

**Pollinator preferences and relative fitness of *Nicotiana*
alata, *N. forgetiana* and their F₁ hybrids**

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Abstract

We investigated pre- and post-mating barriers to reproduction between two interfertile species, *Nicotiana alata* and *N. forgetiana*. Hawkmoths pollinate *N. alata* while primarily hummingbirds, but occasionally bees and small hawkmoths visit *N. forgetiana*. The species' ranges overlap and some metapopulations are parapatric. F₁ hybrids are easily produced in the greenhouse and although the species grow in similar habitats, hybrids have not been found in nature. Two experiments were conducted near an area of parapatry in Rio Grande do Sul, Brazil to examine reproductive isolation and hybrid fitness: 1) experimental sympatric plots containing both species and 2) plots of both species plus F₁ hybrids. In 1998 and 1999 in the mixed species plots, hawkmoths showed strong preference for *N. alata* but visited *N. forgetiana* on average 16.5% of the total visits. The hawkmoth visits to *N. forgetiana* were not due to a few individuals who 'majored' on *N. forgetiana*, rather all hawkmoths had similar preferences. Hummingbirds were not observed in our plots in 1998 but in 1999 only visited *N. forgetiana*. The plants in the mixed species plots produced an average (over four plots in two years) of 9.5% hybrid offspring, significantly less than the frequency of interspecific pollinator movements. Ninety-seven percent of the F₁ hybrids produced had *N. forgetiana* as their seed parent, suggesting an asymmetry in the post-pollination processes that may contribute to the incomplete species boundary. In plots with F₁ hybrids and both parental species, hawkmoths preferred *N. alata* in two of three plots while hummingbirds preferred *N. forgetiana* in all plots. F₁ hybrids competed well for pollinator visitation; receiving as many or more pollinator visits as the parental species in two of three plots. Hybrid plants were equally fit as the parent species in several life history traits; they

survived as well, received as many visits from pollinators and produced an equal number of flowers and at least as many fruit as the parent species. Pollinator discrimination among species is strong but is an imperfect pre-mating isolation mechanism. Pollinator discrimination among parental species is lower when F_1 hybrids are present. No post-mating hybridization barriers were detected.

Introduction

The genetic integrity and phylogenetic independence of closely related species depends on the strength of reproductive isolation between the species (Mayr 1963, Dobzhansky 1970). Genetic drift, natural selection or sexual selection can culminate in pre-mating or post-mating isolation mechanisms (Mayr 1963, Dobzhansky 1970, Coyne and Orr 1989, Coyne 1992, Rice and Hostert 1993). The evolution of barriers to hybridization can be facilitated by geographic isolation or by other factors that minimize gene flow between the incipient species. If pre-mating isolating mechanisms are effective, then little or no cost in reproductive potential is incurred when and where the incipient species come into contact. However, post-mating isolation mechanisms may be more costly to organisms if resources are expended on sterile or inferior hybrids (Howard 1993, Rice and Hostert 1993, Rundle and Schluter 1998). Hence, traits that augment pre-mating reproductive isolation should be strongly favored by selection (Dobzhansky 1970). Pre-mating isolation mechanisms need not evolve in direct response to selection against hybridization. Isolation mechanisms could arise as by-products of divergence achieved in allopatry, which subsequently confer isolation upon secondary contact.

Divergence of floral traits is thought to be one means by which pre-mating reproductive isolation is maintained or reinforced among closely related animal-pollinated plant species (Grant 1949, 1981, 1994; Levin and Watkins 1984; Vickery 1995; Schemske and Bradshaw 1999). Floral divergence may prevent matings either mechanically, by restricting access to certain pollinators or via differential pollen placement, or ethologically, due to the flowers' influence on the behavior of their pollinators (Grant 1994).

Evolution of premating hybridization barriers is not necessarily accompanied by evolution of postmating barriers (Galiana et al. 1996). Premating barriers are often incomplete so that some hybrids are produced (Dobzhansky 1951). In this scenario, the fate of the two incipient species depends on the relative fitness of the hybrid, i.e., on the strength of post-mating hybridization barriers. If hybrid fitness is relatively low, then individuals waste reproductive potential contributing to hybrid production and traits promoting premating isolation could diverge further. If hybrid fitness is relatively high, and barriers to gene flow are low, then the incipient species may be reamalgamated upon secondary contact. Or, the hybrids may occupy a new position on the adaptive landscape and, with sufficient ecological or genetic barriers to introgression, be maintained as a separate entity (e.g., Part III of Grant 1981; Rieseberg et al. 1995).

In this paper, we examine both premating and postmating isolating mechanisms in two florally divergent but interfertile species of wild tobacco, *Nicotiana alata* and *N. forgetiana*. First, we examine plant/pollinator interactions and their potential role in premating isolation. We also estimated the survivorship and fitness of F₁ hybrids in a natural setting, including the F₁ hybrids' ability to compete for pollinator services with the parental species. We initiated *in situ* experiments under nearly natural conditions to address two questions:

- 1) Do pollinators discriminate between different floral forms and hence contribute to reproductive isolation of *N. alata* and *N. forgetiana*?
- 2) Are F₁ hybrids less fit than pure parental types in a natural environment and can F₁ hybrids compete for pollinator services?

Materials and Methods

Experimental system

Nicotiana alata and *N. forgetiana* are ruderal species that grow in well-drained soil in rock outcrops, roadsides, and disturbed areas in southern Brasil. *Nicotiana alata* and *N. forgetiana* are well-supported members of *Nicotiana* Section *Alatae*, based on *nrITS* sequence data (Buckler et al., 1997; Ippolito, 2000). Morphological and crossability studies (Goodspeed 1954; Wunsch, Ippolito and Holtsford, unpublished data) suggest that *N. alata* and *N. forgetiana* are each others' closest relatives, although the *nrITS* data are ambiguous on this point (Ippolito, 2000). The species are at the same ploidy level ($n=9$) and vigorous fertile hybrids are easily produced in the greenhouse, but hybrid formation has not been documented in nature despite the proximity of some interspecific populations (Goodspeed 1954, Ippolito and Holtsford, pers. obs.). 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). We have seen *N. forgetiana* and *N. alata* individuals within 300m of each other but sympatric populations or putative hybrids have not been found.

