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POLLINATOR PREFERENCES FOR *NICOTIANA ALATA*, *N. FORGETIANA*, AND THEIR F₁ HYBRIDS

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Abstract.—The role of pollinators in plant speciation and maintenance of species boundaries is dubious because most plant species are visited by several types of pollinators, and most pollinator species visit several species of plants. We investigated pollinator preferences and their efficacy as ethological isolation mechanisms between two interfertile species, *Nicotiana alata* and *N. forgetiana* and their F₁ hybrids. Hawkmoths pollinate *N. alata*, while primarily hummingbirds and occasionally small hawkmoths visit *N. forgetiana*. F₁ hybrids are easily produced in the greenhouse and although the species grow in similar habitats, hybrids have not been found in nature. In Rio Grande do Sul, Brazil, near where both species are found, experimental plots were studied containing both species, and both species plus F₁ hybrids. In the mixed-species plots, hawkmoths showed a strong preference for *N. alata*. Hummingbirds were less common and only visited *N. forgetiana*. Hybrid seed was produced but plants made significantly fewer hybrid offspring than predicted by the frequency of interspecific pollinator movements. *Nicotiana forgetiana* was the seed parent of 97% of the F₁ offspring, suggesting an asymmetry in pollen delivery or postpollination processes. In plots containing F₁ hybrids plus both parental species, hawkmoths preferred *N. alata* and undervisited the other two phenotypes, except that in the third plot they visited hybrids in proportion to the hybrid frequency. Hummingbirds strongly preferred *N. forgetiana* in all plots but also visited F₁ hybrids in proportion to their frequency in the third plot. Overall, F₁ hybrids were well pollinated and were frequently visited immediately before or after one of the parental species. Thus hybrids could facilitate gene flow between the parental species. We conclude that pollinator discrimination among species is strong but is an imperfect isolation mechanism, especially if hybrids are present.

Key words.—Floral evolution, hybridization, plant-pollinator interaction, pollinator preference, reproductive isolation.

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The genetic integrity and phylogenetic independence of closely related species depends on the strength of reproductive isolation between the species (Mayr 1963; Dobzhansky 1970). Recently, floral traits that may contribute to, or even cause, reproductive isolation have drawn renewed attention from plant evolutionists. One source of this rekindled interest can be traced to the *Drosophila* community's reexamination of the genetics of speciation (Coyne and Orr 1989; Coyne 1992; Rice and Hostert 1993). Grant's (1994) review pointed out that floral traits have long been suspected of contributing to the genetic isolation between species; surely botanists already knew the functions of some "speciation genes".

Another impetus for studying plant-pollinator interactions in the context of speciation came when Ollerton (1996) and Waser (1998) pointed out that many or most plants appear to have a generalized association with their pollinators. Plants often attract many types of pollinators, and individual pollinators often visit many species of plants. Generalized plant-pollinator associations should not lead to effective isolating mechanisms. In a generalized system, selection on floral traits would be diffuse or nonexistent—unless selection occurs consistently by all members of the entire pollinating fauna (Waser 1998, 2001). So plant evolutionists are beginning to take a closer and more experimental approach to try to understand flowers' and pollinators' roles in establishing and maintaining plant species boundaries (e.g., Bradshaw et al.

1995; Fulton and Hodges 1999; Schemske and Bradshaw 1999; Wolf et al. 2001).

One difficulty in interpreting floral isolation is that traits that apparently isolate plant species may or may not have evolved for that purpose. Traits that promote isolation may evolve in direct response to selection (i.e., reinforcement, Dobzhansky 1970; but see Marshall et al. 2002), or through a correlated response to selection on associated characters (e.g., Rice and Hostert 1993). Further, the evolution of traits that reduce or prevent hybridization can be facilitated by geographic isolation or other extrinsic isolating mechanisms, such as reproduction in different microhabitats or at different times. Traits that may seem to have evolved as isolation mechanisms could have evolved in allopatry (Grant 1994). For example, plant populations may evolve to specialize on different pollinators that are common in different regions. This scenario is a case of adaptive speciation (sensu Dieckmann et al. 2004), mediated by adaptation to pollinators, but not *sympatric* adaptive speciation—in which speciation itself is adaptive in order to minimize hybridization. If these populations or species make secondary contact, and attract different pollinators, we might erroneously conclude that the different floral forms evolved in response to the plants' interactions, rather than adaptation to pollinators during the time they spent in allopatry (Grant 1993). Although the historical question of the origin of floral differentiation is difficult to answer with certainty, it is interesting to ask how well floral differences function as barriers to hybridization (Waser 2001).

Production of hybrid plants is contingent on the degree of

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TABLE 1. Floral traits of *Nicotiana alata*, *N. forgetiana*, and their F₁ hybrids (mean [SE]). Plants described here are those in Figure 3.

Trait	<i>N. alata</i> N = 45	F ₁ hybrid N = 31	<i>N. forgetiana</i> N = 38
Anther-stigma separation	0.13 (0.04)	0.08 (0.009)	0.11 (0.01)
Stigma insertion	0.15 (0.02)	0.07 (0.009)	0.05 (0.02)
Mouth diameter	0.70 (0.02)	0.65 (0.02)	0.62 (0.03)
Floral limb width	4.57 (0.14)	3.45 (0.13)	2.45 (0.13)
Floral tube length	5.98 (0.14)	4.44 (0.14)	3.76 (0.15)
Color	white	pink	red

pollinators' discrimination of particular floral types and the mechanics of pollen transfer (Grant 1994), as well as post-pollination processes (e.g., Emms et al. 1996). Unless hybrids become immediately isolated genetically from their parents (e.g., as in allopolyploidy) then the hybrids may become a bridge between partially differentiated gene pools, and floral differentiation may be lost, at least locally. A stable hybrid zone might arise (e.g., Campbell et al. 1997), a new hybrid species could form (Rieseberg et al. 1995; Rieseberg 1997), or genes from one species might be introgressed into the other species. If hybridization is predominantly in one direction due to the ethological or mechanical details of pollen transfer or postpollination biases in successful seed set, then one species' genes could be absorbed entirely by the other species (i.e., genetic swamping; Ellstrand and Elam 1993).

