MATING SYSTEMS IN *NICOTIANA LONGIFLORA* AND *N. PLUMBAGINIFOLIA*: THE EFFECT OF INTERSPECIFIC INTERACTIONS

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

PLANT MATING SYSTEMS IN *NICOTIANA LONGIFLORA* AND *N. PLUMBAGINIFOLIA*: THE EFFECT OF INTERSPECIFIC INTERACTIONS

Presented by Dulce M. Figueroa-Castro

A candidate for the degree of Doctor of Philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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To my mom,
for her unconditional support and lovely heart,
which constituted the fuel that kept me going every minute of this journey.
Let your light and love show me the path… mi muñequita linda…

To my dad,
for teaching me about constancy and hard work,
indispensable for this journey and for life in general.
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To my brothers, David, Pepe, and Oscar
for their unconditional love and support

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that allowed me to keep smiling even in the distance

To the upcoming newborn nephew,
who represents a miracle of hope and light.

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CHAPTER 4.

PLANT MATING SYSTEMS IN *Nicotiana longiflora* AND *N. plumbaginifolia*: THE EFFECT OF INTERSPECIFIC INTERACTIONS

Dulce M. Figueroa-Castro

Dr. Timothy Holtsford, Dissertation Supervisor

Abstract

The research presented here was focused on the effects of interactions between *Nicotiana longiflora* and *N. plumbaginifolia* on their mating systems. First, I conducted a series of observations and pollination experiments in natural populations to determine interpopulational variability in traits associated with mating system and to ask if corolla length, anther-stigma distance and pollen-ovule ratio are good predictors of mating system. Results showed significant interpopulational variability in floral traits of the outcrossing *N. longiflora*, but not in the selfer *N. plumbaginifolia*. Pollination experiments showed that seeds were set primarily via pollinators and self-fertilization in *N. longiflora* and *N. plumbaginifolia*, respectively. Only corolla length and pollen:ovule ratio proved to be relatively good predictors of mating system (estimated as percentage self seed set). According to Cruden's (1977) classification of mating systems based on pollen:ovule ratios, both *Nicotiana* species are considered between facultative and obligated autogamous.

Second, after demonstrating that pollen:ovule ratio, the most common estimator of plant mating systems, varies among populations, then I determined if this variability

also exists in the realized mating systems (i.e., outcrossing rates) estimated via molecular markers. Because *Nicotiana longiflora* and *N. plumbaginifolia* might be interacting with each other and those interactions might affect their plant mating systems, estimates of outcrossing rates in allopatric and sympatric populations were compared. I also evaluated the effect of interspecific interactions on seed set as a surrogate of fitness and genetic differentiation among the populations studied. Results showed that *N. longiflora* has greater genetic diversity, outcrossing rates and heterozygosity as well as lower fixation indices, biparental inbreeding and genetic differentiation than *N. plumbaginifolia*. Sympatry showed a negative effect on *N. longiflora* fitness and *N. plumbaginifolia* outcrossing rate. An increase in genetic diversity was detected in sympatric *N. plumbaginifolia* populations, suggesting the occurrence of hybridization with *N. plumbaginifolia* being the maternal parent.

Finally, I explore the importance of post-pollination mechanisms determining offspring paternity in natural population of both *Nicotiana* species. First I determined pollen tube growth rates for outcross- and self- pollen of each species. Then, I conducted two- (outcross- vs. self-pollen, and outcross- vs. heterospecific- pollen) and three-donor (self- vs. outcross- vs. heterospecific- pollen) hand pollinations in each species and I determine offspring paternity using molecular markers. Results showed that outcross-pollen has a greater growth rate than self-pollen in *N. longiflora* but not in *N. plumbaginifolia*. In addition, most *N. longiflora* was set by outcross-pollen, thus indicating the occurrence of cryptic self-incompatibility in this species. On the other hand, *N. plumbaginifolia*'s offspring were equally set by self and outcross pollen in two-donor crosses; however, in three-donor hand pollinations, self- and heterospecific-pollen

were equally successful at siring offspring. Results suggest that hybridization might occur in sympatric natural populations, with *N. plumbaginifolia* acting as the maternal parent.

Overall, this research strongly supports that interactions between *N. longiflora* and *N. plumbaginifolia* are occurring in sympatric natural populations at the present time. In sympatry, asymmetrical hybridization is a possibility, but a decrease in outcrossing rates in *N. plumbaginifolia*, as well as strong preference for outcross pollen in *N. longiflora*, might be acting as isolation mechanisms.

Chapter 1. Introduction and study system

The evolution and ecological functioning of plant mating systems is determined by both genetic and ecological factors. In particular, the evolution of self-fertilization is influenced by the breakdown of self-incompatibility, the magnitude of inbreeding depression and pollen and ovule discounting as well as the transmission advantage of selfers over outcrossers (Jain, 1976; Lande and Schemske, 1985; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987; Chang and Rausher, 1998; Barrett, 2002). Ecological factors also influence the evolution of selfing, such as in small populations or in the periphery of populations or any situation in which pollinator and/or mate availability limit seed set (Fausto *et al*., 2001). Other scenarios where pollinators are scarce, such as in cold or rainy seasons, or where pollinators' efficiency is low could also select for selfing (Jain, 1976; Waser, 1978; Piper *et al.*, 1986; Affre and Thompson, 1997; Fausto *et al*., 2001; Barrett, 2002; Gómez, 2002; Elle and Carney, 2003; Steven and Waller, 2004). The reproductive assurance hypothesis proposes that the evolution of self-fertilization is a mechanism to insure reproduction only when conditions are unfavorable for outcrossing and has been widely accepted as an explanation to the evolution and maintenance of selfing and mixed mating systems (Jain, 1976; Wyatt, 1983; Holsinger, 1996; Fausto *et al*., 2001; Bianchi *et al*., 2005; Lobo *et al*., 2005; Coates *et al*., 2006; Dahr *et al*., 2006; Gutierrez-Marcos *et al*., 2006; Sanders and Sipes, 2006; Zhang *et al*., 2006; Lewinsohn and Tepedino, 2007).

