

Biological Sciences, Evolution

Pollinator-mediated floral evolution and the potential for speciation in *Nicotiana*.

Anthony Ippolito, Christina Merrels, G. Wilson Fernandes† and Timothy P. Holtsford

Division of Biological Sciences, University of Missouri, Columbia, MO 65211-7400, USA., † Departamento de Biología Geral, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, CP 486, Belo Horizonte, Minas Gerais 30161-970, Brasil.

Corresponding author:

Tim Holtsford

E-mail: HoltsfordT@missouri.edu

105 Tucker Hall
University of Missouri
Columbia, MO 65211-7400
573-882-2988
573-882-0123, fax

. The role of pollinators in plant speciation is a topic of much debate, especially when divergence is proposed to have occurred sympatrically (1,2). *Nicotiana alata* and *N. forgetiana* are interfertile sister species pollinated by hawkmoths and hummingbirds, respectively. We studied five experimental F_2 populations where the species' ranges overlap in southern Brazil (3,4) to measure the effect of pollinator-mediated mating structure on floral evolution. The natural setting allowed the operation of natural ecological processes, including potentially conflicting selection by other potential pollinators and selection on characters genetically correlated with floral traits. Pollinators showed divergent preferences for larger whiter versus smaller redder flowers. The evolutionary consequences of pollinator mediated mating were estimated by comparing twelve F_3 populations descended from the F_2 by natural pollination (F_3 nat) to control F_3 s produced by random mating among the same F_2 individuals (F_3 ran). Genetic differences in corolla tube length were consistent with pollinator preferences. Pollinators also increased correlations among floral traits, tending to re-associate trait values of the parental species. Further floral divergence and ultimately speciation could arise due to the positive feedback loop of pollinators favoring extreme phenotypes that would increase reproductive isolation each generation.

Despite the longstanding view that plant-pollinator interactions have contributed to the diversity of species and floral morphologies of flowering plants, evidence that pollinators' floral preferences can drive plant speciation is dubious (1,2). Floral divergence and speciation are often thought to occur in allopatry, especially in geographically isolated populations (4,5,6,7,8). Sympatric speciation is theoretically possible (9,10) but empirical evidence is correlative; the evolution of traits contributing to reproductive isolation is not actually observed. The strongest cases for sympatric speciation are made when historical information allows important ecological events to be inferred, e.g., the date of introduction of alternative host species (11) or the ages and water levels of lakes (12,13). Sympatric divergence has also been documented, though rarely, in laboratory experiments (14,15) but the ecological context of these experiments is much simplified and determined by the investigators.

Sympatric divergence may occur if selection for divergence is strong and mate choice is genetically correlated with the factor that is promoting divergence (5, 6). Because pollinators serve a dual role as agents of selection on floral traits and vectors of gene flow (16), speciation may follow a single variation model (14); i.e. pollinators affect the frequencies of genes that cause both phenotypic divergence and reproductive isolation. If a few genes with large effects control floral traits that affect plant-pollinator interactions then floral evolution and speciation may proceed rapidly (17). However, a simple genetic architecture does not guarantee rapid evolution. If most pollinators are generalists then assortative pollination is improbable because different preferences by different pollinators would produce diffuse or conflicting selection (2,18). Even if pollinators' preferences are strong and heritability of floral traits is high (19,20), floral evolution may

be opposed by conflicting selective pressures (e.g., florivory, water relations) or limited by developmental or epistatic constraints (21,22).

Discriminating pollinators contribute to reproductive isolation of the sister species *Nicotiana alata* and *N. forgetiana* (4). *Nicotiana alata* flowers are white, have a strong odor, and a long narrow corolla tube. *Nicotiana forgetiana* has red flowers that rarely have a detectable odor and have shorter broader floral tubes. The species have the same chromosome number and hybrids are vigorous and fertile through at least four generations. *Nicotiana alata* is pollinated by large hawkmoths, primarily *Agrius cingulata* and *Eumorpha labruscae* (Sphingidae). *Nicotiana forgetiana* is pollinated by various hummingbird species, most commonly, *Chlorostibulon aureoventris* but is occasionally visited by Halictid bees and small hawkmoths (4).