Our overall goal is to experimentally determine the potential for plant-pollinator relationships to affect reproductive isolation of *Nicotiana alata* and *N. forgetiana*. These species are closely related and interfertile and found within 300 m of each other in southern Brazil. These species are divergent in floral phenotypes and pollinator associations (primarily hawkmoths for *N. alata* and hummingbirds for *N. forgetiana*, Goodspeed 1954; Ippolito 2000). Because we cannot be sure whether the floral phenotypes arose to diminish hybridization or became seemingly adapted to different pollinators in allopatry, we have approached the problem from two sides. In this paper, we ask whether pollinator preferences act to preserve these species' integrity. In a future paper, we ask whether pollinators' behaviors can actually cause a divergence of floral form.

Our first goal here was to determine whether pollinators discriminated among these species during foraging bouts in experimental sympatry plots. Our knowledge of pollinators' roles as agents of plants' gene flow is limited by the relatively small number of studies on incipient (or sister) species that incorporate pollinator observations with estimates of realized gene flow (Galen et al. 1987; Campbell et al. 1997, 2002; Leebens-Mack and Milligan 1998). Pollinators' behavior need not have exclusive preferences to impede gene flow, as long as individual bouts are made with floral constancy. Therefore, we were interested in not only overall preferences, but also plant-to-plant movements. Next, we grew seeds from our experimental sympatry plots to see whether interspecific pollinator movements resulted in interspecific gene flow. Finally, we grew both species with F₁ hybrids to determine whether the presence of the hybrids facilitated further gene flow across the species boundary, another aspect of pollinator-mediated isolation on which we have limited information (Leebens-Mack and Milligan 1998; Campbell et al. 1998).

MATERIALS AND METHODS

Study Species

Nicotiana alata is broadly distributed throughout southern Brazil, northeastern Argentina, and southern Paraguay (Goodspeed 1954). *Nicotiana forgetiana* is known only from the most southerly state of Brazil, Rio Grande do Sul, and is found from the coastal mountains westward into the southern high plateau. The range of *N. forgetiana*, though incompletely known, appears to be nearly surrounded by that of *N. alata*. Although no other *Nicotiana* species occur in the study area, *N. alata*'s range overlaps with several other *Nicotiana* species in other parts of its range. *Nicotiana alata* and *N. forgetiana* both grow in similar habitats in rocky cliffs and outcrops, and along rocky stream sides in southern Brazil. Both species also grow along roadsides and invade disturbed areas, where they can be prolific. No apparent differences exist in the microhabitats or soil types where the species grow. Although populations grow within a few kilometers of each other at several sites, and scattered individuals have been found within 300 m of each other, we know of no sympatric populations (A. Ippolito, G. W. Fernandes and T. P. Holtsford, pers. obs. over four years). The source populations for the experiments described below are approximately 100 km from each other, but plants of both species are found scattered all along the intervening stretch of highway (BR 116).

Nicotiana alata and *N. forgetiana* are members of a well-supported clade, *Nicotiana* section *alatae*, based on nuclear ribosomal internal transcribed spacer (*nrITS*) sequence data (Buckler et al. 1997; Ippolito 2000; Chase et al. 2003), though the *nrITS* trees can not resolve relationships among *N. alata*, *N. forgetiana*, nor three other species within *alatae*. Morphology, cytogenetics (Goodspeed 1954), and biogeography suggest that *N. alata* and *N. forgetiana* are each other's closest relatives. The species are of the same ploidy ($n = 9$) and vigorous fertile hybrids are easily produced in the greenhouse.

The parent species and F₁ hybrids are self-incompatible and thus seed set is dependent on pollinator visitation. *Nicotiana alata* flowers are white, have a strong scent (Raguso et al. 2003), and have a long narrow corolla tube (Table 1). The pollinators are large hawkmoths (Sphingidae), primarily *Agrius cingulata* and *Eumorpha labruscae* (A. Ippolito, G. W. Fernandes and T. P. Holtsford, pers. obs. from at least 120 hours over four years). *Nicotiana forgetiana* has red flowers that produce much less scent (Raguso et al. 2003) and the floral tube is shorter and broader. Hummingbirds, most commonly *Chlorostibulon aureoventris*, pollinate *Nicotiana*

forgetiana but we have once witnessed a visit by a Halictid bee.

Nicotiana forgetiana's flowers are crepuscular whereas those of *N. alata* are nocturnal. Flowers of *N. forgetiana* generally open in the late afternoon, approximately 1–2 h before the flowers of *N. alata*, and close after sunrise about one hour after those of *N. alata*. However, on cloudy or cool days, flowers of both species will remain open for longer periods of time. The ancestral pollination syndrome within section *alatae* is unclear—four species are predominantly moth pollinated and four are predominantly bird pollinated. The most closely related group to section *alatae* is section *suaveolens* (Ippolito 2000; Chase et al. 2003), a clade whose white, scented flowers suggest moth pollination. That, coupled with the observation that *N. forgetiana*'s range seems to be encompassed by that of *N. alata*, suggests that hummingbird pollination in *N. forgetiana* evolved from a hawkmoth-pollinated ancestor, which was likely *N. alata* itself or its immediate progenitor.

Study Site and Experimental Sympatry Plots

Experimental plots were sown at an agricultural field station maintained by the Conselho de Desenvolvimento Comunitario de Campestre (CDCC) and at a nearby private orchard (Fazenda Tiscornia) in Rio Grande do Sul, Brazil (51°06'47" W; 28°05'13" S). Both sites are about 10 km from the place where individuals of the two species have been found within 300 m of each other. Vegetation at these sites is similar to nearby natural roadside populations. We grew seeds of the parental species in a completely randomized design in flats in the CDCC greenhouse, and transplanted them into 1 L bags when rosettes were approximately 5 cm in diameter. When seedlings began to produce inflorescence shoots, we transplanted them into the ground at CDCC or Fazenda Tiscornia. Plants were watered but some plants died in both years (summers of 1997–1998, 1998–1999) due to drought.

We transplanted 20 plants of each species in each of two common-garden plots, denoted Asp and Rasp in 1998 and House and Down in 1999. Plants that died after transplanting were not replaced. The arrangement of plants within plots was randomized and interplant distances were also randomized and mimicked those of nearby natural populations, between 30 cm and 1 m apart.

Pollinator Observations

We observed pollinators during peak activity from late afternoon until midnight (when hawkmoths visits had declined to nearly zero), and from dawn until flowers closed for the day, for at least three days per plot. Only one plot could be observed each night. The focal plot rotated every night until approximately 40 observer hours were spent in each plot. We recorded, on microcassette recorders, the identity of each plant visited and the number of flowers visited per plant for all plants visited by a pollinator, until the pollinator left the plot or was otherwise lost. Our attempts to mark individual pollinators were not successful. The same individual pollinators may have made more than one foraging bout.