Most studies concerning the evolution of plant mating systems are focused on their genetic causes and consequences, usually on artificial arrays of plants and based only on the female component of plant fitness (Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987; Holtsford and Ellstrand, 1990; Jarne and Charlesworth, 1993; Husband and Schemske, 1996). Other studies have addressed the importance of ecological factors, such as pollinator and mate availability, on shaping plant mating systems (Eckert and Schaeffer, 1998; Lu, 2000; Elle and Carney, 2003; Kalisz and Vogler, 2003). However, there is a lack of studies addressing the importance of plant-plant interactions on mating system evolution.

The importance of interactions between plant species in the context of pollination may be indirect or direct. Indirect interactions are mediated through pollinator's foraging patterns between the species sharing pollinators (Rathcke, 1983, 1988a; Waser, 1983). Direct interactions take place after pollination and include the rate at which pollen tubes grow in the styles, interactions between pollen grains and the styles as well as interactions among conspecific and heterospecific pollen grains (Murfett *et al*., 1996; McClure *et al*., 2000; Hancock *et al*., 2003; Sanchez *et al*., 2004). Together, pre- and post-pollination interactions between plants might determine the final output of the interaction, such that it could be negative, when the interaction is competitive, or positive, when the interaction is facilitative (Rathcke, 1983; Waser, 1983). In any case, the interactions might be determinant for plant fitness, mating system, or both. For example, if the coexistence of the species and the interactions between them increase the possibilities of interspecific fertilizations, isolation barriers between the species might be

reinforced. Nevertheless, if seeds sired by interspecific pollen grains experience hybrid vigor, then hybridization may be advantageous.

Despite the relevance of interactions between species on plant mating systems, there is a lack of research on the field. Probably the most important factor that has prevented addressing the role of interactions on plant mating systems is the need of heritable polymorphic codominant markers that allow the differentiation between pollen donors and paternity determination (Bernasconi, 2003). In the current research I address the importance of interspecific interactions between *Nicotiana longiflora* and *N. plumbaginifolia* using codominant cleaved amplified polymorphic sequences (CAPS) markers. *Nicotiana longiflora* and *N. plumbaginifolia* are sister (Ippolito, 2000; Chase *et al*., 2003; Clarkson *et al*., 2004; Lim *et al.* 2006), self-compatible species, intercrossable with each other (Chapter 4). These species have contrasting mating systems, *Nicotiana longiflora* is predominantly outcrosser whereas *N. plumbaginifolia* presents a mixed mating system (Chapter 3). Both allopatric and sympatric populations of the two species are found in northern Argentina. In sympatry, the flowering seasons of both *Nicotiana* species overlap, and share floral visitors (Chapter 2). Therefore, heterospecific pollinations might take place and unless there are mechanisms of isolation between the species, hybridization is a possibility. The research that I present here addresses both the effects of interactions between species mediated through pollinators and post-pollination processes that might influence paternity of sired seeds.

Interactions mediated through pollinators

Indirect interactions between plants for pollinators take place when two or more plant species have overlapping flowering seasons and share pollinators. Competition mediated through pollinators occurs when the presence of one species decreases pollinator visits to another species or when both species suffer a decrease in visits because of each other's presence (Brown and Kodric-Brown, 1979; Pleasants, 1980; Kephart, 1983; Rathcke, 1983, 1988a). Because visitation rates are negatively affected by the presence of a second species, both female and male fitness for either one or all the species are also affected, especially when pollinators are limited (Rathcke, 1983, 1988b; Kephart, 1983; Waser, 1983; Campbell, 1985; Feisinger and Tiebout III, 1991; Fishman and Wyatt, 1999; Moragues and Traveset, 2005). If the abundance of pollinators is not limited, plant reproductive success may not be affected by pollinator preferences (Rathcke, 1983). The net impact of competition on plant reproductive success will depend on the relative frequencies of the coflowering species and the foraging patterns of pollinators (Fishman and Wyatt, 1999).

If the presence of one species has a positive effect on pollinator visitation to a second species, there is a facilitative interaction. In this case and under the assumption that there is not pollen limitation, plant fitness increases as a consequence of a higher frequency of visits when the second species is present with no cost to the first species (Rathcke, 1983; Feldman *et al*., 2004; Moeller, 2004; Moragues and Traveset, 2005).

Post-pollination interactions

Direct competitive interactions between plants occur after pollen deposition on stigmas, when pollen grains grow through the style in order to fertilize ovules. In this case, interactions can be conspecific as well as inter-specific. Interactions among conspecific pollen tubes in the style might lead to the occurrence of cryptic self-incompatibility (the advantage of outcross- over self-pollen); whereas interactions among heterospecific pollen tubes might lead to unilateral incompatibility (heterospecific fertilizations between maternal self-compatible and paternal self-incompatible species) and asymmetric hybridization (the species whose pollen tubes grow faster might fertilize ovules from the other species) (Emms *et al*., 1996; Murfett *et al*., 1996; McClure *et al*., 2000; Hancock *et al*., 2003; Sanchez *et al*., 2004; Martin and Willis, 2007; Lee *et al*., 2008).

Intraspecific competition among pollen grains occurs when pollen from more than one donor (i.e., different genotypes) from the same species are deposited on the stigma and interact while growing in the style. Donors whose pollen grains germinate and/or grow faster will be superior and probably will fertilize more ovules than those with slow-growth pollen grains (Bateman, 1956; Mulcahy, 1979; Lassere *et al*., 1996; Lee *et al*., 2008). In self-compatible species, interactions between self- and outcross- pollen are of particular interest because of the possibilities of weak incompatibility reactions, also known as cryptic self-incompatibility (Bateman, 1956; Bowman, 1987; Casper *et al.*, 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al*., 1990; Cruzan and Barrett, 1993; Rigney *et al*., 1993). Thus, outcross-pollen over-grows self-pollen when competing in the style, so that self-fertilization is prevented yet allowing it whenever outcross pollen

is not available (Bateman, 1956; Bowman, 1987; Casper *et al*., 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al*., 1990; Cruzan and Barrett, 1993; Rigney *et al*., 1993; Travers and Mazer, 2000; Kruszewski and Galloway, 2006).