We estimated the net effect of pollinator-mediated mating structure on floral evolution in five F₂ populations in which we measured six floral traits and observed pollinator behavior. If the floral morphologies of the parent species are on or near their adaptive peaks, then our experimental populations' phenotypes were centered in the valley between the species' peaks (23). If pollinators were responsible for the location of the adaptive peaks on the phenotypic landscape, then hawkmoths should prefer to visit, and thereby increase the fitness of, plants similar to *N. alata* while hummingbirds should benefit plants with flowers similar to *N. forgetiana*.

Materials and Methods

Plant material and fieldwork. *Nicotiana alata* and *N. forgetiana* are members of *Nicotiana* Section *Alatae* based on nuclear ribosomal internal transcribed spacer (*nrITS*) sequence data (4), morphology and cytology (3). The *nrITS* data do not resolve most

relationships within Section *Alatae*, but crossing data strongly suggest that *N. alata* and *N. forgetiana* are sister species. The only other candidate for a sister species of either of focal species is *N. bonariensis* (3,4). Crosses between *N. alata* and *N. forgetiana* produced 42% of the number of seeds of conspecific crosses (average of 179 crosses in both directions). *Nicotiana bonariensis* crossed with *N. alata* or *N. forgetiana* produced only 11%, and 24% as many seeds as conspecific crosses, respectively (N= 206 and 175, Wunsch, Ippolito & Holtsford, unpubl.).

Field experiments were done at a field station operated by the Conselho de Desenvolvimento Comunitario de Campestre (CDCC), at 51° 06'47.3"W; 28° 05'13.8"S. CDCC is 30 km south of the *N. alata* source population and 70 km north of the *N. forgetiana* population. Both species are found scattered all along the intervening highway (BR 116) and we have seen *N. forgetiana* and *N. alata* individuals within 300m of each other. Sympatric populations or putative hybrids have not been found. Two F₂ populations, evaluated in 1997-8, descended from crosses among 90 F₁ individuals that were descended from random crosses among 15 plants of each species used as a male and female parent 3 times each. Three F₂ populations, grown in 1998-9 descended from 30 plants of each species crossed to five plants of the other species. One F₁ plant from each of the resulting 300 families was crossed once each as a male and female parent with one other randomly chosen F₁ plant. F₂ seeds were bulked and chosen at random. We propagated plants in plastic greenhouses and transplanted seedlings into the ground when rosettes were c. 6 cm diameter. Populations were sown in weedy old-field habitat that was indistinguishable in vegetation from natural *Nicotiana* populations found along

roadsides and in pastures. After seedling and transplant mortality, the five F_2 plots had 177, 149, 137, 106 and 103 flowering plants.

We observed F_2 plots for at least four days each from dawn until midmorning when the flowers closed, and again from floral opening in late afternoon until hawkmoth activity had declined to less than one visit per plot per hour. Experimental populations in 1997-1998 flowered when most other hummingbird-visited plant species had ceased flowering and hummingbird sightings were rare. In 1998-99, flowering was nearly synchronous with nearby natural populations but hummingbirds were still rare.

Floral traits. In the 1997-1998 season F_2 flowers were measured with digital calipers. Thereafter, digital pictures were taken and measured using Canvas (v. 6.0, Deneba Systems Inc.). We scored six traits: floral tube length, front-view (limb) width, diameter of the floral tube opening, anther-stigma separation, stigma insertion within floral tube and, in the three populations from 1998-1999, corolla color. Color was scored on a scale from 0 = white to 7 = red by comparing corolla limb color with Pantone color standards. Averages of three flowers per plant were used for analysis.

Analysis.

Pollinator preferences and their correlation with floral traits in the F_2 .

Pairwise correlation analyses were performed among the six floral traits and four measures of pollinator visitation frequency (Table 1). For each F_2 plant, visitation frequencies were estimated in two ways: 1) the observed frequency of visits to plant_{*i*} / the total number of observed per-plant visits, and 2) the observed number of visits to flowers on plant_{*i*} / total number of observed visits to all flowers on all plants. The second measure is a per-plant visitation frequency weighted by the number of flowers visited per plant.