To test for pollinators' preferences, we used contingency tables to compare the frequency of pollinator visitation to a given plant species with the frequency of that plant species in the plot. In this approach, preference may be obscured if different individual pollinators have preferences for different plant species' flowers. If half the hawkmoths preferred *N. alata* and half preferred *N. forgetiana*, then the analysis of all floral visits may not detect a preference, even though individual moths are showing preference. To test for this possibility (i.e., the tendency for individuals to "major" on a particular flower type, that is, to show floral constancy), we compared pairs of plant-to-plant movements to the expectation based on the overall preference of each pollinator type (following Stanton et al. 1989). If the frequency of visits to *N. alata* (a) = $visfreq(a)$ and the frequency of visits to *N. forgetiana* (f) = $visfreq(f)$, then the expected pairwise transitions for a-a, a-f, f-a and f-f are: $visfreq(a)^2$, $visfreq(a) \times visfreq(f)$, $visfreq(f) \times visfreq(a)$, and $visfreq(f)^2$, respectively. If pollinator movements between plants conform to these expectations, then their plant-to-plant movements can be viewed as a simple extension of their overall preference for floral types. If pollinators made more homotypic plant-to-plant movements than expected, then that would be evidence of floral constancy, or the tendency of individuals to major on one floral type.

Rate of Hybridization

To estimate the frequency of hybridization in the experimental sympatry plots, we collected all fruit produced in each year and pooled the seeds from each plant. For each plant, 10 seeds were randomly drawn, sown, and grown to flower. Because F_1 hybrids are intermediate in floral traits, including color (Table 1), they were easily distinguished from the parental species. We recorded the number of hybrid and pure offspring produced by each plant and averaged these to estimate hybridization frequency.

Parental Species plus F_1 Hybrid Plots

Our 1998 sympatry plots yielded hybrid plants, so in 1999 we also investigated whether the presence of hybrids with both parental species might facilitate interspecific gene flow. We grew plants as outlined above and transplanted 15 plants of each phenotype (both parental species and their F_1 hybrids) to three common garden plots, denoted Hill, Mitt, and Lixo in 1998–1999. Plants that died after transplanting were not replaced. The arrangement of plants within plots was randomized and interplant distances mimicked those of nearby natural populations. We observed pollinators during the time that flowers were open on at least three days per plot. We tested for floral preference and constancy as in the experimental sympatry plots above, except that there were three plant types.

Analyses

Contingency tables were analyzed using G -statistics, with Williams' correction (Sokal and Rohlf 1981, p. 706). All our analyses make use of external hypotheses for the expected frequencies. To predict pollinators' visitation frequency we

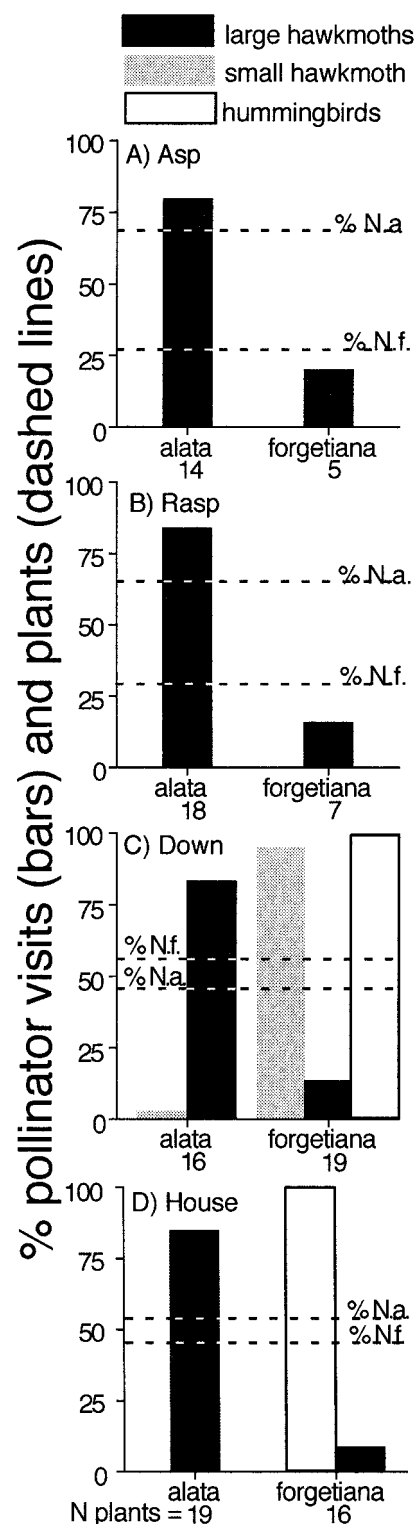
used the frequencies of the different types of plants in the plots. Preferences were then used to generate plant-to-plant transition expectations to test for floral constancy. Pollinators' interspecific movements became the expectation for the numbers of hybrid seed produced. Because the expectations were not drawn from the contingency tables themselves (i.e., we did not want to test the observed table vs. all other tables with the same fixed marginal totals), we could not use Fisher's exact tests (Sokal and Rohlf 1981, p. 739). If expected frequencies in any cell were less than five we did not analyze the data. If expected frequencies were greater than five for all cells but one *observed* cell was zero, we substituted 0.01 for the zero to estimate an approximate *G*. These approximate *G*s were very similar to χ^2 estimates (in which zeroes in observed cells are not a computational problem). In one case (preference tests for plots including F_1 hybrids), we present a χ^2 analysis to allow the contributions from individual cells to be visualized, though the conclusions were no different from the *G* test.

RESULTS

Pollinator Preference in Experimental Sympatry Plots

Two species of large hawkmoths were common in the plots, *Agrius cingulata* and *Eumorpha labruscae*—the same species that were common in nearby natural populations. At least three other putative hawkmoth species were observed but we failed to catch or identify them. The total number of hawkmoth visits and bouts observed were 212 visits distributed over 11 bouts in the Asp plot, 489 visits over 52 bouts in the Rasp plot, 120 visits over nine bouts in the House plot, and 58 visits over 12 bouts in the Down plot. Large hawkmoths overvisited *N. alata* and undervisited *N. forgetiana*, relative to the plant species' frequencies, in all four plots in both years (Fig. 1, for the Asp, Rasp, Down, and House plots, respectively, $G = 4.20$, $df = 1$, $n = 212$ visits, $0.05 > P > 0.025$; $G = 37.95$, $df = 1$, $n = 489$, $P < 0.0001$; $G = 37.32$, $df = 1$, $n = 58$, $P < 0.0001$; $G = 64.97$, $df = 1$, $n = 120$, $P < 0.0001$). Despite their preference for *N. alata*, large hawkmoths also visited *N. forgetiana*—on average, 16.5% of the 879 visits we observed in all the experimental sympatry plots.

