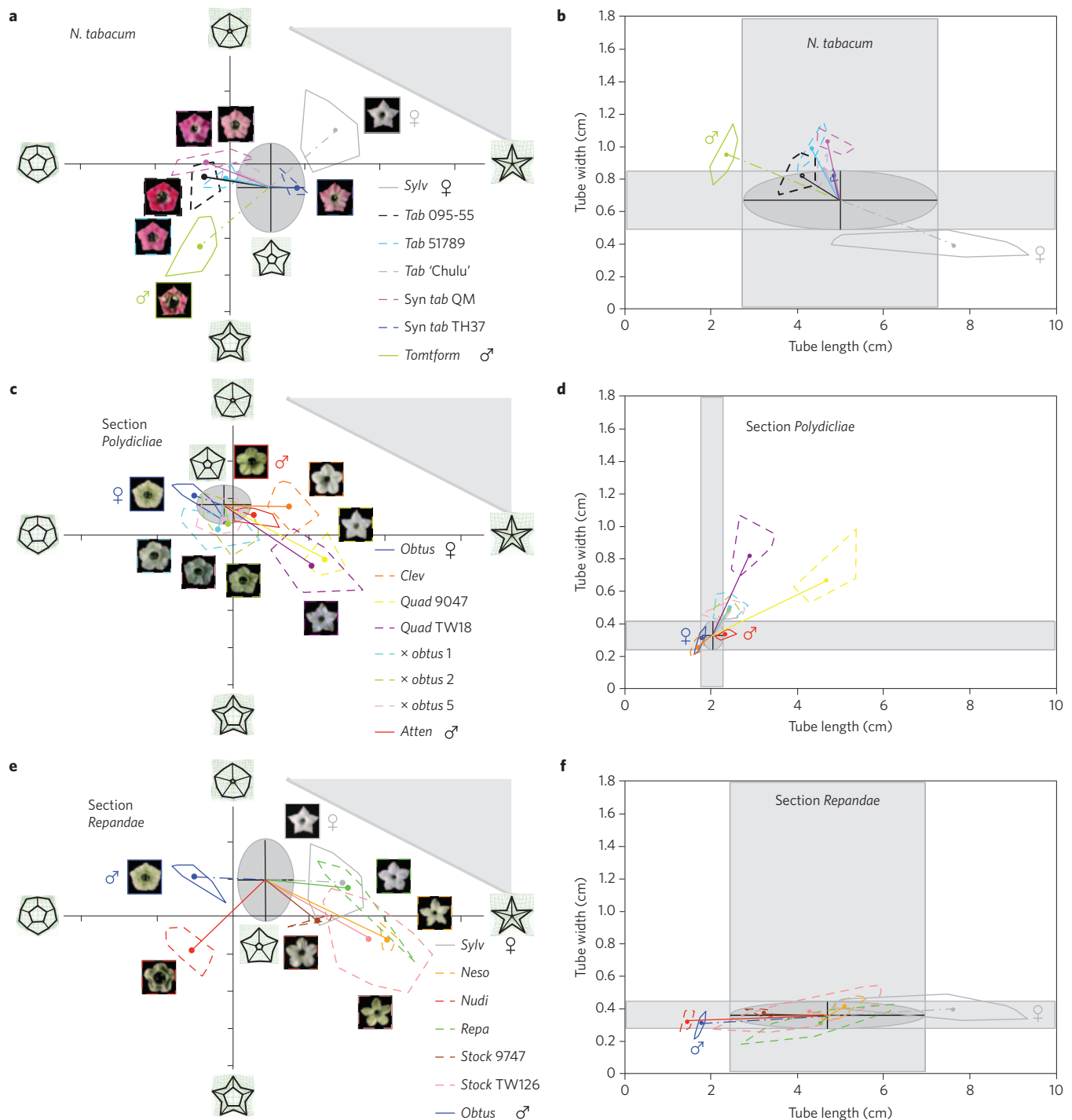


Figure 3 | Allotetraploid sections and their diploid progenitors in the floral limb morphospace and in tube length and width. a,b, *N. tabacum*. c,d, Section *Polydicliae*. e,f, Section *Repandae*. These figures compare polyloid morphology to that of their diploid progenitors and progenitor midpoint range (the expected polyloid morphology based on that of its progenitors). Convex polygons include the flower averages of each accession; diploids have solid lines and allotetraploids have dashed lines. The grey triangle in the top right-hand corner of the floral limb shape plots represents physically impossible shapes. The images at the extremes of the axes show the relative warps of the landmarks at those points. Grey ellipses and boxes represent the progenitor midpoint range. Flower photographs represent each accession and the coloured outline corresponds to the colour of the convex polygon. ♀ and ♂ symbols denote maternal and paternal progenitors, respectively. Accession abbreviations are found in the Methods.

Q3

1 more divergent from the progenitor midpoint than younger ones.
 2 There was no significant relationship between distance from the