Estimating a true per-flower correlation between visitation and phenotype was not possible because not all individual flowers that were visited could be measured. We used a three-flower average for the phenotypes of all plants. Therefore the sample sizes in Table 1 are the number of plants in the F_2 , not the number of visits observed. For hummingbird visitation only F_2 -3 is presented because that is the only population where hummingbirds were observed in numbers marginally sufficient for analysis ($N=74$ observed per-plant visits). If we had analyzed hummingbird visitation data over all five F_2 plots then of the 672 plants for which we have tube length data, almost 600 plants would have had zero hummingbird visits recorded. Color was not scored in two F_2 populations so the sample sizes for color correlations are smaller than for the other traits. Because 45 pairwise comparisons were made, α was reduced to 0.01.

The distributions of floral phenotypes and pollinator visitations were divided into ten bins each containing 10% of the range of F_2 phenotypes. The distributions were analyzed in 10×2 contingency tables using the F_2 population phenotypes as the external hypothesis for the expected visitation frequency to plants in each bin, where the expected number $[N]$ of visits to plants in bin_{*i*} =

$$N[\text{visits expected}_{\text{bin}=i}] = (N[F_2 \text{ phenotypes}_i] / N[F_2 \text{ phenotypes}_{\text{total}}]) \times N[\text{visits observed}_{\text{total}}].$$

Contributions from each *i*th bin ($([\text{obs}_i - \text{exp}_i]^2 / \text{exp}_i)$) to the overall χ^2 were evaluated by determining whether that value alone would have been significant with $df=1$. Because ten comparisons were made for each pair of distributions, individual bins' χ^2 were considered significant if $p < 0.01$. Finally, the observed/expected visitation rate was regressed on tube length to test the null hypothesis that this slope=0, the no-preference expectation (Fig. 1c, f).

Evolutionary response in the F₃.

To estimate the effects of pollinator-mediated mating on floral evolution we measured the genetic response in the F₃ generation. We collected all seeds produced from naturally pollinated flowers in the F₂ plots (F₃nat) and control seeds from the same F₂ plants but from flowers that were hand-pollinated to randomly chosen mates (F₃ran). The net effects of any (unobserved) generalist pollinators or other selective agents, observed or not, are included in the F₃nat. F₃ran populations controlled for all unmeasured evolutionary forces and constraints, and for genetic segregation that would confound F₂-F₃ comparisons. Environmental effects were randomized by the planting designs of the F₃ populations. Therefore, differences in floral trait distributions between F₃nat and F₃ran are genetically based and can only be due to the different mating structures applied to the F₂ (natural vs. random) since the genetic and ecological histories of the F₃ populations are otherwise identical. This approach is not designed to estimate selection *per se* but rather net evolutionary change.

The magnitude of pollinator-driven floral evolution was estimated for the trait most highly correlated with pollinator visitation of those we measured, corolla tube length. Pollinators' influence on correlation structure among trait is analyzed below but we did not combine the floral variables into composite variables, as in a principle components analysis, nor did we analyze evolution of a multi-dimensional phenotype. Studying evolution of only one trait is a conservative approach that avoids potentially inflating pollinators' inferred roles in causing floral evolution of a suite of traits in a hypothetical ancestral species. Gametic-phase disequilibrium (D) among floral trait genes would certainly be greater in our F₂ populations than in the ancestral species since there has only

been one generation of random mating (F_1 to F_2) to disrupt statistical associations between floral trait alleles. Strong D in the F_2 might lead to a larger multivariate evolutionary response than would be possible if the floral trait alleles were in gametic phase equilibrium, as would be expected in a highly outcrossing ancestral species (both extant species are self-incompatible).

The F_3 trait distributions were analyzed similarly to the F_2 traits/pollinator visitation data, with the F_3 ran serving as the null hypothesis for the distribution of the F_3 nat. Twelve replicate F_3 populations were analyzed together by removing the among-replicate variance with ANOVA and using the residuals. Six pairs (F_3 ran and F_3 nat) of F_3 populations were evaluated in Missouri in 1998 (three blocks each descended from F_2 -1 and F_2 -2) and three F_3 pairs were evaluated in Missouri in 1999 (one pair from F_2 -3, F_2 -4 and F_2 -5). Three F_3 pairs were evaluated in Brasil in 1999-2000 (one pair from F_2 -3, F_2 -4 and F_2 -5). There was significant variation in corolla tube length among the replicates ($F_{11,1331} = 7.13$, $p < 0.0001$), and pollination treatments (natural vs. random, $F_{1,1331} = 4.94$, $p = 0.03$). The replicate by treatment interaction was not significant ($F_{11,1331} = 1.48$, $p = 0.13$). Therefore, treatment by environment interactions did not compromise the genetic response evaluations in Missouri and replicates could be fairly combined after accounting for the among-replicate variation. Standardized F_3 trait values (Fig. 1g-i) are the residuals from a one-way ANOVA, using replicate as the only independent variable, plus the grand trait mean across all plots. F_2 trait values were not standardized because 1) they were all grown *in situ* and 2) the floral phenotypes, including any environmentally based variation, were what the pollinators acted on.