In 1997–1998, we did not observe any hummingbirds visiting experimental plots. In 1998–1999, the glittering-bellied emerald hummingbird, *Chlorostibulon aureoventris*, was the only hummingbird species observed. The total number of visits and bouts to the House and Down plots by hummingbirds were 39 visits over nine bouts and 11 visits over two bouts, respectively. The hummingbirds we observed had an unwavering preference for *N. forgetiana* (Fig. 1C,D, for the Down and House plots respectively ($G = 37.32$, $df = 1$, $n = 11$, $P < 0.0001$ and $G = 60.11$, $df = 1$, $n = 39$, $P < 0.0001$). In the Down plot, we observed one species of small hawkmoth (probably *Callionima nomius*) that preferred *N.*



(as a percentage of total visits) to each plant species by large hawkmoths (black), hummingbirds (white), and small hawkmoths (gray). The lines indicate the percentages of *N. alata* (%N.a.) and *N. forgetiana* (%N.f.) plants in the plots. Pollinator preferences were tested ($G_{df=1}$) by comparing observed visitation frequencies (bars) to the expectation that plant species would be visited in proportion to their presence in the plots (lines). The hawkmoths in the Asp plot overvisited *N. alata* and undervisited *N. forgetiana* at $P < 0.05$. All other tests were significant ($P < 0.0001$).

FIG. 1. Pollinator visits to *Nicotiana alata* and *N. forgetiana* in experimental sympatry plots. Bars indicate the frequency of visits

TABLE 2. Plant-to-plant transitions made by pollinators in plots containing *Nicotiana alata* (alata) and *N. forgetiana* (forg). The hypothesis tested here is that plant-to-plant movements are a simple extension of single-flower preferences. Expected plant-to-plant movement frequencies are based on single-flower-visit preferences (Fig. 1) and are rounded to whole numbers for presentation. If homotypic movements are more frequent than expected, then that provides evidence for floral constancy. The direction of movement is from the species listed on the left of each matrix to the species listed above. *Nicotiana alata* to *N. forgetiana* movements were equal in number to *N. forgetiana* to *N. alata* movements in all cases, because all observed foraging bouts began and ended with *N. alata*. Hummingbird movements were too infrequent for this analysis.

Plot	Pollinators	Plant-to-plant transition matrices						G (df = 1)	P
		Expected		Observed					
Asp	hawkmoths	alata	alata	forg	alata	alata	forg	0.440	≈0.5
		forg	114	29	forg	118	27		
			29	7		27	7		
							sum = 179		
Rasp	hawkmoths	alata	alata	forg	alata	alata	forg	10.305	0.001 < P < 0.005
		forg	280	54	forg	264	65		
			54	10		65	4		
							sum = 398		
Down	hawkmoths	alata	alata	forg	alata	alata	forg	1.923	0.1 < P < 0.5
		forg	38	7	forg	38	6		
			7	1		6	3		
							sum = 53		
House	hawkmoths	alata	alata	forg	alata	alata	forg	3.525	0.1 < P < 0.5
		forg	95	13	forg	92	15		
			13	2		15	0		
							sum = 122		

forgetiana to *N. alata*; 26 of 28 plants visited over two foraging bouts (Fig. 1C, $G = 26.62$, $df = 1$, $n = 28$, $P < 0.0001$).

Tests of Floral Constancy in Experimental Sympatry Plots

Next, we determined whether pollinators' plant-to-plant movements were predicted by their single-visit preferences or if there was evidence for floral constancy. For hawkmoths, in three of four cases, expectations generated from the single-plant-visit frequencies were not rejected; floral preferences were a satisfactory predictor of plant-to-plant movements so there was no evidence of constancy (Table 2). In the Rasp plot the single-plant preference model was rejected, but there was no evidence for floral constancy; heterospecific movements were more frequent than expected.

Hummingbirds only visited *N. forgetiana*, thus, their plant-to-plant movements were completely consistent with their per-visit preferences (i.e., all 41 plant-to-plant transitions observed were *N. forgetiana* to *N. forgetiana*, see also Fig. 1). The single small hawkmoth's plant-to-plant movements differed very little from the predictions based on its single-plant-visit preference, which was strongly biased in favor of *N. forgetiana*. These trends could not be statistically analyzed because few plant-plant movements were observed and because the very strong preference toward *N. forgetiana* meant that expectations involving *N. alata* were often zero, which is a computational problem for both χ^2 and G tests.

Production of Hybrid Offspring in Experimental Sympatry Plots

F₁ hybrids were produced in all four plots, but at a frequency less than that of interspecific pollinator movements. The average percentage of hybrids produced from all four plots was 9.5% whereas interspecific pollinator movements averaged 29.3% of all observed plant-to-plant movements. For each plot in each year, the rate of F₁ hybrid production

was significantly less than the rate of observed interspecific movements made by pollinators (Fig. 2A; Asp, Rasp, Down, House: $G = 28.87$, $df = 2$, $n = 93$ offspring, $P < 0.0001$; $G = 117.75$, $df = 2$, $n = 185$, $P < 0.0001$; $G = 100.29$, $df = 2$, $n = 210$, $P < 0.0001$; $G = 113.72$, $df = 2$, $n = 378$, $P < 0.001$, respectively).

Next, we tested hybrid production separately for each species. Overwhelmingly, *N. forgetiana* was the seed parent of most hybrids produced (97%; see Table 2 for pollinator movements and Fig. 2B for the comparison of movement frequencies to hybrid production). In all plots, *N. alata* produced significantly fewer F₁ hybrids than expected (Fig. 2B, for the Asp, Rasp, Down and House plots, respectively: $G = 26.12$, $df = 1$, $n = 83$ offspring; $G = 62.92$, $df = 1$, $n = 144$; $G = 61.09$, $df = 1$, $n = 161$; $G = 45.92$, $df = 1$, $n = 272$, all $P < 0.0001$).