 3 progenitor midpoint in tube width and polyploid age ($F=2.57$,
 4 d.f. = 1, $P=0.123$; Fig. 5c).
 5 We also hypothesized that the morphological distance between
 6 diploid progenitor means might affect polyploid divergence.

7 However, there was no correlation between progenitor distance
 8 and polyploid distance from the midpoint in any of the traits
 9 examined (data not shown).

Homoploid hybrid floral morphology. Several species in *Nicotiana* 10
 were shown to be homoploid hybrids^{19,23}. However, the progenitors 11

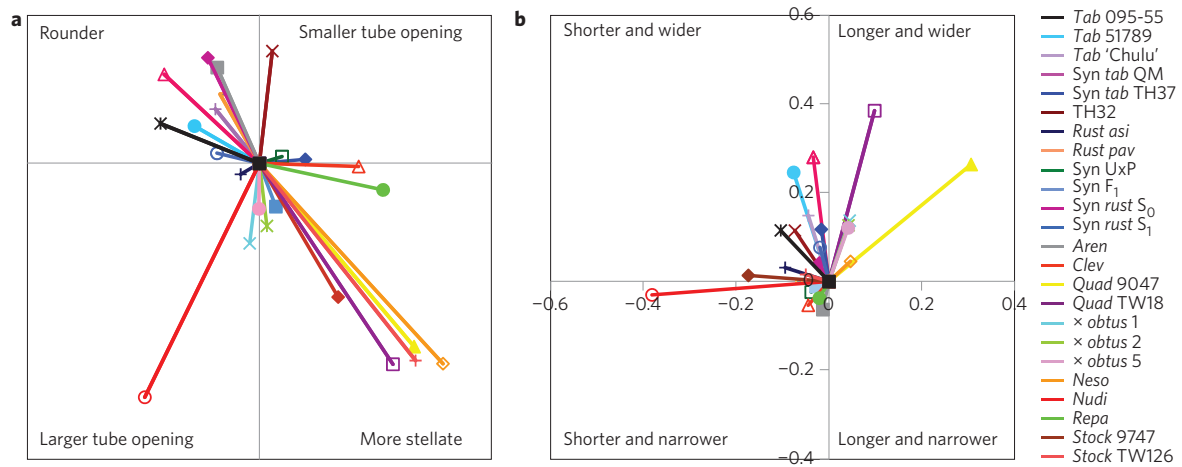


Figure 4 | Directional distance of polyploid means from the progenitor midpoint in floral limb shape and corolla tube length and width. These figures show trends in the floral evolution of polyploids by compiling the magnitude and direction of morphological change from the progenitor midpoint. The direction of morphological change is described by into which quadrant the polyploid falls. **a**, Smaller tube opening, rounder, larger tube opening or more stellate. **b**, Longer and wider, shorter and wider, shorter and narrower, or longer and narrower. Accession abbreviations are found in the Methods.