Phenotypic correlations among floral traits in $F_{3\text{ran}}$ and $F_{3\text{nat}}$ were compared to the correlations in the F_2 using a t-statistic on z-transformed correlation coefficients²⁴. Correlations among floral traits are expected to decay due to genetic segregation but might increase due to pollinator-driven assortative mating. So the *a priori* expectations are 1) floral trait correlations will generally be lower in $F_{3\text{ran}}$ than in F_2 due to segregation, but 2) some correlations may persist due to physical linkage or pleiotropy (e.g., general size loci). Third, floral trait correlations in $F_{3\text{ran}}$ and $F_{3\text{nat}}$ will be similar in magnitude for traits that have no association with pollinator visitation. Fourth, correlations between traits that are correlated with pollinator visitation could be higher in $F_{3\text{nat}}$ than in F_2 or $F_{3\text{ran}}$ due to pollinator-driven assortative mating among plants with preferred phenotypes. Identity disequilibria would arise from pollinator preferences, e.g., alleles for long corolla tubes may be more often found with in gametes with alleles for white corollas (as in *N. alata*) rather than with red alleles. Pollinators may also decrease correlations between two traits if one is preferred and one not, provided physical linkage or pleiotropy between the two traits is not strong.

Results and Discussion

Correlations among floral traits and pollinator visitation. The two measures of pollinator visitation (per plant & per plant weighted by flowers visited/plant) were highly correlated within the two the pollinator groups (Table 1). The hummingbird and hawkmoth relative visitation frequencies were not correlated, suggesting they have different preferences.

Seven visitation/floral trait correlations were significant, including three different floral traits. Hawkmoths preferred broader flowers with longer corolla tubes while hummingbird visitations were positively associated with shorter tube lengths and for one visitation estimate, more pigmented corollas. Color is tantalizingly close to being significantly associated with the other three measures of pollinator visitation, with hawkmoths tending to prefer lower scoring (whiter) flowers.

We chose tube length for the evolutionary response analysis because of its functional relationship with nectar availability and because only tube length was correlated with both visitation measures for both pollinator types. Hawkmoths also preferred broader flowers (larger corolla limbs) and the correlation coefficients of length vs. width with visitation are indistinguishable statistically. The phenotypic correlation between length and width is 0.49, the highest r_p of any pair of traits – which is not surprising since both traits are size-related. Larger corolla limbs may enhance visibility to both types of pollinators. Nevertheless, beyond any overall size preference that may exist, corolla tube length would probably be more important to a hummingbird than limb breadth since tube length influences access to the nectar. Hawkmoths may prefer longer tubes because if hummingbirds are excluded then the standing crop of nectar may be larger. While hawkmoths can apparently obtain nectar from *N. forgetiana*, they had a

significant preference for *N. alata* in four experimental sympatry experiments over two years *in situ* (4). Hawkmoths sometimes place their front legs on the limbs of long-tubed flowers — which seems to stabilize their hovering — but this is not possible when they are foraging on shorter flowers.

Three other analyses suggest hummingbirds and hawkmoths have divergent preferences for corolla tube length. First, the average corolla tube length of hummingbird-visited flowers was smaller than the F_2 population average (Student's $t_{df=213} = 3.12$, $p=0.002$, Fig. 1a) while hawkmoth-visited flowers were longer (Student's $t_{df=3101} = 5.76$, $p < 0.001$, Fig. 1d). Second, the distribution of hummingbird visits to plants in the 10 size bins differed significantly from the distribution of all F_2 plants' phenotypes ($\chi^2_{df=9} = 17.4$, $p=0.015$, Fig. 1b). This result is due primarily to a significant overrepresentation of hummingbird visits to plants with flowers in bin 3 (20th – 30th %-iles). Smaller size classes were generally preferred and larger ones avoided but not by a significant margin. Hawkmoths dominated the observed pollinator pool, accounting for 96.2% of the 2431 visits observed. Hawkmoths under-visited smaller flowers while larger flowers were over-visited ($\chi^2_{df=9} = 231.1$, $p<0.001$, Fig. 1d,e). Plants with flowers in the three smallest bins were under-visited, the fourth and fifth bins were visited in approximate proportion to their frequency, and three of the four largest bins were over-visited. Finally, the ratio of observed/expected hummingbird visits to plants in each bin was significantly negatively correlated with corolla length (Fig. 1c), while net pollinator preference ratio (Fig. 1f) and hawkmoth preference ratio (not shown) were positively correlated with tube length.