For *N. forgetiana* seed parents, only in the Rasp plot was the number of hybrid offspring produced significantly less than the expectation based on the rate of a to f movements ($\chi^2 = 75.69$, $df = 1$, $n = 41$ offspring, $P < 0.0001$). In the Asp and Down plots, the number of hybrids produced was statistically indistinguishable from that expected based on pollinator movements (Fig. 2C, $G = 2.18$, $df = 1$, $n = 10$, $G = 4.27$, $df = 1$, $n = 49$, respectively, both $P > 0.10$). In the House plot, the proportion of hybrids produced exceeded the rate of a to f pollinator movements ($G = 4.27$, $df = 1$, $n = 106$, $P < 0.01$).

Pollinator Preferences in Plots with F₁ hybrids and Both Parental Species

In 1998–1999, F₁ hybrids were planted along with parental species in three plots at the Fazenda Tiscornia. We observed 392 hawkmoth visits distributed over 18 foraging bouts in the Hill plot, 341 visits (18 bouts) in the Mitt plot, and 168 visits (seven bouts) in the Lixo plot. Hummingbirds were

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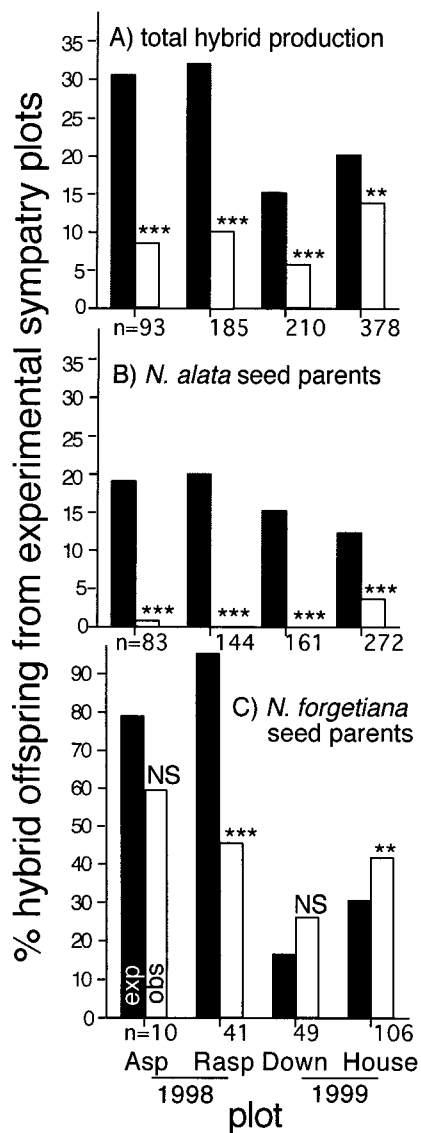


FIG. 2. Comparison of observed hybrid production (obs; white bars) with expectations (exp; black bars) based on the number of pollinator movements observed between parent species in each of the experimental sympatry plots. For (A) the expected percentage was the fraction of interspecific plant-plant movements by all pollinators regardless of the direction of the movement. For (B) the hybridization expectation was estimated as number of *Nicotiana forgetiana* to *N. alata* movements (f-a) divided by the total number of movements to *N. alata* (f-a + a-a). For (C) the expectation was a-f/(a-f + f-f). Sample sizes (n) of progeny are given below the white bars. The sample sizes of pollinator movements, from which the expectations were inferred, are given in Table 2. Levels of statistical significance ($G_{df=2}$ for A; $G_{df=1}$ for B and C) are abbreviated as NS, not significant; ** $P < 0.001$; *** $P < 0.0001$.

more common in plots that included F₁ hybrids. However, we cannot test the hypothesis that the presence of the F₁ hybrids affected hummingbird visitation, since each type of plot (parental species vs. parental species + F₁ hybrids) was only grown in one site (CDCC vs. Fazenda Tiscornia). *Chlorostibulon aureoventris* was the most common hummingbird observed but at least two other unidentified putative species were also seen. We observed 39 visits by hummingbirds

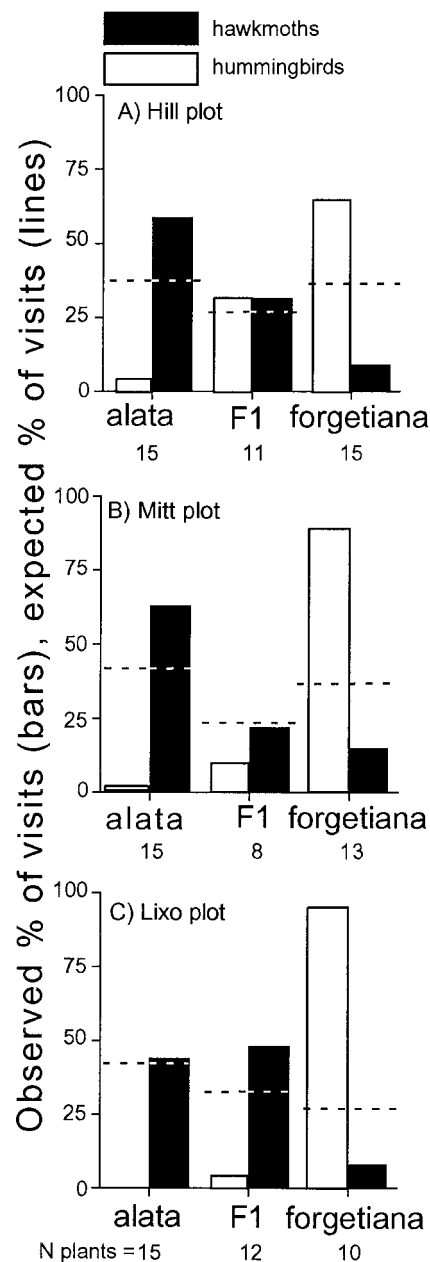


FIG. 3. Frequency of pollinator visits (bars) to *Nicotiana alata*, *N. forgetiana*, and their F₁ hybrid in mixed plots. The number of plants in each group is presented below each plot. Bars indicate the frequency of visits to each plant type by large hawkmoths (black) and hummingbirds (white), expressed as percentages of these pollinators' total visits. Dashed lines indicate the percentage of each type of plant in each plot, which formed the null hypothesis for pollinator visitation rate to each type of plant. Observed visitation rates (bars) were significantly different ($G_{df=2}$) from expected rates (lines) in all cases at the $P < 0.001$ level.

(three bouts) in the Hill plot, 18 visits (11 bouts) in the Mitten plot, and seven visits (three bouts) in the Lixo plot.