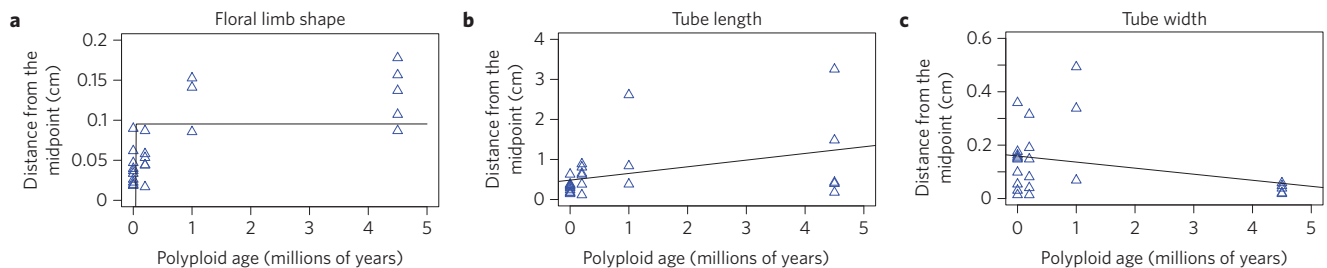


Figure 5 | Distance from the polyploid mean to the progenitor midpoint against estimated polyploid age for floral limb shape, corolla tube length and corolla tube width. **a**, Floral limb shape. An asymptotic curve was fitted to the data, which shows that distance from the progenitor midpoint in floral limb shape increases rapidly with polyploid age. **b**, Corolla tube length. Regression analysis shows that polyploid distance from the progenitor midpoint in tube length is positively associated with polyploid age. **c**, Corolla tube width. Regression analysis shows no significant relationship between polyploid distance from the progenitor midpoint in tube width and polyploid age.

1 of these species were not represented by a single extant taxon;
 2 instead, it seemed that they were formed before the diversification
 3 of some of the sections of *Nicotiana*. Thus, we compared
 4 homoploid hybrid morphology with the total range of floral
 5 morphology found in the multiple species of their progenitor
 6 sections (Supplementary Fig. 3). In floral limb shape, half of
 7 homoploid hybrids were overlapping with their progenitor range
 8 and half were transgressive (Table 2 and Supplementary Figs 2
 9 and 3). In tube length, 83% of homoploid hybrids were
 10 overlapping and 17% were distinct (Table 2 and Supplementary
 11 Figs 2 and 3). In tube width, all homoploid hybrids overlapped
 12 with their progenitor range (Table 2 and Supplementary Figs 2
 13 and 3). Most homoploid hybrids were transgressive or distinct in
 14 at least one trait, whereas only 17% overlapped with their
 15 progenitor range in all traits examined.

16 Discussion

17 Our results show that divergence in floral morphology increases
 18 with polyploid age (Fig. 5) but not with increased morphological
 19 divergence between progenitor diploids (data not shown). In
 20 addition, we show that most polyploids are distinct or transgressive
 21 in at least one floral trait examined (Table 2) and that polyploids
 22 tend to have shorter and wider corolla tubes than expected (Fig. 4b).

23 **Floral evolution following polyploidy.** Divergence in floral limb
 24 shape proceeds rapidly following allopolyploidization and tends to

increase over time (Fig. 5a). Divergence in tube length in 25
 polyploids also tends to increase over time (Fig. 5b), but without 26
 the initial rapid increase. Divergence in tube width, however, is 27
 not associated with polyploid age (Fig. 5c). Differences in the 28
 overall patterns of floral morphological divergence in polyploids 29
 among characters suggest differing constraints on morphological 30
 trait evolution. Floral limb shape seems to be the most 31
 evolutionarily labile character as only 50% of polyploids overlap 32
 with their progenitor midpoint range in floral limb shape 33
 compared with 77% and 68% in tube length and width, 34
 respectively (Table 2). Floral limb shape can also be highly 35
 variable within some species (for example, *N. glutinosa*)²⁴, 36
 providing further evidence that the evolution of floral limb shape 37
 is less constrained. Potentially the large variability in shape 38
 among and within species arises because the floral limb is the 39
 most prominent feature of the flower for attracting pollinators, 40
 and it may be beneficial to be less constrained to facilitate rapid 41
 response to changing selective pressures. 42