Evolutionary response

The correlation structure of floral traits will affect the progress of divergence and speciation. Evolution may proceed more quickly when a trait is controlled by fewer effective segregating factors (i.e., higher genetic correlations among floral traits). Conversely, the potential range for evolutionary response will be higher if the genes controlling the various aspects of floral form are many and non-interacting (25). While the intrinsic correlations due to pleiotropy and physical linkage influence evolutionary potential, the correlation structure can also evolve due the mechanics of the mating process.

Pollinator-mediated mating structure influenced the phenotypic correlation structure among floral traits with the net effect of preserving or strengthening the correlations in the F_2 (Table 2). The absolute magnitude of the phenotypic correlations was greater in the F_3 ran than in the F_2 (paired $t_{df=14} = 3.622$, $p < 0.0028$ [26]) where the correlations were on average larger than the F_3 ran ($t_{df=14} = 3.401$, $p < 0.0043$). Eight of fifteen pairs of floral traits were significantly correlated in the F_2 . Segregation broke down 5 of these associations; only three pairs of traits were significantly correlated in F_3 ran. Pollinator-mediated mating preserved six of eight correlations and created one new correlation. Further, the correlation coefficients between all three traits that were correlated with pollinator visitation were larger in F_3 nat than in F_3 ran (tube length/limb width: $r_p=0.51$ vs. 0.43 , tube length/color: $r_p=-0.35$ vs. -0.24 , limb width/color: $r_p=-0.17$ vs. -0.09 , $p<0.01$). Therefore, pollinators had a significant diversifying effect on the correlation structure of floral traits, tending to promote the re-association of parental trait values.

Floral tube length evolution — The F_2 phenotypic landscape was centered between the parental phenotypes for all traits that were correlated with pollinator visitation. Further, the F_2 bridged the valley between parental values of tube length and limb width. Mean tube length in the F_2 is intermediate between the parent species (Fig. 1d) but 6.8% of F_2 plants had floral tube lengths within the range of *N. forgetiana* while 2.8% were within the range of *N. alata*.

F_3 nat plants had significantly longer average standardized floral tubes than the F_3 ran controls (Student's $t_{df=1353} = 4.17$, $p = 3.2 \times 10^{-5}$, Fig. 1g). More importantly, the differences between F_3 nat and F_3 ran distributions correspond well with pollinators' preferences, especially those of the more common hawkmoths (Fig 1d-f). Significant contributions to the differences between the observed F_3 nat distribution and the expectation generated by F_3 ran were found in bin 4 (deficit of plants in the 30-40th%-iles) and bins 8 & 9 (abundance of plants in the 70-90th %-iles, overall $\chi^2_{df=9} = 132.8$, $p < 0.001$, Fig. 1h). The largest and smallest flowers were also over-represented, although small expectations for counts in these bins render these conclusions tentative. Intermediate tube lengths decreased in frequency (bin 4, Fig. 1h) and in relative proportion (Fig. 1i), presumably because neither pollinator type favored these phenotypes. The smaller flowers preferred by hummingbirds (Fig. 1a-c) did not decrease in frequency (Fig. 1h) and increased in relative proportion (Fig. 1i), despite being under-visited by hawkmoths relative to their abundance in the F_2 (Fig. 1d-f), suggesting that our observations may have underestimated the true frequency of hummingbird visits.

The genetic response is not expected to match the pollinators' preferences exactly because the traits are unlikely to have a heritability of 1.0 and because other selective

forces may be operating. Further, visitation frequency may not be highly correlated with the strength of selection. The influences of pollinator-mediated mating structure may include not only visitation frequency but the degree of assortative mating, distance flown between plants (which may be correlated with genetic distance between plants), and variation in the size and composition of pollen loads due to the mechanics of pollen transfer.