In order to test if outcross pollen has an advantage over self pollen (i.e., cryptic self-incompatibility) it is necessary to demonstrate not only siring differences among pollen types, but also that those differences are due to variability in pollen tube growth rate and not due to selective seed abortion (Bateman, 1956; Walsh and Charlesworth, 1992). Thus, in exploring the existence of cryptic self-incompatibility it is necessary to determine both pollen tube growth rate and offspring paternity via molecular markers (Bateman, 1956; Walsh and Charlesworth, 1992; Eckert and Allen, 1997). Because cryptic self-incompatibility depends upon the availability of outcross pollen, its occurrence might vary in time and space within and across populations. However, most studies on cryptic self-incompatibility have been conducted on single populations (Travers and Mazer, 2000).

Interspecific pollen tube competition might occur when species growing in sympatry share pollinators so that heterospecific pollen grains can be deposited on the stigmas. Under this scenario, intraspecific pollen tubes are expected to be favored for ovule fertilization and thus, hybridization would be diminished or prevented (Smith, 1968; Carney *et al*., 1994; Emms *et al*., 1996). However, if pollen tube growth is correlated with style length, and the sympatric species have contrasting style lengths, then pollen tubes from the long-style species are expected to have an advantage to fertilize the ovules of the short-style species (Diaz and Macnair, 1999). Therefore, it is expected that whenever pollen tubes from long-style species fertilize ovules of short-style species,

asymmetric hybridization might be relatively common (Emms *et al*., 1996). However, if short style species are not able to support greater tube rates than those carried out by their own pollen as it has been recorded for *Mimulus nasutus*, then hybridization will be prevented (Diaz and Macnair, 1999). In single donor hand pollinations, Lee *et al*. (2008) found that *Nicotiana plumbaginifolia* flowers pollinated with conspecific pollen sired a similar amount of seeds as flowers pollinated with *N. longiflora* pollen. On the other hand, *N. longiflora* showed a substantial decrease in seed set when pollinated with *N. plumbaginifolia* pollen. Therefore, their results suggest that asymmetric hybridization might occur between these species.

Direct pollen-pollen interactions might also facilitate ovule fertilization by particular pollen genotypes. This phenomenon, known as mentor effect, occur when mixtures of pollen from different donors are deposited on the stigmas, such that one donor mediates the growth of different pollen types (Cruzan, 1989; De Nettancourt, 2001), or pollen tube growth rate of one donor is partially dependent on another donor's presence (Cruzan, 1990). In this way, genotypes that otherwise would be rejected or self-incompatible will be successful at fertilizing ovules.

Implications of interactions

Plant-plant interactions not only are important for their effects on both fitness and mating systems, but also have implications for population structure, because pollinator activities determine gene flow within and among populations. In populations where pollinators are not limited, outcrossing species have high gene flow within and among populations, and thus low differentiation among populations (Schoen, 1982; Holtsford

and Ellstrand, 1989; van Treuren *et al*., 1993; Williams *et al*., 2001; Casiva *et al*., 2004; DeWoody *et al*., 2004; Iddrisu and Ritland, 2004; Ledig *et al*., 2005). Because these species have high outcrossing rates, low fixation indices and high heterozygosity levels are also common (Ellstrand *et al*., 1978; Ennos, 1981; Shea, 1987; van Treuren *et al*., 1993; Gaiotto *et al*., 1997; Kittelson and Maron, 2000; Lee *et al*., 2000; Williams *et al*., 2001; Rajora *et al*., 2002; Galloway *et al*., 2003; DeWoody *et al*., 2004; Iddrisu and Ritland, 2004; Travis *et al*., 2004; Ledig *et al*., 2005). On the contrary, primarily selfing species have low outcrossing rates, low heterozygosity as well as high fixation indices, and because the gene flow between and among populations is strongly limited, high differentiation among populations (Ennos, 1981; Schoen, 1982; Holtsford and Ellstrand, 1989; Agren and Schemske, 1993; Penteado *et al*., 1996). In sympatric populations, if the presence of species B facilitates pollinator visitation to species A then, an increase in heterozygosity and outcrossing rates as well as a decrease in population differentiation will be expected, compared with estimations from allopatric populations. However, an increase in pollinator visits might also lead to higher hybridization rates, and, consequently, to the development of isolation mechanisms between the species. If on the other hand, the presence of species B has a competitive effect on species A, then, a decrease in outcrossing rates and heterozygosity (Bell *et al*., 2005) together with an increase in population differentiation will be expected, compared with estimations from allopatric populations.

Questions addressed in the current dissertation

The research that I present here addresses the effects of interactions between *Nicotiana longiflora* and *N. plumbaginifolia* on realized mating system. These species are each other's closest relatives (Ippolito, 2000; Chase *et al*., 2003; Clarkson *et al*., 2004; Lim *et al.* 2006) and co-occur in sympatry in northwest Argentina, where their flowering seasons overlap and hawkmoths visit both species. Therefore, both direct and indirect interactions between them might occur and thus affect plant mating structure. In order to explore this possibility, and because plant mating systems are associated with floral traits that promote either self or outcross pollination (Jain, 1976; Steven and Waller, 2004; van Kleunen and Ritland, 2004), in Chapter 2 I ask if floral traits associated with mating system (corolla length, anther-stigma distance and pollen:ovule ratio) vary among populations and if variation is a consequence of sympatry. Then, by conducting pollination experiments to determine seed set via pollinators and selfing, I asked whether floral traits are good predictors of plant mating system estimated as seed-set via selfing.

Having tested the existence of interpopulational variability in mating system estimated as pollen:ovule ratio, in Chapter 3 I focus on two parameters that might be affected by indirect interspecific interactions between the study species: seed set (as an estimator of female fitness) and realized mating systems. Here I ask, do interspecific interactions mediated through pollinators affect plant seed set and outcrossing rates of the *Nicotiana* species? If that is the case, is the interaction positive (facilitative) or negative (competitive)? And, because gene flow in plants is determined in part by pollinator movements, then I ask, how does the interaction between *N. longiflora* and *N. plumbaginifolia* affect population structure for these species?