The parent species and F_1 hybrids are self-incompatible so seed set is dependent on pollinator visitation. *Nicotiana alata* flowers are white, have a strong odor, and have a long narrow corolla tube (Table 1). *Nicotiana alata* is pollinated by several species of hawk moths (Sphingidae). *Nicotiana forgetiana* has red flowers that rarely have a detectable odor and a short floral tube. Several hummingbird species pollinate *N. forgetiana* and infrequently solitary bees and small hawkmoths visit also. Generally,

flowers of *N. forgetiana* close after sunrise about 1 hour later than flowers of *N. alata* and open in the late afternoon about an hour before the flowers of *N. alata*. However, on cool days, flowers of both species will remain open for longer periods of time. F₁ hybrids between the species are fertile and vigorous, and produce vigorous F₂ hybrids and backcrosses. F₁ hybrids have intermediate floral trait values in traits which are associated with hawkmoth and hummingbird pollination syndromes, and traits that may effect transfer of pollen, except for anther-stigma distance in which the value is smaller than either parent (Table 1).

Study site

Experimental plots were set up at a field station maintained by the Conselho de Desenvolvimento Comunitario de Campestre (CDCC) approximately 51° 06'47.3"W; 28° 05'13.8"S and at a nearby private farm. Both sites are about 10 km from the place where occasional individuals of both species have been found within 300m of each other. The vegetation surrounding CDCC is similar to that in nearby natural populations: roadside weeds, pastures, and second-growth *Aurecaria* forest. We germinated parental and F₁ hybrid seeds in speedling flats in a polyhouse. One hundred twenty seedlings of each species and F₁ hybrids were transplanted into 1-liter bags when rosettes were c. 5 cm in diameter. When seedlings began to produce inflorescence shoots, we transplanted seedlings into the ground at CDCC or the farm. Plants were watered sparingly, as not to lose individuals to a severe drought.

Reproductive isolation experiment

To investigate whether *N. alata* and *N. forgetiana* attract different pollinator types, we transplanted 20 plants of each species in each of two common garden plots in two consecutive years, 1998 and 1999. Plants that died after transplanting were not replaced. The plots were approximately 300 m from one another to minimize inter-plot gene flow. The arrangement of plants within plots was randomized and interplant distances mimicked those of nearby natural populations, between 30cm – 1m apart. 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. We recorded, on micro-cassette recorders, the identity of each plant visited and the number of flowers visited per plant for all observed pollinator foraging bouts.

To determine whether pollinators in the experimental plots moved randomly with respect to species, we compared the observed number of all pairwise plant-plant movements with two kinds of expected transition frequencies. The first expectation is that pollinators have no preferences and so plant-plant movements would be determined by the frequencies of *N. alata* and *N. forgetiana*. If the frequency of *N. alata* = $plfreq(a)$ and the frequency of *N. forgetiana* = $plfreq(f)$, then the expected pairwise transitions for a-a, a-f, f-a and f-f are $plfreq(a)^2$, $plfreq(a)plfreq(f)$, $plfreq(f)plfreq(a)$, and $plfreq(f)^2$, respectively (Table 2). We refer to the first expectation as “plantfreq”. Deviations from the plantfreq expectation in the direction of more homotypic movements can be

interpreted as evidence for pollinator preference. The second expectation for plant-to-plant movements was based on the overall single-plant visitation frequency of each pollinator type to *N. alata* or *N. forgetiana*, hereafter termed “visitfreq” (following Stanton et al. 1989). If the frequency of visits to *N. alata* = $visfreq(a)$ and the frequency of visits to *N. forgetiana* = $visfreq(f)$, then the expected pairwise transitions for a-a, a-f, f-a and f-f = $(visfreq(a))^2$, $visfreq(a)visfreq(f)$, $visfreq(f)visfreq(a)$, $(visfreq(f))^2$, respectively. If pollinator movements between plants conform to the visitfreq expectation, then their plant-to-plant movements can be viewed as a simple extension of their overall preference for floral types. If pollinators had made more homotypic plant-to-plant movements than expected based on their overall preferences, then that would be evidence of floral constancy, or the tendency of individuals to major on one floral type.

To estimate the frequency of hybridization in the experimental plots, we collected all fruit produced and pooled the seeds from each plant. For each plant, 20 seeds were randomly drawn, sown, and grown to flower. Because F_1 hybrids are intermediate in floral traits (Table 1) including color, 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 get a per-plot estimate of hybridization frequency.

F₁ Hybrid Fitness Estimation

We estimated hybrid fitness in common garden plots in Brasil by comparing hybrid survival at various life history stages and two components of reproduction relative to the parental species (Table 3). We recorded seed germination and survivorship in the CDCC polyhouse, and survivorship after transplanting into three field plots. We

transplanted 15 plants of each type (two spp. & F₁ hybrids) into three randomized common garden plots in 1999. Plants that died were not replaced. The plots were located in habitat similar to that of the roadside/fallow fields in which *N. alata* and *N. forgetiana* are commonly found. Our experiments were designed to test speciation scenarios in the habitats where hybridization is most likely to occur, along roadsides and in disturbed agricultural settings. The soils at these sites are more acidic and less well drained than the natural rock outcrop habitats (D. A. Tiscornia, pers. comm.). Spacing within blocks mimicked the density of natural populations, as in experiment 1.