The evolution we observed in tube length and the correlation structure among floral traits can only be due to pollinator-mediated mating structure. Genetic drift is unlikely since the aggregate population sizes are moderately large ($N > 650$). We do not assert that these species arose via pollinator-mediated speciation, but rather that this scenario is plausible. The genetic architecture of our experimental populations almost certainly differed in the amounts or kinds of genetic variation present in the true ancestor of these taxa. Further, the ecological circumstances during speciation are unknowable. Seasonal and regional variation in pollinator-driven evolution is likely to be high. Our experiments were done in only one environment (albeit an appropriate one) in two years. Given hummingbirds' preferences (Table 1, Fig. 1a-c) we expect that in seasons or regions where hummingbirds are more common we would detect a larger hummingbird effect. Campbell et al. (27) also reports divergent selection on *Ipomopsis* flowers by hawkmoths and hummingbirds, but the hawkmoths' seasonal rarity meant that hummingbirds dominated the pollinator-driven selection.

Corolla tubes are functionally similar to nectar spurs in that both structures influence which pollinators can profitably visit flowers and where pollen is deposited on the pollinators. Therefore, differences in tube or spur morphologies may reduce gene

flow between plants with flowers of different sizes and eventually lead to speciation. The correlation between polymorphism for nectar spurs and increased species diversity in many plant groups suggests that spurs are a key innovation that fosters speciation (28). Our data provide experimental evidence that polymorphic corolla tubes may also contribute to angiosperm species diversity.

Because pollinator-mediated mating tended to preserve or increase correlations between floral trait associations similar to those of the parental species, the potential for subsequent divergence of floral traits is even more likely. Evolutionary response of a suite of traits will be accelerated when the traits are correlated (14). Because the traits whose phenotypic values and correlation structure evolved are the same traits which pollinators preferred in the F_2 , the stage is set for a positive feedback loop between floral evolution and reproductive isolation, mediated by pollinator preferences.

Acknowledgements

Financial support was provided by the UM Research Board and NSF (DEB-9727037). Daniel Tiscornia, Dimas Eleizer, the community of Campestre da Serra, Ivan Romano, Vitorli & Vera Machado, Roberto & Rose Paim and João Renato Stehmann, kindly provided assistance with field logistics. For field help and digital flower measurements we thank Bruce Moreira, Michael Wunsch, Jana U'Ren, Jessica Cuba, Amy Gooch, Carrie Turner, Allena Volskay, Liz Staley, Esther Stroh, Madalyn Painter, Sonia Vasquez and Travis Plume. Candi Galen, Robin Kennedy, Ray Semlitsch, Bruce McClure, and Sarah Mathews commented helpfully on the manuscript.

1. Grant, V. (1994) *Proc. Natl. Acad. Sci.* **91**, 3-10.
2. Waser, N.M. (1998) *Oikos* **81**, 198-201.
3. Goodspeed, T. H. (1954) *The Genus Nicotiana*, (Chronica Botanica, Waltham, MA, USA).
4. Ippolito, A. (2000) *Systematics, floral evolution and speciation in Nicotiana*. Ph.D. thesis, (University of Missouri, Columbia, MO USA).
- 5 Grant, V. (1993) *Proc. Natl. Acad. Sci. USA* **93**, 7729-7733.
- 6 Qian, H. & Ricklefs, R.E. (2000) *Nature* **407**, 180-182.
- 7 Mayr, E. (1942) *Systematics and the Origin of Species*, (Columbia , New York).
- 8 Reiseberg, L. H. & Brouillet, L. (1994) *Taxon* **43**, 21-32.
9. Kondarashov, A. S. & Kondrashov, F. A. (1999) *Nature* **400**, 351-354.
10. Dieckmann, U. & Doebeli, M. (1999) *Nature* **400**, 354-357.
11. Bush, G. L. & Smith, J. J. (1998) *Researches on Population Ecology* (Kyoto). **40**, 175-187.
12. Johnson, T. C., Scholz, C. A., Talbot, M. R., Kelts, K., Ricketts, R. D., Ngobi, G., Beuning, K. (1996) *Science*, **273**, 1091-1093.
13. Sturmbauer, C. & Meyer, A. (1992) *Nature* **358**, 578-581.
14. Rice, W. R. & Hostert, E. E. (1993) *Evolution* **47**, 1637-1653.
15. Higgin, M. Chenoweth, S. & Blows, M. W. (2000) *Science* **290**, 519-521.
16. Waser, N. M. (2001) in *Cognitive Ecology of Pollination*, eds. Chittka, L. & Thomson, J. D. (Cambridge University Press, Cambridge UK), pp. 318-335.