Chapter 4 addresses the effect of direct pollen-pollen interactions within and between the *Nicotiana* species. First, I ask, do interactions among self- and outcross-pollen grains within species lead to cryptic self-incompatibility for one or both species? Then, because the study species coexist in sympatry in natural populations, hybridization is a possibility, I ask, do inter-specific interactions with two- and three- donor hand pollinations lead to hybridization between the *Nicotiana* species?

Finally, in Chapter 5 I conclude by summarizing the main findings and discussing the ecological and evolutionary implications of plant-plant interactions in general and, between *Nicotiana longiflora* and *N. plumbaginifolia* in particular. In addition, I propose questions that need to be addressed in order to fully understand the role of interactions between *N. longiflora* and *N. plumbaginifolia* and their mating system evolution.

Study system

Nicotiana longiflora and *N. plumbaginifolia* (Solanaceae) are sister species forming a strongly supported clade within section *Alatae* (Ippolito, 2000; Chase *et al*., 2003; Clarkson *et al*., 2004; Lim *et al*., 2006). Both species are self-compatible and inter-crossable in the greenhouse (East, 1916; Goodspeed, 1954). However, they have contrasting floral morphology that might affect their mating systems (Fig. 1). *N. longiflora* is a herbaceous perennial or vigorous annual, 0.5 to 1.0 m in height (Goodspeed, 1954). Its flowers open at night, releasing fragrance (Goodspeed, 1954). The tubular part of the *N. longiflora* corolla is 40-120mm long, 1.5-2.5 mm wide, pale yellow, oily grey or purplish externally; the limb is 12-25 mm wide (Fig. 1; Goodspeed, 1954). This species produce abundant nectar with 21.3 ± 0.3% solids in sucrose-dominant nectar

(Kaczorowski, 2005). These floral and nectar traits suggest *N. longiflora* is an outcrosser. This species inhabits open fields, stream banks and roadsides in South America, in northern Argentina, Paraguay, Uruguay, southeastern Brazil and south Bolivia (Goodspeed, 1954; Fig. 2).

Nicotiana plumbaginifolia is an annual plant, with 0.3 to 1m in height, with nocturnal anthesis (Goodspeed, 1954). The tubular part of the corolla is 25-35 mm long and 1.5-2.0 mm wide, greenish, ivory or purplish in color; the limb is 10 mm wide (Fig. 1; Goodspeed, 1954). *N. plumbaginifolia* nectar has 19.3 ± 1.1% solids in sucrose-rich nectar (Goodspeed, 1954; Kaczorowski, 2005), which is within the range recorded for hawkmoth pollinated species (Baker and Baker, 1982). A study on floral organ development of these two species showed a negative anther-stigma distance for one-day old *N. plumbaginifolia* flowers and high levels of autogamy, suggesting it is a primarily selfer species (Soule, 2007). *N. plumbaginifolia* is distributed in South America east of the Andes from northwestern Argentina to southwestern Brazil, north Peru and Ecuador, Guatemala, Cuba, Mexico and southeastern U.S.A. (Goodspeed, 1954; Fig. 2).

Both *N. longiflora* and *N. plumbaginifolia* co-occur sympatrically at north of Argentina (Goodspeed, 1954; Fig. 2). Self-fertilization before flower anthesis in *N. plumbaginifolia* may provide a mechanism of reproductive isolation between the species when they coexist in sympatry. In populations where both of the species grow, it is probable that inter-specific interactions mediated through pollinators occur. Moreover, such interactions may be an evolutionary force shaping mating system evolution of these species.

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Figure 1. The study species. *N. longiflora* has long corolla length and has been considered as outcrosser, whereas *N. plumbaginifolia* has short corolla length and small anther-stigma distance, suggesting it is a selfer.

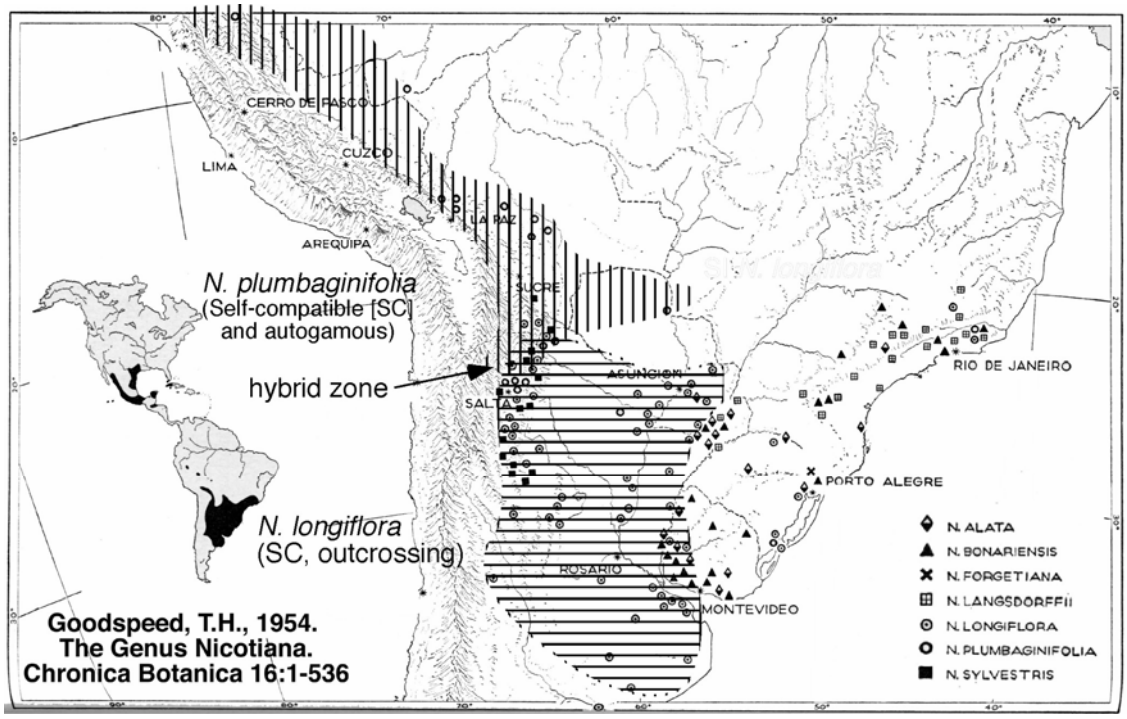

Figure 2. Distribution of *Nicotiana* species in South America (Goodspeed, 1954).