43 Tube length is an important factor in pollination in species with
 44 nectar rewards, and it has been proposed that a fit between corolla
 45 tube or nectar spur length and pollinator tongue length improves
 46 both pollen removal and deposition^{25,26}. Indeed, coevolution
 47 between floral tube/spur length and tongue length, as proposed by
 48 Darwin²⁷, has been reported in plant interactions for hawkmoths²⁵,
 49 long-tongued flies²⁸ and hummingbirds²⁹. Within species (rep-
 50 resented by a single population), floral traits that are involved in

1 the fit between flower and pollinator are reported to have the smal-
 2 lest amount of phenotypic variation³⁰. Pollinator-mediated selection
 3 on corolla tube length has been suggested from studies on six popu-
 4 lations of *Nicotiana glauca*, and the strongest directional selection
 5 was proposed to occur in populations where there was the greatest
 6 mismatch between flowers and pollinators³¹. Taken together, this
 7 evidence suggests that tube length can be constrained by pollina-
 8 tor-mediated selection and is most likely to be under tight develop-
 9 mental control. Studies using *N. alata*, *N. forgetiana* and their
 10 hybrids show that corolla tube length, stamen length and carpel
 11 length are correlated¹². In all natural polyploids examined here,
 12 stamens and carpels reach to the mouth of the corolla tube, even
 13 though some progenitors have exerted stamens. Synthetic *N.*
 14 *tabacum* QM is the exception (its paternal progenitor has exerted
 15 stamens), and the extent of stamen and carpel exertion varies
 16 among synthetic lines (E.W. McCarthy *et al.*, unpublished data).
 17 However, natural *N. tabacum* has inserted reproductive organs,
 18 perhaps suggesting that this trait is under selection in this species.

19 Tube width is also likely to be under pollinator-mediated selec-
 20 tion. Relative to hummingbirds, bumblebees prefer wider corolla
 21 tubes^{10,32}. Narrower corolla tubes in hummingbird-pollinated
 22 *Penstemon* species are suggested to be due to both 'pro-bird' and
 23 'anti-bee' adaptations¹⁰. In contrast, relative to hawkmoths, hum-
 24 mingbirds select for wider corolla tubes^{6,7}. However, studies on
 25 flowers visited by short-billed and long-billed hummingbirds
 26 suggest that tube width is under greater selection from pollinators
 27 with shorter tongues and that changes in tube width may be a
 28 response to increases in corolla tube length³³. Potentially, the evo-
 29 lution of a wider tube makes it possible for pollinators with
 30 shorter tongues to reach the nectar reward.

31 The divergence distance of the polyploids from the progenitor
 32 midpoint in tube width is not associated with polyploid age,
 33 largely because the oldest polyploids examined here have little diver-
 34 gence from expectation and the younger polyploids have a large
 35 range in divergence distances (Fig. 5c). The four allotetraploids of
 36 section *Repandae*, the oldest polyploids examined here, had a
 37 single origin^{16,19,20}. One of these, *N. nudicaulis*, has a transgressively
 38 short corolla tube length, suggesting that even short-tongued polli-
 39 nators can gain access to the nectar. The other three species,
 40 *N. repanda*, *N. nesophila* and *N. stocktonii*, are likely to be pollinated
 41 by nocturnal hawkmoths because they have long-tubed, white
 42 flowers that open at night. However, field pollination studies in
 43 *Nicotiana* are limited to a few diploid species, so the pollinators of
 44 these species have not been documented. *Nicotiana nesophila* and
 45 *N. stocktonii* are endemic to the isolated Revillagigedo Islands off
 46 the Pacific coast of Mexico²⁴. Hawkmoths are present on these
 47 islands^{34,35}, but hummingbirds have not been recorded in recent
 48 surveys³⁶. There was a single potential sighting of a hummingbird
 49 in a survey from 1897³⁷. The lack of hummingbirds may have
 50 resulted in reduced selective pressure for wider tubes^{6,7}.