We measured two fecundity variables: the number of open flowers on each plant on one day approximately ten days into peak flowering and the total number of fruit produced at the end of the flowering season. Cumulative fitness estimates were calculated for the two fecundity variables by multiplying survivorship (0 or 1) by the fecundity variable. Because distributions of fitness estimates were non-normal, we tested for fitness differences among groups with the nonparametric Kruskal-Wallis Test.

We observed pollinators during the time that flowers were open on at least three days per plot. To determine whether pollinators in the experimental plots moved randomly with respect to either species or F₁ hybrid, we compared the observed number of all pairwise plant-plant transitions with expectations based on plant type frequency, “plantfreq”, and overall single-plant visit frequency per pollinator, “visitfreq”, as in experiment 1.

Results

Pollinator preference in the mixed parental plots

Two species of hawkmoths were common in the plots, *Agrius cingulata* and *Eumorphia labruscae*. The hawkmoths visited *N. alata* significantly more than *N. forgetiana* in both years (Fig. 1). For hawkmoth visitation, in the Asp, Rasp, Down, and House plots, respectively, N = 212 plant visits observed, $X^2_{df=1}=16.1$, $p < .0001$; N=489, $X^2=76.2$, $p < .0001$; N=58, $X^2=62.8$, $p < .0001$; and N=120, $X^2=102$, $p < .0001$. On average for all plots, large hawkmoths visited *N. forgetiana* 16.5% of the total number of visits. In 1998, we did not observe any hummingbirds visiting experimental plots. In 1999, the Glittering Bellied Emerald hummingbird, *Chlorostibulon aureoventris*, was the most common hummingbird in the plots but several other unidentified species were also observed. The hummingbirds were only observed visiting *N. forgetiana* (Fig. 1b). For hummingbird visitation, in the Down and House plots respectively, N= 11, $X^2_{adj}=9.17$, $p < .01$ and N=39, $X^2=45.5$, $p < .0001$. In the Down plot in 1999, we infrequently (two bouts) observed one species of small hawkmoth (*Callionima nomius*) that visited *N. forgetiana* more than *N. alata*; 26 of 28 plants visited in two foraging bouts (Fig. 1). The small hawkmoth significantly preferred *N. forgetiana*: $X^2= 16.58$, $p < .0001$.

While pollinators showed detectable overall *visitation* preferences (all single-plant visits considered together (Fig. 1), we wished to determine whether their plant-to-plant *movements* were also biased. The number of plant-plant transitions observed was not always equal to two less than the number of plants visited because we occasionally lost track of a pollinator during a plant-plant movement. We compared the observed number of all pairwise plant-plant transitions with expectations based on plant species' frequency in each plot, *plantfreq*, and overall visitation frequency to each plant species, *visitfreq*. Hypotheses based on *plantfreq* expectations were rejected in all seven tests (done

separately for each pollinator type in each of four plots, Table 2), with all movement patterns deviating in the direction of more homotypic and fewer heterotypic plant-to-plant transitions. This result shows pollinators have preferences not only for which plants they visit (Fig. 1), but that these preferences also affect their plant-to-plant movements (Table 2). Further, in six of seven tests, the visitfreq expectations were not significantly different from the pollinators' observed plant-to-plant transitions (Table 2). This suggests that plant-to-plant transitions were a simple extension of the pollinators' overall visitation biases, and that different individual pollinators were not 'majoring' on different plant species. The one exception to this finding was that, even though they had significant preference for visiting *N. alata* (plantfreq hypothesis rejected), the hawkmoth movements in the Rasp plot had more heterotypic moves and fewer homotypic moves than their visitation frequencies alone would suggested (visitfreq hypothesis also rejected).

In 1999, hummingbirds only visited *N. forgetiana* in all plots and one small hawkmoth, *Callionima nomius* visited *N. forgetiana* 26 of 28 visits in two bouts. Due to the low number of observations, we could not statistically analyze the hummingbird or small hawkmoth observation data. Fisher's exact test is only applicable when expectations are drawn from the contingency tables themselves (vs. all other tables with the same fixed marginal totals), not external hypotheses. Pooling categories did not increase our expected values sufficiently; many expectations were zero or one. This low sample size was less of a problem in the overall preference analysis (Fig. 1) because the observations were only split into two species categories, rather than four transition

categories. Nevertheless, the data, though limited, are nearly unwavering in their support for the conclusion that both hummingbirds and the small hawkmoth *Callionima nomius* prefer *N. forgetiana* (Table 2).

Production of Hybrid Offspring in Mixed Parental Plots

The average percent of hybrids produced from seed gathered from all four plots was 9.5% (Fig. 2a). For each plot in both years, the rate of F₁ hybrid production was significantly less than rate of interspecific movements that pollinators made in the field (Fig. 2a, $X^2_{df=2}$ and p values for the Asp, Rasp, Down, and House plots are 22.59, p <.0001; 233, p <<<.0001; 103.7, p <<<.0001; and 14.1, p <.001, respectively).