Chapter 2. Floral traits and mating systems in sister species of *Nicotiana*: interpopulations variability and sympatry effects

Abstract

Mating systems of Angiosperms are important determinants of population genetic structure and evolutionary potential. *Nicotiana longiflora* and *N. plumbaginifolia* are self-compatible, but *N. longiflora* is a presumed outcrosser, whereas *N. plumbaginifolia* is a selfer. I studied the natural interpopulational variability and sympatry effects on floral traits associated with mating system (pollen:ovule ratio, herkogamy, and flower size). I also estimated seed set by selfing as well as via pollinators using pollination treatments. The study populations included two sympatric and ten nearby allopatric populations. I found that all measured floral traits varied significantly among populations of *N. longiflora*, but not among *N. plumbaginifolia* populations. According to Cruden's (1977) classification of mating systems, both *Nicotiana* species are predicted to be intermediate between facultative and obligate autogamous. However, pollination treatments indicate that *N. longiflora* is primarily an outcrosser. Seed set attributable to pollinators was significantly higher in *N. longiflora* than in *N. plumbaginifolia* populations. Pollination treatments showed that most seed set in *N. plumbaginifolia* populations was produced through self-pollination. I did not detect an effect of sympatry in either species for any of the floral traits studied. Since floral visitors showed a strong preference for *N. longiflora* in sympatry, selfing in *N. plumbaginifolia* assures reproduction. Corolla length and

pollen:ovule ratio were significantly correlated with the percentage of self-seeds estimated by pollination treatments.

Key words: floral traits, interpopulational variability, mating system evolution, *Nicotiana longiflora*, *Nicotiana plumbaginifolia*, pollen:ovule ratio, sympatry effects.

Introduction

The evolution of self-fertilization has occurred multiple times in entomophilous angiosperms (e.g., Levin, 1972; Vogler and Kalisz, 2001; Barrett, 2002; Kalisz and Vogler, 2003). Shifts in plant mating systems are linked to changes in those floral traits promoting either self or outcross pollination (Jain, 1976; Steven and Waller, 2004; van Kleunen and Ritland, 2004).

Outcrossing plants usually have a high production of large, showy flowers with relatively long flowering times, are protandrous with anthers and stigma spatially separated and high production of pollen grains compared to ovules. In comparison, selfing species generally have smaller, less attractive flowers, shorter flower longevity, less temporal and spatial separation of male and female functions and lower pollen:ovule ratios (Cruden, 1977; Armbruster, 1985; Morgan and Barrett, 1989; Dole, 1992; Parker *et al*., 1995; Karron *et al*., 1997; Chang and Rausher, 1998; Sun, 1999; Fishman et al., 2002; Suso et al., 2003; Steven and Waller, 2004; van Kleunen and Ritland, 2004).

The evolution of plant mating systems is determined by both genetic and ecological factors. In particular, the evolution of self-fertilization is influenced by the breakdown of self-incompatibility, the magnitude of inbreeding depression and the

transmission advantage of selfers over outcrossers because they transmit their genes through outcross pollen in addition to self- pollen and ovules (Jain, 1976; Lande and Schemske, 1985; Schemske and Lande, 1985; Charlesworth and Charlesworth, 1987; Chang and Rausher, 1998; Barrett, 2002). Moreover, in Solanaceae of the genus *Lycopersicon*, it has been shown that genes related with the breakdown of self-incompatibility and changes in floral traits associated with mating systems are clustered altogether, suggesting they are a functional unit (Bernacchi and Tanksley, 1997; Chen and Tanksley, 2004). Ecological factors might also influence the evolution of selfing, such as in small populations or in the periphery of populations, where fewer mates or pollinators could drive selection for selfing (Fausto *et al*., 2001). Other scenarios where pollinators are scarce, such as in cold or rainy seasons, or where pollinators' efficiency is low could also select for selfing via reproductive assurance (Jain, 1976; Waser, 1978; Piper *et al.*, 1986; Affre and Thompson, 1997; Fausto et al., 2001; Barrett, 2002; Gómez, 2002; Elle and Carney, 2003; Steven and Waller, 2004).

Competitive interactions between pairs of species sharing pollinators can also influence the evolution of selfing (Waser, 1978; Carter and Murdy, 1986; Piper *et al*., 1986). Competition for pollinator services, when sympatric species share floral visitors, leads to a decrease in visitation rates and consequent plant fitness for at least one of the species sharing pollinators (Rathcke, 1983; Waser, 1983; Campbell, 1985), especially if the pollinator fauna are highly limited (Fishman and Wyatt, 1999). If, on the other hand, pollinators are not limited, plant reproductive success will not be affected by pollinator preferences (Rathcke, 1983). Therefore, the net impact of competition on plant reproductive success will depend on the relative frequencies of the co-flowering species

and the foraging patterns of pollinators (Fishman and Wyatt, 1999). Competitive interactions are particularly important when the species interacting are inter-crossable, sister species; such as *Nicotiana longiflora* Viviani and *N. plumbaginifolia* Cavanilles in northwest-Argentina (East, 1916; Goodspeed, 1954; Pandey, 1979). When interfertile species co-occur, hybridization can be common unless there are premating reproductive isolation barriers operating. The evolution of both floral traits and/or mating systems may contribute to reproductive isolation (Carter and Murdy, 1986).