We analyzed pollinator visitation in parental + hybrid plots similarly to the sympatry plots, using the frequencies of the three plant types as the null hypothesis for pollinator visitation frequency. In all cases, the no-preference hypothesis was rejected (Fig. 3). The cell-by-cell contributions to

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TABLE 3. Preference tests in plots containing both parental species and F₁ hybrids. For hawkmoths, hummingbirds, and all pollinators grouped, the expected frequencies (exp) of visits to each plant type are compared to the observed frequencies (obs). Expected frequencies are based on the frequency of plants in each class (parental species or F₁). χ^2 statistics are broken down to show the contribution of each cell to the total χ^2 . *G*-statistics yield the same conclusions but do not illustrate the cell-by-cell contributions as easily. Preferences for, or discrimination against, parental species (see [obs - exp]²/obs column) are always greater than the hybrids' contributions to the χ^2 , with one exception: hawkmoths in the Lixo plot.

	No. plants	Hummingbirds			Hawkmoths		
		obs	exp	$\Sigma(\text{obs} - \text{exp})^2/\text{obs}$	obs	exp	$\Sigma(\text{obs} - \text{exp})^2/\text{obs}$
Hill plot							
<i>N. alata</i>	15	3	14.27	8.90	225	143.47	46.33
F ₁ hybrid	11	12	10.45	0.23	119	105.06	1.85
<i>N. forgetiana</i>	15	24	14.27	6.63	48	143.47	63.53
Total	41	39	39	$\chi^2 = 15.76$ $P = 3.78 \times 10^{-4}$	392	392.00	$\chi^2 = 111.71$ $P = 5.53 \times 10^{-25}$
Mitt plot							
<i>N. alata</i>	15	1	33.78	31.81	216	142.61	37.76
F ₁ hybrid	8	8	17.98	5.54	73	75.92	0.11
<i>N. forgetiana</i>	13	72	29.24	62.53	53	123.46	40.21
Total	36	81	81	$\chi^2 = 99.87$ $P = 2.05 \times 10^{-22}$	342	342.00	$\chi^2 = 78.09$ $P = 1.10 \times 10^{-17}$
Lixo plot							
<i>N. alata</i>	15	0	7.71	7.71	74	68.21	0.49
F ₁ hybrid	12	1	6.16	4.32	81	54.43	12.97
<i>N. forgetiana</i>	10	18	5.13	32.29	13	45.36	23.09
Total	37	19	19	$\chi^2 = 44.32$ $P = 2.38 \times 10^{-10}$	168	168.00	$\chi^2 = 36.55$ $P = 1.16 \times 10^{-8}$
All plots		All pollinators					
<i>N. alata</i>		45		519	410.06		28.94
F ₁ hybrid		31		294	270.00		2.13
<i>N. forgetiana</i>		38		228	360.94		48.96
Total							$\chi^2 = 80.04$ $P = 4.17 \times 10^{-18}$

the χ^2 statistics indicate that both pollinator types had strong preferences for or against the parental plant species, but discrimination effects with regard to F₁ hybrids were smaller (Table 3). The one exception to this pattern was in the Lixo plot, where hawkmoths overvisited the hybrid but only visited *N. alata* in approximate proportion to *N. alata*'s frequency in the plot (Table 3). Hummingbirds preferred *N. forgetiana* to *N. alata* in all plots. Hybrids received at least one visit from a hummingbird in all plots and in the Hill plot, hummingbirds visited hybrids in approximate proportion to the hybrid's frequency (Fig. 3 and Table 3).

Plant-to-Plant Transitions in Plots with F₁ Hybrids and Both Parental Species

Pollinators' plant-to-plant movements (Table 4) were tested against the expectation that movements were simple extensions of single-plant preferences (Fig. 3). In one case this null hypothesis was not rejected (the Lixo plot). Hawkmoths in the Mitt plot exhibited a small but significant tendency toward floral constancy; *alata*-to-*alata* (a-a) and *forgetiana*-to-*forgetiana* (f-f) transitions were overrepresented, whereas a-f and f-a movements were underrepresented. In the Hill plot, simple preference expectations were not a satisfactory model for hawkmoth movements but the pattern did not resemble constancy; hybrid-to-hybrid movements were overrepresented, but a-a and f-f movements were underpre-

sented and a-f and f-a moves were overrepresented (Table 4).

Although we observed an average of approximately 41 hummingbird movements per plot, hummingbirds' strong aversion to *N. alata* meant that many cells involving *N. alata*'s expectations were zero, which precluded contingency table analyses. Nevertheless, hummingbird plant-to-plant movements were almost identical to the expectations based on their preferences. Hummingbirds seemed to make their plant-to-plant movements according to their overall preferences (which were statistically detectable; Table 3, Fig. 3), not the identity of the last plant type they visited.

The presence of F₁ hybrids tended to erode the partial ethological barrier between *N. alata* and *N. forgetiana*. Hummingbirds never visited *N. alata* in plots containing only the two parental species ($n = 2$ plots where hummingbirds were observed). But in plots that included F₁ hybrids, we observed four hummingbird visits to *N. alata* (of 139 total). When F₁s were present with both parental species, interspecific gene flow could result from *N. alata* to *N. forgetiana* (and reciprocal) movements, and from backcrosses to either parent (F₁ × parental species and reciprocal). Almost one-third of hummingbirds' movements in the plots containing F₁ hybrids could have caused interspecific gene flow (29.84%, $n = 3$ plots, SD = 23.59, Table 2 vs. Table 4). However, no hummingbird movements could have resulted in interspecific

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TABLE 4. Plant-to-plant transitions made by pollinators in plots containing *Nicotiana alata* (alata), *N. forgetiana* (forg), and their F₁ hybrids (F₁). Expected plant-to-plant movement frequencies are based on single-flower-visit preferences (Fig. 3) and have been rounded to whole numbers for presentation. The hypothesis tested here is that plant-to-plant movements are a simple extension of single-flower preferences. If homotypic movements are more frequent than expected, then that provides evidence for floral constancy. The direction of movement is from the species listed at left to the species listed above the matrices.