17. Bradshaw, H. D. Jr., Wilbert, S. M., Otto, K. G. & Schemske, D. W. (1995) *Nature* **376**, 762-765.
18. Ollerton, J. (1996) *J. Ecology* **84**, 767-769.
19. Schemske, D.W. & Bradshaw H.D. Jr., (1999) *Proc. Natl. Acad. Sci. USA* **96**, 11910-11915.
20. Galen, C. (1996) *Evolution* **50**, 120-125.
21. Galen, C. (1999) *Oikos*, **85**, 426-434.
22. Galen, C. (2000) *Amer. Natur.* **156**, 72-83.
23. Armbruster, W. S. (1990) *Amer. Nat.* **135**, 14-33.
24. Sokal, R.R., Rohlf, F.J. (1981) *Biometry*, 2nd ed., (Freeman, San Francisco).
25. Falconer, D.S. , Mackay, T.F.C. (1996) *Introduction to Quantitative Genetics*, 4th ed., (Longman, Essex).
26. SPSS (1999) *SPSS Base 10.0 Applications Guide*, (SPSS Inc., Chicago).
27. Campbell, D. R., Waser, N. M., Meléndez-Ackerman, E. J. (1997) *Amer. Natur.* **149**, 295-315.
28. Hodges, S. A. (1997) *Intl. J. Plant Sci.* **158**, S81-S88.

Table 1. Correlations among pollinator visitation estimates and floral traits. Visitation estimates are abbreviated as: Hawkmoth (HM) and hummingbird (HB) percent visits per plant (% vis/pl), and percent visits per plant weighted by the number of flowers visited per plant (% vis/pl-flr). Stig ins = stigma insertion; anth-stig = anther-stigma separation; mouth diam = diameter of corolla tube opening. Cells contain, from top to bottom, Pearson's correlation coefficient (r), the probability $r=0$, and the number of plants in the sample. Bold type indicates that a correlation coefficient is significant at $p < 0.01$.

Table 2. Effect of pollinator-mediated mating on the phenotypic correlation structure among floral traits in three populations (F_{3nat} , F_2 , F_{3ran}). Pearson's correlation coefficient (r), its probability of being zero $p[r=0]$, and the sample size, N = the number of plants in the sample for each of five floral traits in three experimental populations. or

indicates a difference in the significance or sign of in r_p between the F_2 and F_{3nat} or F_{3ran} (24). *: $p < 0.01$, **: $p < 0.005$, ***: $p < 0.001$

Figure 1. Pollinator visitation and genetic response to pollinator mediated mating. The bins on the y-axes contain 10% of the range of F_2 phenotypes. **a**, Hummingbird preferences *in situ* in F_2 -3, the only population where hummingbirds were regularly observed. Black outline: relative frequencies of corolla lengths of all plants (N=137). Grey bars: hummingbird-visited plants (N=78 observed visits). **b**, Hummingbird preference presented as the difference in the absolute frequencies of corolla lengths of hummingbird-visited plants minus corolla lengths of all F_2 plants. **c**, Hummingbird preference presented as the ratio of the relative frequencies of corolla lengths of visited plants vs. corolla lengths of all plants. **d**, The relative frequencies of corolla lengths of all plants in 5 F_2 populations and all pollinator-visited plants (N=672 plants [black outline], N=2431 visits [grey bars], 96.2% of which were by hawkmoths). Triangles indicate the phenotypic means of *N. forgetiana*, F_1 hybrids and *N. alata*, from left to right. **e**, **f** are plotted as in **b**, **c** but for all pollinator visits. **g**, Corolla lengths of F_3 plants descended from naturally pollinated F_2 plants (F_3 nat, N=681, grey bars) and a control population of F_3 plants descended from hand pollination among randomly-chosen F_2 plants (F_3 ran, N=673, black outline). **h**, The genetic response expressed as the difference of the standardized relative frequencies (F_3 nat - F_3 ran). **i**, The genetic response expressed as the ratio of the standardized relative frequencies (F_3 nat / F_3 ran). The dashed lines represent null hypotheses that the two distributions in the top row of panels are identical (grey = observed; black outline = expectation). Asterisks in indicate that a bin contributed

significantly to the overall χ^2 (** = $p < 0.01$, *** = $p < 0.001$). Parentheses indicate that expected counts in that bin were below five.