Although different populations experience diverse ecological pressures, few studies have addressed the interpopulational variability and sympatry effects on floral traits associated with mating systems among interfertile species (Gottlieb and Bennett, 1983; Armbruster, 1985; Carter and Murdy, 1986; Morgan and Barrett, 1989; Dole, 1992; Stewart *et al*., 1996; Herlihy and Eckert, 2005). Most studies on interpopulational variability in reproductive traits have analyzed the variability of secondary sexual traits (associated with attractiveness), including anther-stigma distance, resources allocated to androecium, gynoecium and perianth, flower production, inflorescence duration, nectar spur length, days to flowering, corolla length, pistil length and stamen length (Armbruster, 1985; Morgan and Barrett, 1989; Dole, 1992; Herlihy and Eckert, 2005). The relative production of pollen versus ovules is an important trait because it predicts self-fertilization rate (Cruden, 1977); however, few studies have evaluated pollen versus ovule interpopulational variability. Herlihy and Eckert (2005) found significant among-population variability in ovule production, whereas Stewart *et al*. (1996) did not find significant variability in pollen:ovule ratio among populations. Cruden (1977) found significant biological interpopulational variability in pollen:ovule ratio in three out of 17

species. Studies evaluating sympatry effects on floral trait variability are also scarce. Armbruster (1985) showed significant effects of sympatry on gland size of *Dalechampia scandens* when co-occurring with other five species of *Dalechampia*; however, the direction of the change depended upon inflorescence size of the sympatric species. Carter and Murdy (1986) found a significant increase in ovule production and anther-stigma distance in sympatric populations of two species of *Talinum*. However, the direction of change for petal length, style length and pistil length was opposite between species when comparing sympatric versus allopatric populations.

The lack of studies addressing interpopulational variability and sympatry effects in floral traits associated with mating systems and the context-dependent results found by other researchers, prompt the necessity for new studies, particularly focusing on natural populations of closely related taxa with contrasting mating systems (Mazer and Hultgard, 1993; Parker *et al*., 1995; Affre and Thompson, 1997, 1998).

Nicotiana longiflora and *N. plumbaginifolia* are an excellent system in which to explore the effect of species interactions on the floral traits associated with mating systems. They are self-compatible, sister species, with contrasting mating floral morphology and mating systems, and inter-crossable in the greenhouse (East, 1916; Goodspeed, 1954; Ippolito, 2000; Chase *et al*., 2003; Clarkson *et al*., 2004; Lim *et al*., 2006). Their ranges of distribution overlap, so that both allopatric and sympatric populations can be studied (Goodspeed, 1954). In this study, I determine how floral characters vary among natural populations of these *Nicotiana* species and how well they predict mating system estimated as the percentage of self-seeds obtained with pollination treatments. The particular goals are: i) to develop regression models useful for the

estimation of number of pollen grains and ovules for the indirect estimation of pollen:ovule ratios for the study species; ii) to quantify variability in pollen:ovule ratio, corolla length and anther-stigma distance among populations within and between species, iii) to determine the effect of sympatry on the variability of floral traits and pollinator preferences, iv) to estimate variability among populations in maximal seed set, seed set by pollinators, self-seeds via pollination treatments, pollinator-assisted seed-set and visitation patterns; and v) to determine if floral traits (corolla length, anther-stigma distance and pollen:ovule ratio) are accurate predictors of mating system variation.

Methods

Study species

Nicotiana longiflora and *N. plumbaginifolia* (Solanaceae) are sister species, forming a strongly supported clade within section *Alatae* (Ippolito, 2000; Chase *et al*., 2003; Clarkson et al., 2004; Lim *et al*. 2006). These species are self-compatible and inter-crossable in the greenhouse (East, 1916; Goodspeed, 1954). *Nicotiana longiflora* flowers are 40-120mm in corolla length, with 21.3 ± 0.3% solids in sucrose dominant nectar (Kaczorowski, 2005). On the other hand, *N. plumbaginifolia* has shorter flowers, ranging from 25 to 35 mm in length, with 19.3 ± 1.1% solids in sucrose-rich nectar (Goodspeed, 1954; Kaczorowski, 2005). In both species, nectar concentration fits within the range for hawkmoth pollinated species (Baker and Baker, 1982). However, nectar production and the ratio of sucrose to glucose+fructose are lower for *N. plumbaginifolia* (Kaczorowski, 2005). A study on floral organ development of these two species showed a negative anther-stigma distance for one-day old *N. plumbaginifolia* flowers and high levels of

autogamy, whereas *N. longiflora* showed a rather low percentage of autogamy (Soule, 2007). These *Nicotiana* species co-occur in sympatry at North-Argentina, with the allopatric range of *N. longiflora* to the South and Southeast of the continent, and that of *N. plumbaginifolia* to the North (Goodspeed, 1954). The co-occurrence of the two species in sympatric populations and the overlap in their flowering seasons suggests that interactions mediated through pollinators might have occurred in the past and may still be happening.

Development of regression models for gamete number estimations

Plants growing in natural populations were used to develop regression models that relate floral morphology to pollen and ovule number so that estimations of pollen:ovule (P:O) ratio in many plants per population in several populations could be obtained. In 2005, between nine and 15 plants in each of five natural populations, including both sympatric and allopatric populations, were used to develop the regression models (total N= 69 plants). One to two flowers with undehiscent anthers per plant were measured for corolla length, anther-stigma distance (ASD), ovary width and anther width and length. These variables were chosen because they showed the highest significance in regression models previously developed from plants grown in the greenhouse (data not shown). Anther width and length were estimated from two anthers per flower. Anthers were individually stored in uncapped 1.5 ml microcentrifuge tubes until dry, whereas ovaries were stored in 70% ethanol. Both anthers and ovaries were brought back to the laboratory for pollen and ovule counts. Ovaries from each flower were dissected under a stereoscopic microscope. Ovules from a quarter of the ovary were placed in water and then counted under the

microscope. The number of ovules counted was then multiplied by four to obtain the estimated total number of ovules per flower. Pollen grain number was estimated with an Elzone 280 PC particle counter, previously calibrated with 40μm beads, which are in the range of pollen grain size for both *Nicotiana* species (15-45μm). 1.5 ml of 2% saline solution were added to each tube, anthers were dissected with forceps and sonicated for 4min in a Mini Ultrasonik Ney sonicator. Tubes were then rinsed into a 20ml sample vial which was left from eight hours to overnight to set. Pollen estimations were done after sonicating each 20 ml vial for 1min. Five estimations were made for each tube, the highest and lowest scores were discarded and the three other measurements were used to get the average number of pollen grains per anther, which then was multiplied by five (i.e. number of anthers per flower) to estimate the total number of pollen grains per flower.