Plot	Pollinators	Plant-to-plant transition matrices									$G_{df=4}$	P
		Expected			Observed							
Hill	hawkmoths		alata	F ₁	forg		alata	F ₁	forg		10.47	<0.01
		alata	124	65	26	alata	118	66	32			
		F ₁	65	35	14	F ₁	65	42	8			
		forg	26	14	6	forg	29	8	7			
						Total = 375						
Lixo	hawkmoths		alata	F ₁	forg		alata	F ₁	forg		5.12	0.1 < P < 0.5
		alata	32	35	6	alata	35	29	6			
		F ₁	35	38	6	F ₁	32	43	7			
		forg	6	6	1	forg	6	6	0			
						Total = 164						
Mitt	hawkmoths		alata	F ₁	forg		alata	F ₁	forg		10.76	<0.01
		alata	133	45	33	alata	141	45	25			
		F ₁	45	15	11	F ₁	45	15	11			
		forg	33	11	8	forg	25	10	17			
						Total = 334						

gene flow in the plots with only the parental species (0.00 vs. 29.84, Student's $t = 2.19$, $df = 2$, ns). Hawkmoths' interspecific movements constituted 27.53% of plant-to-plant movements in plots containing only the parental species ($n = 4$ plots, $s = 0.045$). In plots that included F₁ hybrids, 66.00% ($n = 3$, $s = 0.130$) of movements could have led to interspecific gene flow—a significant difference ($t = 4.89$, $df = 2$, $P = 0.03$; Table 2 vs. Table 4).

DISCUSSION

Our experimental sympatry plots were designed to assess the role of pollinator preferences in preventing pollen transfer, thus maintaining reproductive isolation between *N. alata* and *N. forgetiana*. Pollinators showed floral preferences that were consistent with the plant species' presumed pollination syndromes (83.5% [$n = 756$] of all observed visits by large hawkmoths were to *N. alata* and 100% [$n = 43$] of visits by hummingbirds were to *N. forgetiana*; see also Fig. 1). Hybrids were produced in these plots so that pollinator-mediated assortative mating is an imperfect prereproductive isolating mechanism. Hybrids were produced in lower frequency than the interspecific movements by pollinators, suggesting a role for postpollination processes in the species boundary (Fig. 2), but biases in the mechanics of pollen transfer cannot be ruled out. In plots that included hybrids with both parental species, the hybrids attracted both hummingbirds and hawkmoths in moderate frequencies (Table 3), allowing for backcrossing and introgression (Table 4).

Floral Preferences among Parental Species

Large hawkmoths preferred *N. alata* but visited both species. Hummingbirds visited *N. alata* very rarely (only when F₁s were also present) and were likely excluded from the nectar reward of most *N. alata* flowers by the length of the corolla tube. Understanding hummingbirds' preferences for *N. forgetiana* seems relatively simple. We estimated the bills of the hummingbirds that visited our plots to be about 4 cm

long—about 2 cm too short to extract nectar from the average *N. alata* flower (Table 1). Hummingbird visits were so rare in our plots that we did not risk driving them away by trying to net them to measure their bills.

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. The correlation between polymorphism for nectar spurs and increased species diversity in many plant groups suggests that polymorphic spurs are a key innovation that fosters diversification (Hodges 1997). Corolla tube length differences should logically play the same role, although corolla length evolution may be more constrained by pleiotropic effects on size of other floral organs.

Large hawkmoths are capable of retrieving nectar from either *N. alata* or *N. forgetiana*; however, they prefer *N. alata* flowers. *Nicotiana alata* flowers are larger and their white color may make them more apparent than *N. forgetiana* flowers. Since floral limb width and floral tube length are positively correlated, hawkmoths may not be choosing longer-tubed flowers per se, but larger flowers (Table 1). In *N. alata* × *N. forgetiana* F₂ populations, the phenotypic correlation between corolla tube length and limb width is $r = 0.51$ ($n = 618$; Ippolito 2000).

Large hawkmoths may not be able to efficiently manipulate the shorter flowers of *N. forgetiana*. When hawkmoths visit *N. alata*, their proboscis usually appeared fully inserted and they often steadied their hovering by placing their front two legs on the lower limb of the flower. When feeding on *N. forgetiana*, hawkmoths' bodies remain too far from the flower to steady their flight with their front legs. The seemingly more awkward feeding may be uncomfortable and more energetically demanding. However, in *Aquilegia pubescens*, artificially shortening floral spur lengths did not affect hawkmoth visitation (Fulton and Hodges 1999).

Hawkmoths may choose *N. alata* flowers if they have

learned that nectar rewards are greater in the larger flowers. But in greenhouse conditions, the two species produced an equivalent nectar reward (R. L. Kaczorowski and T. P. Holtsford, unpubl. data). *Nicotiana alata* produced a greater volume of nectar but at a lower concentration than *N. forgetiana* (15.61 μ l [SE 1.00] at 23.48% w/w [SE 0.37], total reward = 3.99 mg sucrose equivalents versus 6.68 μ l [0.57] at 47.56% [2.07], total reward = 3.73 mg sucrose equivalents, respectively; $F = 0.97$, $df = 1,30$, $P = 0.33$). More dilute nectar may be easier for lepidopterans to extract from a flower (Heyneman 1983), but this assertion has been questioned (May 1985). Sugar and amino acid concentration and composition, as determined by high performance liquid chromatography, were also very similar in the greenhouse (R. L. Kaczorowski, M. C. Gardener, and T. P. Holtsford, unpubl. data). The standing crop of nectar might have been greater in *N. alata* than *N. forgetiana* due to hummingbirds' foraging on *N. forgetiana*. Hummingbird activity was rare, however, so this explanation alone seems unlikely to account for hawkmoths' preference.

Color may also play a role in pollinator preferences. We know of no experiments that show hawkmoths prefer white flowers, but the association of hawkmoth pollination and white- or pale-colored flowers is very strong (Baker 1961; Grant 1983), except for diurnally active hawkmoths (Kelber 1996). Flowers of *N. forgetiana* are red, and although hummingbirds may not have an innate preference for red (Stiles 1976), they can quickly learn to associate various colors with nectar rewards (Goldsmith and Goldsmith 1979; Campbell and Waser 1997; Meléndez-Ackerman et al. 1997).

Scent characteristics may make *N. alata* more attractive than *N. forgetiana* to hawkmoths. In the glasshouse, *N. alata* flowers produced approximately 16-fold more scent compounds per flower than *N. forgetiana* (or a sixfold difference per dry mass of floral tissue; Raguso et al. 2003). Feeding by the hawkmoth *Manduca sexta* required both an attractive odor to be present near a patch of flowers and some visual target: either an unscented paper flower, or a scented flower of *Oenothera neomexicana* (Raguso and Willis 2002). These data suggest that scent may act as a long-range cue to help moths find patches of flowers but may be less important for choosing individual flowers once a group of plants is located. Floral scent has been implicated as a basis for hawkmoth choice in *Aquilegia* but direct evidence is thus far lacking (Hodges et al. 2002).