	HM %vis/pl	HB % vis/pl	HB %vis/pl-flr	stigma ins	anth-stig	mouth diam	limb width	tube length	color
(1) correlations among visitation variables			(2) visitation & floral trait correlations						
Hawkmoth	0.8874	-0.0315	-0.0448	-0.0426	-0.0264	0.0194	0.2261	0.2579	-0.1014
%vis/plant	0.0001	0.6939	0.5761	0.2761	0.5003	0.6182	0.0001	0.0001	0.0619
	775	158	158	655	653	662	652	672	340
Hawkmoth		-0.0485	-0.0506	-0.0643	-0.0043	0.0065	0.2317	0.2588	-0.1049
% vis/plant		0.5455	0.5275	0.1002	0.9127	0.8681	0.0001	0.0001	0.0533
wtd by flrs		158	158	655	653	662	652	672	340
Hummingbird			0.90789	0.17248	-0.12768	0.14165	-0.11175	-0.22971	0.3071
% vis/plant			0.0001	0.0471	0.143	0.1039	0.2038	0.0069	0.0003
			158	133	133	133	131	137	135
Hummingbird				0.13502	-0.06258	0.04167	-0.17268	-0.26789	0.186
% vis/plant				0.1213	0.4742	0.6339	0.0486	0.0016	0.0308
wtd by flrs				133	133	133	131	137	135

	anther-stigma distance			mouth diameter			limb width			corolla tube length			Color, 1-7, red to white				
	Pop	r	p(r=0)	N	r	p(r=0)	N	r	p(r=0)	N	r	p(r=0)	N	r	p(r=0)	N	Pop
	F₃nat	0.2145	0.0001	667	0.0426	0.2910	617	-0.1145	0.0047	607	-0.3312	0.0001	668	0.1455	0.0014	477	F₃nat
stigma		↑ ***			↓ ***			↓ **			ns			ns			
insertion	F₂	-0.0254	0.5151	652	-0.2236	0.0001	642	-0.2890	0.0001	635	-0.3186	0.0001	655	0.1475	0.0075	327	F₂
		ns			↓ ***			↓ ***			↓ ***			ns			
	F₃ran	-0.0064	0.8702	661	0.0477	0.2355	621	0.0608	0.1327	613	0.0415	0.2866	661	0.0071	0.8785	464	F₃ran
anther					0.0014	0.9719	616	-0.0667	0.1011	606	-0.0524	0.1762	667	0.0389	0.3974	475	F₃nat
stigma					ns			ns			ns			ns			
distance					0.0383	0.3330	640	-0.0290	0.4657	633	-0.0473	0.2278	653	-0.0970	0.0803	326	F₂
					ns			ns			ns			ns			
					-0.0674	0.0941	618	-0.0817	0.0439	609	-0.0492	0.2075	657	-0.0380	0.4157	462	F₃ran
mouth								-0.0627	0.1082	658	0.0320	0.4230	629	0.0495	0.2611	518	F₃nat
diameter								↓ ***			ns			ns			
								0.2866	0.0001	648	0.0881	0.0237	659	0.0638	0.2455	333	F₂
								ns			ns			ns			
								0.2663	0.0001	670	0.0199	0.6191	628	0.0265	0.5508	511	F₃ran
limb											0.5127	0.0001	618	-0.1720	0.0001	510	F₃nat
width											ns			ns			
											0.4906	0.0000	649	-0.0781	0.0013	324	F₂
											ns			ns			
											0.4257	0.0001	619	-0.0868	0.0516	503	F₃ran
corolla														-0.3596	0.0001	485	F₃nat
tube														ns			
length														-0.2787	0.0001	337	F₂
														ns			
														-0.2427	0.0001	469	F₃ran