Simple linear, quadratic and cubic regression analysis between floral traits measured and number of pollen grains and ovules per flower were modeled using SAS. The best models were chosen based on the highest determination coefficients adjusted (Adj. R^2) for degrees of freedom (SAS 9.1.). Stepwise regression analyses were also applied to assure that single traits were the best predictor variables of gamete number.

Interpopulational variability and sympatry effects on floral traits

In 2005, twelve natural populations were located in northern Argentina (Fig. 1, Table 1). Six populations were composed of only *N. longiflora*, four others contained only *N. plumbaginifolia* and two populations included both species in sympatry. Eleven of the populations were small enough that population size was determined by counting all

individuals in the population. In the large population Río, population size was estimated by counting the number of individual plants every other meter along a transect extending through the whole population and then multiplying that number by two to get an estimate of the total size of the population.

In each population, 1-2 flowers per plant with mature but still undehiscent anthers were collected to measure floral traits. Developmental stage of flowers measured in the field was similar to the one of flowers used for the development of regression models. Plants were chosen according to the availability of flowers with undehiscent anthers, so that between 11 (in Ledesma) and 33 (in Canal) plants per population were sampled. In all the populations but Río all of the plants with flowers available at the moment of the collections were sampled. In the large Río population, plants were chosen at random from throughout the population. Collected flowers were measured *in situ* for corolla length, anther-stigma distance, anther length, anther width and ovary width. All traits were measured within 24 hours prior to flower anthesis. Anther length and width were then used as predictor variables for the estimation of gamete number using the regression models.

In order to test for interpopulational variability and sympatry effects on all floral traits (pollen number, ovule number, pollen:ovule ratio, corolla length and anther-stigma distance) two ANOVA tests were performed for each trait. Interpopulational variability was tested including species as a fixed effect and population nested within species. To determine sympatry effects on floral traits I pooled data from all allopatric and the two sympatric populations and included species and sympatry as fixed effects in the model. *Post-hoc* Tukey analyses were performed to identify significant differences among

groups. The interpopulational variability and sympatry effect analyses could not be combined because populations are nested within the species variable for the first test, and confounded within the sympatry/allopatry category in the second test.

Natural seed set, selfing, and pollinator effectiveness

Spontaneous self-pollination and pollinator effectiveness were estimated with pollination treatments applied on October-November 2005 to plants in eight natural populations (four pure *N. longiflora*, 3 pure *N. plumbaginifolia* and 1 with both species in sympatry) located in northwest Argentina (Table 1). Sample sizes of Oran and San Pedro populations, which are both located within towns, were greatly reduced between treatment application and fruit collection by human interference, and therefore were excluded from statistical analyses. Results then, are from six populations (*N. plumbaginifolia*: Milagro and Ledesma; *N. longiflora*: Católica, Río, and Tanque; both species: Mango). Four treatments per plant were applied (Table 2): i) anther emasculation, which allowed only outcross- fertilization (seed set attributed to pollinators), ii) anther emasculation + pollen supplementation to estimate maximal outcross seed-set, iii) bagged, which allowed just for spontaneous self- pollination, iv) natural (open) pollination. Flowers were marked with wire and average ovary width per plant was used as the predictor variable of number of ovules to estimate percent ovules that set seed in individual fruits. Emasculations were conducted prior to anther dehiscence, within 24 h before anthesis. Pollen addition was made by rubbing the anthers of at least two flowers from different plants in the same population on the stigmas of the experimental flowers within 24 h after anthesis. Plastic bags with small holes made with

a regular sewing needle were used for the bagged treatment. Fruits were collected before dehiscence (one and two weeks after pollination treatments were applied in *N. plumbaginifolia* and *N. longiflora*, respectively). Seed number per fruit was estimated through weighing 100 seeds per fruit and then extrapolating to the total number of seeds per fruit. An analytical balance was used to weight the seeds. Percent seed set was estimated as number of seeds per average number of ovules per flower. An ANOVA test (using SAS 9.1) with species as a fixed factor and population nested within species was applied to determine differences among populations and all four pollination treatments.

Percent of seed-set due to selfing was estimated by subtracting seed set obtained in the emasculation treatment from seed set in the control treatment (Table 2). Assisted selfing (pollinator assisted self seed set; Table 2) was estimated as the difference between total selfing and spontaneous selfing (bagged treatment). In Ledesma, the bagged treatment was not applied. Plants used for treatments were the same as those for which pollen grains and ovules per flower were estimated. Interpopulational variability in seed set attributed to pollinators, total selfing, and pollinator assisted selfing was tested with ANOVA tests with species as fixed factor and population nested within species. Sympatry effects were analyzed with another ANOVA including species and sympatry as fixed effects. To improve data normality, percent seed set was transformed as arcsin $(x)^{0.5}$ (Zar, 1999). All analyses were conducted in SAS 9.1.

Pollinator observations

Observations of pollinators were conducted on October-November 2005, in five natural populations (Mango, Río, Orán, Católica, and Rodrigo). Number of visits and patterns of

movements among flowers and plants were recorded. Observations of pollinators were conducted between 2000 and 2330 hours, the period of highest activity of pollinators in other *Nicotiana* species from section *Alatae* (Ippolito *et al.*, 2004). Because some populations were found within towns, observations for several nights could not be conducted, and in Oran the regular 3.5 h observation time per night conducted in all other populations was not completed. Therefore, total observation time was highly variable among populations: Mango: 1080min; Río: 1441min; Rodrigo: 1080min; Católica: 450min; Orán: 100min. Because no visits were recorded in the Rodrigo, Catolica and Oran populations, I excluded them from any analysis.