51 Pollinators can also use shape cues as they forage. Bees can learn
 52 to distinguish between different convex shapes³⁸ and prefer
 53 symmetry to asymmetry³⁹. Hawkmoths prefer flowers with more
 54 dissected outlines (as opposed to convex)⁴⁰. Shape, however, does
 55 not seem to be the most important cue in determining pollinator
 56 preference. Colour cues are more important than shape for bees⁴¹,
 57 and diurnal hawkmoths respond to both size and colour before
 58 shape while foraging⁴². Shape also appears to be less important
 59 than size for nocturnal hawkmoths because a preference for more
 60 deeply dissected floral outlines in *Nicotiana* section *Alatae* is no
 61 longer observed when floral shapes are standardized for surface
 62 area⁴³. These observations that shape cues play a minor role in
 63 determining pollinator behaviour compared with other floral
 64 cues are consistent with our results that floral limb shape in poly-
 65 ploids is the most evolutionarily labile of the characters examined
 66 here (Table 2).

**Polyploids tend to evolve features associated with generalist
 pollination.** Most polyploids have shorter and wider corolla tubes
 than expected by their progenitor midpoints (Fig. 4b), indicating
 a trend towards the evolution of features associated with more
 generalist pollination (although the pattern of floral evolution in
 polyploids differs somewhat if ancestral reconstructions of the
 progenitor morphologies are considered, polyploids still diverge
 towards shorter and wider tubes more often than is observed in
 diploids; E. W. McCarthy *et al.*, unpublished data). It was
 previously found⁴⁴ that the number of species of floral visitors
 decreases as corolla tubes get longer but increases as tubes get
 wider, suggesting that having shorter and wider corolla tubes can
 in fact lead to more generalist pollination. Generalist pollination
 may be advantageous to polyploids, especially during
 establishment in a new or distinct niche from that of their
 progenitors. Generalism may increase the probability that a
 species will extend its range into new habitats and may decrease
 the likelihood of extinction^{45–47}. The adaptation of specialism to
 certain pollinator types can hinder subsequent shifts to other
 pollinators, suggesting that specialism can be an evolutionary
 dead-end^{48–50}. However, reversals in specialization do occur⁵¹, and
 in some plant systems there is no evidence to link pollinator
 specialization to decreased diversification⁵⁰. Nevertheless,
 polyploids tend to evolve shorter and wider tubes, suggesting that
 allopolyploidy may provide an escape from specialization in
 pollination (dead-end or otherwise), allowing lineages to diversify
 by means of hybridization and WGD.

Methods

Plant growth. We grew plants in a greenhouse with a 16:8 h light/dark cycle that was
 kept between 10 and 30 °C year round with heaters, midterms and fans. The
Nicotiana accessions examined here are found in Supplementary Table 1.

Flower photographs. We took photographs at anthesis with a Canon PowerShot
 A520. *Nicotiana otophora* is characterized by senescence of the floral limb before full
 anthesis; therefore, it was photographed when the flower opened, but before the
 stamens dehisced. We took front and dissected view photographs for each flower
 (five replicates for each type per flower, five flowers per plant and five plants per
 accession), unless five plants were unavailable, in which case we took five to ten
 photographs for as many plants as possible (Supplementary Table 1).