The small hawkmoth that preferred *N. forgetiana* may have been drawn into the plot by the odor of *N. alata* and, being restricted from the nectar reward of *N. alata* flowers, took advantage of *N. forgetiana* flowers. We did not catch this *Callionima* individual but presume from its small body size that its proboscis was too short to profitably visit *N. alata*.

Despite the clear preferences exhibited by both hawkmoths and hummingbirds, interspecific movements accounted for 29.3% of the plant-to-plant movements in the experimental sympatry plots (Table 2). Interspecific movements were mainly attributable to large hawkmoths, with the small hawkmoth species accounting for 1% of the interspecific movements in the Down plot. But if hybrids are present, hummingbirds may also contribute to interspecific gene flow, primarily by backcrossing among hybrids and *N. forgetiana* (Ta-

ble 3, Fig. 3). Similarly, pollen flow between *Ipomopsis aggregata* and *I. tenuituba* in experimental arrays was rare but pollen flow among hybrids and the parental species was as common as conspecific pollen flow (Campbell and Waser 1997).

Rate of Hybridization

Hybrid plants accounted for 9.5% of all offspring from the experimental sympatry plots, significantly less than the average rate of interspecific movements, 29.3% (Fig. 2A). Pollen transfer mechanics may diminish the opportunity for these species to hybridize. Since the floral tubes of *N. forgetiana* are shorter than tubes of *N. alata*, we might expect to find more *N. forgetiana* pollen distally and more *N. alata* pollen proximally along the hawkmoths' probosci, as in hummingbird-mediated pollen flow in *Ipomopsis* (Wolf et al. 2001). But hawkmoths often recoil their probosci when flying between plants so that each segment of the proboscis can contact other sections. The pollen of these species is indistinguishable in external characteristics; thus, we could not ascertain how pollen transfer mechanics affect the probability of interspecific pollination.

Postpollination processes may also contribute to the lower overall frequency of hybrid production relative to the frequency of interspecific visits. Mixed pollen loads from intra- and interspecific sources may compete in the style, or interspecific pollen may simply grow at a slower rate. Studies in several genera confirm that conspecific pollen can have an advantage over heterospecific pollen (e.g., Arnold et al. 1993; Carney et al. 1994; Rieseberg et al. 1995; Hauser et al. 1997). Primary hybridization rate may be different from the realized hybridization rate because we counted hybrids after they had flowered. Our seed storage, germination, and culture techniques were as benign as possible (following Durbin 1979) to try to minimize selection's effects on estimated hybridization rate. But selection could have acted in the style or during seed filling, seed storage, germination, or prereproductive growth. Nevertheless, the realized hybridization rate was poorly predicted by pollinator movements and was nearly unidirectional.

Direction of Hybridization

Nicotiana forgetiana was the seed parent of the vast majority of F_1 hybrid seed (97%; Fig. 2). Differences among species in the ability of pollen to grow through the longer style of *N. alata* may account for our finding that shorter-styled *N. forgetiana* plants produce more F_1 hybrids. We are currently investigating this possibility. But postpollination processes might not be necessary for asymmetric pollen and gene flow. The anthers of *N. alata* are approximately four times larger than anthers of *N. forgetiana* so that on any one visit to *N. alata*, a large hawkmoth might pick up a larger pollen load than on any one visit to *N. forgetiana*. Because large hawkmoths account for almost all of the interspecific movements, and only 16.5% of their visits were to *N. forgetiana*, pollen loads probably consisted overwhelmingly of *N. alata* pollen. Although the mechanics of pollen transfer and postpollination processes may have reduced hybridization below the level predicted by pollinators' interspecific

movements, almost 9.5% of offspring produced in the mixed parental plots were F₁ hybrids.

Asymmetry in the direction of hybridization or backcrossing is not unusual. In some cases asymmetrical gene flow is due to the mechanics of pollen transfer (e.g., Campbell et al. 1998; Wolf et al. 2001). But postpollination processes are often partially responsible for asymmetric gene flow, especially when the more successful pollen donor has longer styles, as is the case here (e.g., Levin 1978; Gore et al. 1990; but see Emms et al. 1996 for an example in which a short-styled species was more successful in hybrid crosses than its longer-styled congeners).

Pollinator Preference among Parents and Hybrids

Our second set of experiments was designed to determine whether F₁ hybrids would share pollinators with the parent species, thereby facilitating interspecific gene flow. F₁ hybrids competed well with parental species for pollinator services (Fig. 3); they were not consistently discriminated against (Table 3), nor did they attract novel pollinators. We only observed hawkmoths and hummingbirds coming to our plots and their movements did not constitute a rigid breeding barrier; visits to hybrids and parental species were common for both pollinator types (Table 4). Similarly, in *Iris* and *Ipomopsis*, hybrids did not suffer from low rates of pollinator visitation and, when grown with parental species, backcrossing was likely (Wesselingh and Arnold 2000; Campbell and Waser 1997).

Because F₁ hybrids can attract both types of pollinators, backcrossing is possible to both parental species. The rate and direction of introgression would depend not only on the tendency of pollinators to visit different kinds of flowers (Fig. 3) but also, critically, on the numbers of plants and pollinators of each type. Our experiments were conducted with approximately one-third of each floral phenotype but initial frequencies of F₁ hybrids in natural situations could be much lower. In our experimental sympatry plots, realized hybridization rate was 9.5%—perhaps that would have been a more appropriate proportion for the F₁ hybrids in the second set of field experiments. Interspecific gene flow in our experimental sympatry plots was biased toward *N. forgetiana* but in other seasons or locations, for example, with different pollinator pools, the situation could be quite different.

Hybrids between *N. alata* and *N. forgetiana* are vigorous and fertile at least through the F₃ generation (Ippolito 2000), so it appears likely that gene flow across the “species” boundary has been or will be important. The consequences of ongoing gene flow may be reflected in the lack of phylogenetic resolution between these species using *nrITS* (Ippolito 2000; Chase et al. 2003) or intersimple sequence repeats (M. J. Murfett and T. P. Holtsford, unpubl. data). Another possibility is that these species arose recently and have not had time to diverge appreciably except for floral traits. Ongoing interactions between these species and their pollinators might have been important in determining their current floral morphology, or we may be studying a secondary contact zone between groups that have diverged in isolation. These species may now be coming into contact as they spread along roadsides and field margins, whereas their rock outcrop

and streamside populations may have been isolated in separate canyons. Ethological prepollination barriers to hybridization are detectable but incomplete.

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