Overwhelmingly, *N. forgetiana* was the seed parent of almost all hybrids produced, 97%, versus 3% for *N. alata*. Therefore, we tested the observed number of hybrids produced to the expectations based on pollinator movements separately for each species. The expected frequency of hybrids mothered by *N. alata* was the ratio of the number of pollinator movements from *N. forgetiana* to *N. alata* to the total number of movements (intra- and interspecific) to *N. alata* plants (Table 2, Fig. 2b). The expected frequency of hybrids mothered by *N. forgetiana* was the ratio of the number of pollinator movements made from *N. alata* to *N. forgetiana* to the total number of movements (intra- and interspecific) to *N. forgetiana* plants (Table 2, Fig. 2c). In all plots, *N. alata* produced significantly fewer F₁ hybrids than expected (Fig. 2b, $X^2_{df=1}$ and p values in the Asp, Rasp, Down & House plots respectively are 17.1, p < .0001, 35.2, p << .0001, 28.4, p << .0001, 20.3, p << .0001). *Nicotiana forgetiana* seed parents were less discriminating than *N. alata* seed parents. Only in the Rasp plot was the number of

hybrid offspring produced significantly less than the expectation based on the proportion of *N. alata* to *N. forgetiana* movements ($X^2_{df=1}=185.6$, $p \ll .0001$). Hybrids were produced at approximately the same rate as *N. alata* to *N. forgetiana* movements in the Asp plot ($X^2_{df=1}=2.18$, $p = 0.14$ [a small number of seed germinated from these plants, $N=10$, which compromised the power of this test]). Hybrid production in the Down and House plots tended to be *more* frequent than pollinator movements suggested (Fig. 2c, $X^2_{df=1}$ and p values in the Down & House plots respectively are 5.76, $p = 0.02$; 3.79, $p = .051$).

Hybrid Vigor

F_1 hybrid plants are as vigorous as both parental species at several life history stages (Data and statistics are in Table 3). The overall cumulative survival of the F_1 in each of three plots was not significantly different from that of either species, although hybrids tended to survive better than *N. forgetiana* (D in Table 3). F_1 hybrids had a higher germination rate and equivalent survival rate after transplantation into 1-liter plastic bags in the CDCC polyhouse. The survival of F_1 hybrids after transplanting into the field was lower than that of either parental species in the Hill and Mitten plots but higher than *N. forgetiana* in the Lixo plot. F_1 hybrids were as fit as the parental species for both fitness estimates —using the average number of flowers on one day during peak flowering or the total number of fruit produced as fecundity estimates. In the Lixo plot, the fitness estimate for the F_1 hybrid with flower number as the fecundity variable was marginally greater than the estimate for *N. forgetiana* (Table 3). All plants produced

normal anthers that dehisced full compliments of pollen, which appeared viable although pollen viability was not tested *per se*.

Pollinator preference in plots with F₁ hybrids and both parental species

To determine whether pollinators visited plants randomly with respect to species or F₁ hybrid, we used a goodness of fit test to compare the observed number of visits to each plant type to the expectation that plants would be visited in proportion to their frequency in each plot. In all cases, this hypothesis was rejected, indicating that pollinators have preferences as to which type of flowers they visit (in the Lixo, Mitten, and Hill plots, respectively, Hawkmoths: $X^2_{df=2}=36.5, 78.1, 112$, Hummingbirds: $X^2_{df=2}=44.3, 99.9, 15.8$, all $p << 0.001$). Large hawkmoths over-visited *N. alata*, under-visited *N. forgetiana*, and visited the F₁ hybrid in approximate proportion to its presence in the Hill and Mitten plots (Fig. 3). In the Lixo plot, large hawkmoths visited *N. alata* at the expected rate, over-visited the F₁ hybrids and eschewed *N. forgetiana* plants. Hummingbirds favored *N. forgetiana* and avoided *N. alata* in all plots. Hummingbirds visited the F₁s in the Hill plot in approximate proportion to their presence, but under-visited the F₁s in the other two plots (Fig. 3).

We also pooled visits of both types of pollinators to get an idea of the hybrids' overall attractiveness to pollinators (Fig. 4). Testing the observed visitation frequencies against the expectation that plant types would be visited in proportion to their presence in the plots, as above, pollinator preferences were again evident ($X^2_{df=2}=79.97, p << .0001$; 16.15, $p < 0.001$; 15.14, $p < 0.001$, for the Hill, Mitten, and Lixo plots respectively). *Nicotiana alata* was visited more than expected in the Hill and Mitten plots and at the expected frequency in the Lixo plot (Fig. 4). *N. forgetiana* was visited less than expected

in all replicates (Fig.4). The F₁ hybrid was visited more than expected in the Hill and Lixo plots but slightly less than expected in the Mitten plot (Fig. 4). So while F₁ hybrids were usually not the favored plant type of either pollinator (Fig. 3), their overall visitation rate was near or above their proportionate representation in the plots (Fig. 4).

The analyses above consider overall visitation rates to each plant type. To examine the plant-to-plant movements, we generated transition expectations using the frequencies of each plant type, “plantfreq” (e.g., $\exp[\text{alata-to-alata moves}] = \text{freq}[\text{alata plants}]^2$), and the overall visit frequency to each plant type, “visitfreq” (e.g., $\exp[\text{alata-to-alata moves}] = \text{freq}[\text{alata visits}]^2$). In all plots, for all pollinators, the plantfreq expectations were rejected, as they were in the overall visitation analyses above, demonstrating pollinator preferences (Table 4). But were plant-to-plant movements a simple extension of pollinators’ overall preferences or was there evidence for floral constancy? For hawkmoth movements in the Mitten plot, homotypic moves were over-represented, (*alata-to-alata* and *forgetiana-to-forgetiana*), and heterotypic (*alata-forgetiana*) moves were under-represented, relative to visitfreq expectations, suggesting a tendency toward floral constancy by individual pollinators. Hawkmoth movements in the Hill plot also showed a slight bias toward more homotypic (in this case, hybrid-hybrid) transitions, and a small deficit of several classes of heterotypic moves, although the visitfreq expectation could not be rejected ($p=0.07$, Table 4). In the Lixo plot, the visitfreq expectation for hawkmoth movements was not rejected ($p=0.51$), indicating that individual hawkmoths were not majoring on particular plant types, i.e. their overall single-visit preferences were adequate predictors of their plant-to-plant movements.