We took front photographs by inserting the corolla tube into a hole in a piece of
 cardboard so that the floral limb laid flat. In some species (*N. setchellii*,
N. langsdorffii and *N. glauca*), the floral limb forms a broad cup that cannot be
 flattened without disrupting the shape, so we placed flowers so the opening of
 the corolla tube lay against the cardboard and photographed directly above the
 corolla opening to minimize distortion of shape. For dissected photographs, we
 dissected flowers, removed the calyx and floral limb, and pinned the corolla
 tube open.

Geometric morphometric analysis. We used 15 landmarks to quantify floral limb
 shape (Fig. 2a). We created a TPS file from front photographs using TPSUtil version
 1.38 (ref. 52). We manually landmarked this series of photographs using TPSDig2
 version 2.10 (ref. 53). We input these landmarked photographs into TPSRelw
 version 1.45 (ref. 54), which calculated a consensus used as the reference specimen.
 TPSRelw aligned all photographs to the reference, calculated partial warp scores for
 each photograph and a principal components analysis was performed on partial
 warp scores to calculate the relative warps of the dataset, which can be used to
 visualize the shape variation present in the dataset.

We imported the principal component scores into Excel and calculated flower
 and accession averages. We used scatterplots of both the first two principal
 components and the third and fourth to examine the clustering of individual flowers
 in the morphospace. We drew convex polygons around flower averages for each
 accession in two-dimensional plots of the morphospace to delimit the portion of the
 morphospace occupied by that accession. We marked the mean position of each
 accession in the two-dimensional morphospace with a circle. We exported images
 showing the relative warps of the landmarks from the extremes of the principal
 components from TPSRelw to visualize the limits of the morphospace.

Metric measurements and analysis. We measured corolla tube length (from
 dissected photographs) and width (from landmarks 5 to 14) using ImageJ version
 1.42q (ref. 55). We calculated flower averages from five replicate photographs. We
 made scatterplots of tube width versus length using flower averages and used convex
 polygons and means to describe each accession.