Hummingbird movement observations were too infrequent to be analyzed rigorously since they had to be split among eight transitions categories, i.e. 8 cells in the contingency tables. However, the hummingbirds' plant-to-plant transition frequencies were very similar to the visitfreq expectations, suggesting that individual hummingbirds were not constant (sensu Stanton et al., 1989), but made their plant-to-plant movements according to their overall preferences, not the identity of the last plant type they visited.

Discussion

Our experimental sympatry plots were meant to assess the role of pollinator preferences in pre-reproductive isolation. Overwhelmingly, pollinators showed floral preferences that were consistent with the plant species' presumed pollination syndromes (Fig 1). 83.5% of all observed visits by large hawkmoths were to *N. alata* and over 99% of visits by hummingbirds and a small hawkmoth (*Callionima denticulata*) were to *N. forgetiana*.

Large hawkmoths are capable of retrieving nectar from either *N. alata* or *N. forgetiana*. However, large hawkmoths may prefer *N. alata* flowers for several reasons. Large hawkmoths may not be able to efficiently manipulate the shorter flowers of *N. forgetiana*. When hawkmoths visit *N. alata*, their probosci are almost fully inserted with their heads roughly 1–2 cm outside of the opening of the flower. While feeding at a flower, hawkmoths steady their hovering flight by placing their front two legs on the lower limb of the flower (Ippolito and Holtsford, pers. obs.). When feeding on *N. forgetiana*, hawkmoths remain roughly 5–7 cm from the flower opening and so are too far from the flower limbs to steady their flight with their legs — their bodies move from side

to side and up and down. The seemingly more awkward feeding at the shorter-tubed flowers may be uncomfortable and more energetically demanding. Hawkmoths may also find flowers of *N. alata* more attractive because *N. alata* flowers are scented while *N. forgetiana* only rarely have a faint scent at dusk. However, scent is probably a long-range cue used to locate patches of flowers rather than choose flowers within a patch (R. Raguso, pers.com.). Flowers of *N. alata* contain more nectar than *N. forgetiana* (standing crop range of 50-90 μ l vs. 5-20 μ l, respectively). The difference in standing crop may be due to nectar production rate or differential depletion of nectar by hummingbirds. We are currently gathering data on these issues, as well as sugar concentration and other nectar constituents.

The floral tube length of *N. alata* exceeds bill and tongue length of hummingbirds, hence, hummingbirds are probably incapable of retrieving a nectar reward from most *N. alata* flowers. Flowers of *N. forgetiana* are red and although hummingbirds do not have a visual acuity for red (Stiles 1976), they can quickly learn to associate various colors with nectar rewards (Goldsmith and Goldsmith 1979, Meléndez-Ackerman et al. 1997). We have observed the same species of hummingbirds as in our experimental plots visiting the rewarding white flowers in nearby populations of *N. mutabilis* while avoiding the older, non-rewarding, pink and red flowers (Ippolito and Holtsford, pers obs., Stehmann et al., in prep.). Infrequently (2 bouts), we observed a small hawkmoth species (*Callionima denticulata*) in one plot that primarily visited *N. forgetiana* (26 of 28 plants visited). In three seasons, *Callionima denticulata* have not been observed in natural populations of *N. alata* or *N. forgetiana*. Because flowers of

N. forgetiana rarely produce a detectable odor, the small hawkmoth may have been drawn into the plot by the strong odor of *N. alata* flowers and being restricted from the nectar reward of *N. alata* flowers, may have taken advantage of *N. forgetiana* flowers. Similar to hummingbirds, probosci lengths of the small hawkmoth is shorter than most floral tubes of *N. alata* so the nectar reward is inaccessible. Infrequently, the small hawkmoths may be able to retrieve a nectar reward from some shorter-tubed individuals of *N. alata*. While hummingbirds and the small hawkmoth species may be mechanically restricted from visiting *N. alata* flowers, large hawkmoths may learn which species is most rewarding.

Although large hawkmoths strongly preferred flowers of *N. alata* (Fig. 1), interspecific movements accounted for 29.3% on average of the total plant-to-plant movements in the mixed parental 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. Hybrid production, then, depends on the tendency of large hawkmoths to move interspecifically, the efficacy of pollen transfer, and post-pollination processes. F₁ hybrid plants accounted for 9.5% of all offspring from the experimental sympatry plots, significantly less than the 29.3% rate of interspecific movements (Fig 2a). Several barriers may diminish the opportunity for these species to hybridize. Pollen from each species might be placed on different parts of the probosci of large hawkmoths. For example, since the floral tubes of *N. forgetiana* are 3-4 cm shorter than tubes of *N. alata*, we might expect to find more *N. forgetiana* pollen toward the tip of the proboscis and more *N. alata* pollen toward the head. We examined several probosci of large hawkmoths and did not find a distinct pattern of pollen placement.

Probosci were evenly or haphazardly covered with pollen. This is not surprising because hawkmoths recoil their probosci completely between flower visits, such that, each segment of the proboscis can contact other sections. Although different segments of the proboscis may carry varying densities of heterospecific pollen, we could not detect a difference because pollen of these species is indistinguishable.

Post-pollination processes may also contribute to the lower 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 grow at a slower rate. Studies in several genera confirm that conspecific pollen can have an advantage over heterospecific pollen when applied in equal amounts (Arnold et al. 1993, Carney et al. 1994, Reisberg et al. 1995, Hauser et al. 1997). We have begun pollen competition experiments between *N. alata* and *N. forgetiana* using 50% conspecific and 50% heterospecific pollen. In glasshouse conditions, pooling crosses in both directions, hybrids constitute approximately 31% of offspring, significantly less than the expected rate of 50% ($n=125$ crosses, $G_{df=1}=10.91$, $p<0.001$, Wunsch, Ippolito & Holtsford), suggesting an important role for post-pollination processes in the hybridization dynamics of *Nicotiana*.

Nicotiana forgetiana was the seed parent of the vast majority of F_1 hybrid seed (97%, Fig. 2). Several explanations can account for this finding. Because large hawkmoths account for almost all of the interspecific movements and 16.5% of their total visits were to *N. forgetiana* plants, pollen loads probably overwhelmingly consisted of *N. alata* pollen. In addition, 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*. Lastly, differences in the ability of pollen to grow through the longer style of *N. alata* and fertilize ovules may also account for our finding that *N. forgetiana* plants produce more F₁ hybrids. Our preliminary data suggest that while *N. forgetiana* pollen is able to grow the entire length of an *N. alata* style, its growth rate to the base of the *N. alata* style is ~11% slower than *N. alata* pollen tubes growing in an *N. alata* style (at 42 hours post-pollination, tube lengths are 45.6 versus 51.2 mm, $t_{df=57} = 4.63$, $p < 0.001$, Wunsch, Ippolito & Holtsford). However, *Nicotiana alata* pollen tubes grow approximately as quickly to the base of an *N. forgetiana* style as do *N. forgetiana* pollen tubes (at 31 hours post-pollination the tube lengths are 27.3 mm versus 27.8 mm, $t_{df=58} = 0.76$, $p=0.45$)

Post-reproductive barriers to hybridization

Although pollinator discrimination and post-pollination processes may reduce hybridization, almost 9.5% of offspring produced in the mixed parental plots were F₁ hybrids. Once hybrids are produced, are there any post-reproductive barriers that would diminish hybrid survival and reproduction? Our data show that at several life history stages, F₁ hybrids have greater or equal survival than either of the parent species (Table 3). F₁ hybrids also have equivalent cumulative estimated fitness as either parental species. Our fitness estimates considered only two components of female reproduction. More detailed fitness estimates, including those related to male function, would be desirable. However, it seems unlikely that F₁s suffer greatly due to their hybrid heritage.

The conditions in the polyhouse, where seeds were germinated and plants were maintained before transplanting into the field, were protected and may partially account

for the high germination rate and survival of all plants. The field conditions in which we transplanted the F₁ hybrids and parent species were very similar to the fallow field and roadside habitats where wild *Nicotiana* are commonly found. These habitats are probably more benign than the rock outcrop and slide areas where wild *Nicotiana* are also found. Under different conditions, perhaps F₁ hybrids would not perform as well as they did in these experiments. We are currently examining the effects of crowding and soil nutrients on relative hybrid fitness. However, these species are spreading along roadsides and in field margins and so our experiments were aimed at simulating the environment where hybridization may be most likely. If F₁ hybrids were formed under these conditions then we might expect them to become established.

In nature, F₁ hybrids must compete with parental species for pollinator services. Since the floral traits of F₁ hybrids are intermediate between *N. alata* and *N. forgetiana* (Table 1), pollinators may discriminate against the new floral morphology or a novel pollinator type that does not visit either parent species may be “captured” (Grant 1981). We only observed large hawkmoths and hummingbirds coming to the parental species/F₁ plots. In two of three plots, large hawkmoths preferred *N. alata* over hybrid or *N. forgetiana* plants (Fig. 3). In the Lixo plot, large hawkmoths did not discriminate between *N. alata* and the F₁ hybrid. In all plots, hummingbirds preferred *N. forgetiana* over F₁ hybrids or plants of *N. alata*. If we sum the total number of visits regardless of pollinator type (Fig. 4), the F₁ hybrid plants received the fewest visits (22%) in the Mitten plot relative to either species (*N. alata* 42% and *N. forgetiana* 36%). In the Lixo plot, F₁ hybrids received more visits (44%) than either parental species (*N. alata* 39.5% and *N.*

forgetiana 16.5%). In the Hill plot, F₁ hybrids received 30% of the visits which was intermediate between the two species (*N. alata* 53% and *N. forgetiana* 17%). In sum, preferences for parental species are diminished when F₁ hybrids are present and hybrids are not consistently discriminated against, nor do they attract novel pollinators. Louisiana *Iris* hybrids also do not suffer from low rates of pollinator visitation (Wesselingh & Arnold, 2000).

Fruit production was not simply related to pollinator visitation rate. Over all plots, hybrid plants produced as many flowers and fruit on average as either species (Table 3). Only in the Lixo plot did F₁ hybrids produce less fruit per plant. Interestingly, F₁ hybrids received the largest share of the pollinator visits in that plot. Conversely, in the Mitten plot, F₁ hybrids received the fewest pollinator visits but produced more fruit than either parental species. In the Hill plot, where visits to F₁ hybrids were intermediate between the parental species, F₁ hybrids produced nearly two times more fruit on average than *N. forgetiana* or *N. alata* (73.5 versus 36.8, and 39.8 fruit/plant, respectively). The lack of correspondence between pollinator visitation and fruit set suggests that pollination was not limiting fruit set in these plots.

In summary, pre-reproductive barriers to hybridization are fairly strong but incomplete, and post-reproductive barriers are weak or absent, at least in our nearly-natural experimental conditions. Pollinators have strong but not exclusive preferences for the parental species when they are intermixed in experimental sympatry plots. We have never found plants of these two species closer than 300m from each other, but that distance by itself would not present much of a barrier to hawkmoths or hummingbirds, which are strong fliers. Unfortunately, the distributions of *N. alata* and *N. forgetiana* are