- 1 **Calculation of progenitor midpoint values.** We used the average of the progenitor
2 means in the morphospace to calculate the progenitor midpoint. We calculated the
3 range expected around the progenitor midpoint based on the ranges of the
4 individual progenitors. We calculated the range of each progenitor for each trait and
5 chose the larger range in each trait for a pair of progenitors to estimate the range
6 around the progenitor midpoint. We delineated these chosen ranges in the
7 morphospace as an ellipse with the progenitor midpoint at its centroid. The chosen
8 range was divided in half and both added to and subtracted from the progenitor
9 midpoint to calculate the ellipse. This 'progenitor midpoint range' is a conservative
10 measurement because it uses the maximum phenotypic variance of the progenitors.
- 11 **Analyses of polyploid divergence.** To analyse the extent of polyploid divergence
12 from expected, we classified polyploids into three categories: overlapping (if a convex
13 polygon enveloping all individuals of the polyploid accession overlapped with the
14 progenitor midpoint range); distinct (if the polyploid polygon did not overlap with
15 the progenitor midpoint range, but was within the combined ranges of the
16 progenitors); or transgressive (if the polyploid polygon fell outside the combined
17 ranges of the progenitors). We analysed tube length and width characters
18 independently because the development of tube length is not genetically linked to
19 that of tube width^{12,22}. Grey boxes delimit the progenitor midpoint range in tube
20 width versus length figures. Owing to the nature of geometric morphometric
21 analyses, principal component (PC) 1 and PC2 do not correspond to individual
22 traits; therefore, we analysed floral limb shape using PC1 and PC2 together as
23 two-dimensional coordinates. We calculated the distance of the polyploid mean
24 from the progenitor midpoint for floral limb shape, tube length and tube width.
25 We plotted the distance between progenitor midpoint and polyploid mean in
26 each floral character against both estimated polyploid age and distance between
27 diploid progenitors in the same character. An asymptotic curve was fitted to the
28 polyploid distance in floral limb shape versus polyploid age plot in Mathematica 5.0
29 (Wolfram Research Inc.). We performed regressions on all other plots using Minitab
30 version 15.1 (Minitab, Inc.) and used a Mood's median test to determine significant
31 differences between medians of each polyploid age group.
- 32 To examine overall trends in the evolution of floral morphology following
33 polyploidy, we plotted the directional distance of the polyploid mean from the
34 midpoint as a vector initiating at the origin, which represents the progenitor
35 midpoint. The figure shows only the direction and magnitude of the change in
36 polyploid morphology because progenitor midpoints from different pairs of
37 progenitors, which are distinct in morphology, were translated to the origin. Because
38 the range in tube length across the dataset is much larger than that of tube width, we
39 standardized the divergence measurements for tube length and width to
40 proportional values (calculated by dividing each species mean by the largest species
41 mean in each character). We used a goodness-of-fit chi-squared test to determine
42 whether the distribution of polyploids into the four quadrants of the graph was
43 significantly different from equal numbers in each.
- 44 **Accession abbreviations.** Accession abbreviations throughout the figures are as
45 follows: *acum*, *N. acuminata*; *aren*, *N. arentsii*; *atten*, *N. attenuata*; *benavid*,
46 *N. benavidesii*; *clev*, *N. clevelandii*; *glau25*, *N. glauca* 51725; *glau51*, *N. glauca* 51751;
47 *glut*, *N. glutinosa*; *knight*, *N. knightiana*; *langs CAM*, *N. langsdorffii* CAM; *langs*
48 8047, *N. langsdorffii* 804750066; *lin TW77*, *N. linearis* TW77; *mier*, *N. miersii*;
49 *mutab*, *N. mutabilis*; *neso*, *N. nesophila*; *noct*, *N. noctiflora*; *nudi*, *N. nudicaulis*; *obtus*
50 *v. obtus*, *N. obtusifolia* var. *obtusifolia* TW143; *obtus v. palm*, *N. obtusifolia* var.
51 *palmeri*; *obtus1*, *N. × obtusata* line 1; *obtus2*, *N. × obtusata* line 2; *obtus5*, *N. ×*
52 *obtusata* line 5; *otoph*, *N. otophora*; *pani*, *N. paniculata*; *pauc*, *N. pauciflora*; *petun*,
53 *N. petunioides*; *plumba*, *N. plumbaginifolia*; *quad 9047*, *N. quadrivalvis* 904750042;
54 *quad TW18*, *N. quadrivalvis* TW18; *raimon*, *N. raimondii*; *repa*, *N. repanda*; *rust asi*,
55 *N. rustica* var. *asiatica*; *rust pav*, *N. rustica* var. *pavonii*; *syn (U×P)*, synthetic U×P;
56 *syn F₁*, synthetic PUE1 F₁; *syn rust S₀*, synthetic *N. rustica* PUE1-R10 S₀; *syn rust S₁*,
57 synthetic *N. rustica* PUE1-R1 S₁; *setch*, *N. setchellii*; *stock 9747*, *N. stocktonii*
58 974750101; *stock TW126*, *N. stocktonii* TW126; *suav*, *N. suaveolens*; *sylv A047*,
59 *N. sylvestris* A04750326; *sylv 6898*, *N. sylvestris* 6898; *tab 095-55*, *N. tabacum* 095-55;
60 *tab 51789*, *N. tabacum* 51789; *tab 'Chulu'*, *N. tabacum* 'Chulumani'; *syn tab QM*,
61 synthetic *N. tabacum* QM; *syn tab TH37*, synthetic *N. tabacum* TH37; *tomtform*,
62 *N. tomentosiformis*; *undu*, *N. undulata*; *wigan*, *N. wigandoides*; TH32, TH32,
63 synthetic *N. sylvestris* × *N. otophora* polyploid.
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 E.M., M.C., S.K., A.R.L. and S.L. designed the research. E.M. collected the data and
 performed most of the analyses. S.L. performed analyses in Mathematica. E.M., A.R.L. and
 S.L. wrote the manuscript with help from M.C., S.K. and A.L.
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