incompletely known (Goodspeed 1954; Daniel Falkenberg, pers.comm.) but they overlap broadly in the Serra Gaucha in the states of Santa Catarina and Rio Grande do Sul. It may be that these species are just now coming into contact with each other as they spread along roadsides and field margins, while their more natural rock outcrop and streamside populations occur in separate canyons. Since pollinator discrimination and post-pollination barriers to hybridization are incomplete, and hybrids are viable and fertile, we may soon witness more widespread hybridization. Hybrids could persist in habitats that are intermediate between those of their parents or in ruderal habitats (Hewitt 1989). Barriers to gene flow such as polyploidy (Grant 1981; Franzke and Mummenhoff 1999), chromosomal rearrangements (Rieseberg et al. 1995; Ungerer et al. 1998), asexual reproduction (Bullini 1994, Emms and Arnold 1997), or the exploitation of a novel pollinator could all contribute to the persistence of hybrids. Whether hybridization between *Nicotiana alata* and *N. forgetiana* will proceed will likely depend on their dispersal capabilities and the availability of colonizable/disturbed habitats. Whether hybridization would lead to the amalgamation of the species or the production of a new hybrid species would depend on the strength of any barriers to gene flow, and the relative fitness of hybrids and the parental species. Our work thus far suggests that introgression is a likely outcome.

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Table 1. Mean (in cm) floral trait values (se) for *N. alata*, *N. forgetiana* and their F₁ hybrid.

trait	<i>N. alata</i>	F1 hybrid	<i>N. forgetiana</i>
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)

Table 2. Pollinator transition frequencies between plants of *N. alata* (a) and *N. forgetiana* (f) in experimental plots in Brazil. Expected frequencies (see Methods) have been rounded to whole numbers for presentation, although analyses were done to four decimal places. Degrees of freedom = 1 for all tests; contingency tables were 2x 2 but are shown as 4 x 1. Where expected frequencies are too small for analysis, (x=unsupported) denotes that the model is supported, (x=unsupported) by visual inspection.

plot	pollinator	obs/expt	transition				X^2	p
			a-a	a-f	f-a	f-f		
Asp								
	<u>hawkmoths</u>							
	observed		118	27	27	7		
	expected (visitfreq)		115	29	29	1	0.44	= 0.51
	expected (plantfreq)		98	34	34	13	10.2	= 0.001
Rasp								
	<u>hawkmoths</u>							
	observed		264	65	65	4		
	expected (visitfreq)		283	52	52	10	9.38	= 0.002
	expected (plantfreq)		207	80	80	31	45.6	<< 0.0001
Down								
	<u>hawkmoths</u>							
	observed		38	6	6	3		
	expected (visitfreq)		37	7	7	1	2.59	= 0.11
	expected (plantfreq)		11	13	13	15	83.4	<< 0.0001
	<u>small hawkmoth</u>							
	observed		1	1	1	24		
	expected (visitfreq)		0	1	1	25		
	expected (plantfreq)		6	7	7	8	x	

Table 2 continued.

hummingbirds

observed	0	0	0	9		
expected (visitfreq)	0	0	0	9		
expected (plantfreq)	2	2	2	3	x	

House

hawkmoths

observed	92	15	15	0		
expected (visitfreq)	94	14	14	1	2.70	= 0.10
expected (plantfreq)	35	31	31	25	134	<< 0.0001

hummingbirds

observed	0	0	0	32		
expected (visitfreq)	0	0	0	32		
expected (plantfreq)	9	8	8	7	x	

Table 3. Performance estimates of *N. alata*, *N. forgetiana* and their F₁ hybrid. Plants were split into three plots upon transplantation into the field — data entries below row C are therefore split into nine columns: 3 plant types in each of 3 plots with separate tests for each plot. Cumulative survival was calculated by multiplying survival at previous stages, i.e. D = A x B x C. The number of flowers (E) were counted on one day approximately 10 days into peak flowering season. Total number of fruit (F) was counted at the end of the season. Fitness estimates were calculated by multiplying the cumulative survival by either fecundity variable, (D)(E) and (D)(F), and because of their non-normal distributions, were compared using X^2 approximations from the nonparametric Kruskal-Wallis Test (PROC NPAR1WAY of SAS). Other X^2 tests are standard (PROC FREQ).

	<i>N. alata</i>	F1	<i>N. forgetiana</i>			
in the polyhouse						
A. germination	72%	87%	59%			
	$X^2_{df=2}=13.3; p<0.001$					
B. survivorship	95%	97%	92%			
	$X^2_{df=2}=1.47; p=0.481$					
in the field						
C. survivorship						
by plot:	hill		mitten		lixo	
species or F1	<i>alata</i>	F1 <i>forg.</i>	<i>alata</i>	F1 <i>forg.</i>	<i>alata</i>	F1 <i>forg.</i>
	100%	73% 100%	100%	60% 87%	100%	80% 73%
	$X^2_{df=2}=11.25; p<0.004$		$X^2_{df=2}=10.49; p<0.005$		$X^2_{df=2}=10.83; p<0.004$	

(Table 3 continued)

D. cumulative survival	0.68	0.62	0.54	0.68	0.51	0.47	0.68	0.68	0.40
(A)(B)(C)	$X^2_{df=2}=0.91; p=0.634$			$X^2_{df=2}=2.5; p=0.28$			$X^2_{df=2}=5.5; p=0.064$		

fecundity variables:

	<i>alata</i>	F1	<i>forg.</i>	<i>alata</i>	F1	<i>forg.</i>	<i>alata</i>	F1	<i>forg.</i>
E. No. flowers/plant	12.1	14.8	9.9	13.5	10.1	16.3	12.1	14.9	11.8
(standard error)	(1.2)	(2.9)	(2.0)	(2.8)	(2.7)	(3.5)	(2.1)	(3.3)	(5.1)
	$X^2_{df=2}=3.40; p=0.182$			$X^2_{df=2}=2.22; p=0.330$			$X^2_{df=2}=1.21; p=0.545$		
F. ave. no. fruit/plant	39.8	73.5	36.8	33.1	45.9	45.2	42.3	39.2	49.5
(standard error)	(6.2)	(8.2)	(7.9)	(4.8)	(8.5)	(8.4)	(10.4)	(7.5)	(11.0)
	$X^2_{df=2}=10.4; p<0.006$			$X^2_{df=2}=2.41; p=0.30$			$X^2_{df=2}=2.31; p=0.891$		

fitness estimates:

	<i>alata</i>	F1	<i>forg.</i>	<i>alata</i>	F1	<i>forg.</i>	<i>alata</i>	F1	<i>forg.</i>
by flowers (D)(E)	8.05	8.43	5.15	8.52	4.76	7.31	8.43	9.94	4.61
	$X^2_{df=2}=3.97; p = 0.279$			$X^2_{df=2}=1.67; p=0.435$			$X^2_{df=2}=5.81; p=0.055$		
by fruit (D)(F)	26.5	45.9	19.7	22.5	22.9	21.1	29.4	26.8	18.0
	$X^2_{df=2}= 3.97; p =0.138$			$X^2_{df=2}= 0.66; p=0.717$			$X^2_{df=2}= 4.24; p=0.120$		

Table 4. Pollinator transition frequencies between *N. alata* (a), *N. forgetiana* (f) and their F₁ hybrid (h) in experimental plots in Brazil. Expected frequencies (see Methods) have been rounded to whole numbers for presentation, although analyses were done to four decimal places. Degrees of freedom = 4 for all X² tests; tables were 3 x 3, but are presented as 8 x 1. χ^2 denotes that the model is supported, (x=unsupported) by visual inspection, but expected frequencies are too small for analysis.

plot	transition									X ²	p
pollinator											
hill											
<u>hawkmoth</u>	a-a	a-h	a-f	h-a	h-h	h-f	f-a	f-h	f-f		
observed	118	66	32	65	42	8	29	8	7		
expected (visitfreq)	123	65	26	65	34	14	26	14	5	8.73	= 0.07
expected (plantfreq)	50	37	50	37	27	37	50	37	50	242	<< 0.001
<u>hummingbird</u>											
observed	1	1	1	1	6	5	1	6	15		
expected (visitfreq)	0	1	2	1	4	7	2	7	14		
expected (plantfreq)	5	4	5	4	3	4	5	4	5	x	
mitten											
<u>hawkmoth</u>											
observed	141	45	25	45	15	11	25	10	17		
expected (visitfreq)	133	45	33	45	15	11	33	11	8	14.2	= 0.006
expected (plantfreq)	58	31	50	31	16	27	50	27	44	11.3	= .02
<u>hummingbird</u>											
observed	0	0	1	1	0	6	0	6	55		
expected (visitfreq)	0	0	1	0	1	6	1	6	54		
expected (plantfreq)	12	6	10	6	3	6	10	6	9	x	

Table 4 continued

lixo

hawkmoth

observed	35	29	6	32	43	7	6	6	0		
expected (visitfreq)	32	35	6	35	38	6	6	6	1	3.32	= 0.506
expected (plantfreq)	27	22	18	22	17	14	18	14	12	85.1	<<<0.01

hummingbird

observed	0	0	0	0	0	1	0	1	14		
expected (visitfreq)	0	0	0	0	0	1	0	1	14		
expected (plantfreq)	3	2	2	2	2	1	2	1	1	x	

Figure 1. Frequency of pollinator visitation to *N. alata* or *N. forgetiana* in experimental sympatry plots. Black bars indicate visits by large hawkmoths, white bars indicate hummingbird visits, and gray bars indicate small hawkmoth visits. The lines indicate the percentages of *N. alata* (%*N.a.*) and *N. forgetiana* (%*N.f.*) plants in the plots. Pollinator preferences were tested ($\chi^2_{df=1}$) by comparing observed visitation frequencies to the expectation that plant species would be visited in proportion to their presence in the plots. All comparisons were significant at the $p < 0.01$ or $p < 0.001$ level, see Results.

Figure 2 Observed (white bars) and expected (black bars) percentage of hybrids produced by plants grown in experimental sympatry plots. A) The expectation was estimated as the fraction of interspecific plant-plant movements by all pollinators, regardless of the direction of the movement. For panels B& C, hybridization expectation was estimated as number of interspecific pollinator movements to *N. alata* (B) or to *N. forgetiana* (C). Numbers of plant-to-plant movements observed are in Table 2. Numbers of offspring grown to estimate the rates of hybrid production are 93 (83 from *N. alata* mothers + 10 from *N. forgetiana*), 185 (146+39), 210 (161 + 49), and 399 (291 +108) for the Asp, Rasp, Down, & House plots, respectively. Levels of statistical significance (χ^2 tests, see Methods) are abbreviated as NS—not significant, * — $p < 0.05$, *** — $p < 0.001$.

Figure 3. Percentage of pollinator visits (bars) to *N. alata*, *N. forgetiana* or their F_1 hybrid in mixed plots. Lines indicate the percentage of each type of plant in each plot, i.e., the expected pollinator visitation rate if pollinators had no preference. Black bars indicate large hawkmoths and white bars indicate hummingbirds. Observed visitation

rates (bars) were significantly different (by $X^2_{df=2}$) from expected rates (lines) in all cases at the $p < 0.001$ level, see Results.

Figure 4. Percentage of all pollinator visits, (pooled over all pollinator types), received by *N. alata* (*N.a.*), *N. forgetiana* (*N.f.*) or their F_1 hybrid in mixed plots. Black bars indicate pollinator visits to *N. alata*, white bars indicate pollinator visits to *N. forgetiana* and gray bars indicate pollinator visits to F_1 hybrids. The dashed lines indicate the expected visitation frequency, estimated from the percentage of each species or F_1 hybrid in each plot. Observed visitation rates (bars) were significantly different from expected rates (dashed lines) in all plots at the $p < 0.001$ level, see Results.