A χ^2 analysis was used to test the hypothesis that number of visits recorded in different populations of *N. longiflora* (Mango and Rio) is a function of both population size and observation time (Fig. 2). Expected number of visits was estimated as: $[(I_{A+B} \text{ X } I_A)/(I_{A+B} + T_{A+B})]$ X # visits; where I_{A+B}= total number of individuals from both population A and B; I_A= number of individuals in population A; T_{A+B}= total observation time for the two populations; and # visits= total number of visits from pollinators to plants in the two populations. A second χ^2 test was applied to test the hypothesis that within the sympatric Mango population, the number of pollinator visits to each *Nicotiana* species is a function of the relative abundance of each species in the population. Expected number of visits was estimated as: (I_A/I_{A+B}) X # visits.

Relationship between mating system and floral traits

Regression models were applied to determine the relationship between floral traits as predictor variables of mating system estimated as seed set via selfing. Corolla length,

ASD and P:O ratio were used as explanatory variables of percent seed set attributed to pollinators and percent self seed-set transformed as arcsin $(x)^{0.5}$ (Zar, 1999). Linear, quadratic and cubic models were applied to data of both species together and the best fit models were chosen based on the highest determination coefficients adjusted for degrees of freedom (SAS 9.1).

Results

Predicting gamete number with regression models

Regression analyses showed that the best fit model for pollen grains was linear, whereas the best fitted model for ovule number was cubic. The product of anther length by width was the best predictor variable for number of pollen grains (#pollen/flower= 37 769+25 330 anther length X width; Adj. R^2= 0.609, Fig. 3a). Anther width was the best predictor for number of ovules per flower [#ovules/ovary= 17 720 + (-30 308) anther width + 18 046 (anther width)^2 + (-3364.62) (anther width)^3; Adj. R^2=0.680, Fig. 3b]. Mean production of pollen grains per flower of *N. plumbaginifolia* and *N. longiflora* was 92 637.59 ± s.e. 1075.19, and 200 798.67 ± 2376.98, respectively. Mean production of ovules per flower for *N. plumbaginifolia* and *N. longiflora* was 1538.48 ± 23.81, and 2367.92 ± 18.88, respectively.

Interpopulational variability of floral traits

All floral traits varied significantly between species and among populations, except ASD, which varied significantly among populations but not between species (Fig. 4, Tables 3,4). Number of ovules, pollen grains, pollen:ovule ratio and corolla length were

significantly higher for *N. longiflora* than *N. plumbaginifolia* (Tables 3,4). Mean P:O ratio for *N. longiflora* and *N. plumbaginifolia* was 84.61 ± 0.65 and 61.73 ± 1.23, respectively. Based on these estimations of pollen:ovule ratio both species are predicted to be intermediate between facultative and obligate autogamous according to Cruden's (1977) mating system classification.

Tukey *post-hoc* tests showed that all floral traits varied significantly among populations of *N. longiflora*, but not among *N. plumbaginifolia* populations. Ovule production in *N. longiflora* populations Tanque and Canal was significantly higher than that in Rio, while the other five *N. longiflora* populations were intermediate between these two groups (Fig. 4a, Table 4). The highest production of pollen grains was recorded in the Tanque population and was significantly different from that in Rodrigo, San Pedro and Rio (Fig. 4c, Table 4). *N. longliflora*'s P:O ratio from Tanque, Caldera, Canal, Catolica, Mango and Rodrigo were significantly higher than all other populations (Fig. 4e, Table 4). *N. longiflora* populations Rio, Tanque, San Pedro, Mango and Canal had the largest flowers, whereas Rodrigo and Catolica had the shortest ones (Fig. 4g, Table 4). Population San Pedro had the greatest anther-stigma distance prior to anthesis, whereas Caldera had the lowest (Fig. 4i, Table 4).

Sympatry effects on floral traits

All floral traits varied significantly between species but were not affected by either sympatry or the interaction between species and sympatry (Fig. 4, Table 5). Mean values for all floral traits were higher for *N. longiflora*, as described in the interpopulational variability analysis (Table 4). Although the ANOVA analysis showed a significant effect

of sympatry on ovule number per flower (Fig. 4b), the *post-hoc* Tukey test did not show significant differences between allopatric and sympatric populations for either species. ANOVA analysis did not show significant effects of sympatry on ASD; however, the *post-hoc* Tukey test showed significant differences among groups for that trait. In allopatric *N. plumbaginifolia* populations, ASD was significantly smaller than in *N. longiflora*. ASD of *N. plumbaginifolia* in sympatric populations did not differ significantly from ASD in *N. longiflora*, nor from that in allopatric *N. plumbaginifolia* populations (Fig. 4j).

Natural seed set, selfing, and pollinator effectiveness

Pollination treatments

Seed set was significantly affected by pollination treatment ($F_{3, 424}$= 73.30, P< 0.0001), site ($F_{5, 424}$= 83.31, P< 0.0001), and the interactions between treatment and site ($F_{14, 424}$= 27.67, P< 0.0001) and species and treatment ($F_{3, 424}$= 4.57, P= 0.0036). Species did not have a significant effect on seed set ($F_{1, 424}$= 2.39, P= 0.123) (Fig. 5). In all populations but *N. plumbaginifolia*-Mango the highest seed set was obtained with control and pollen-added treatments. Seed set for control treatments was significantly higher in Ledesma and *N. longiflora*-Mango, whereas seed set for pollen-added treatments was significantly higher in Catolica. There were not significant differences in seed set between control and added treatments in Milagro, Rio, Tanque and *N. plumbaginifolia*-Mango. In most populations the bagged treatment had significantly lowest seed set, except in *N. plumbaginifolia*-Mango, in which seed set was similar among all treatments. In the

Milagro population of *N. plumbaginifolia*, the bagged treatment had similar seed set to the emasculated treatment (Fig. 5).

Seed set by selfing and pollinators

Total selfing estimated by pollination treatments (assisted + spontaneous; Table 2) was significantly different among populations ($F_{5, 73}$= 15.75, P< 0.0001) but not between species ($F_{1, 73}$= 0.0, P= 0.977). Sympatry ($F_{1, 76}$= 0.64, P= 0.425) and the interaction of sympatry by species did not have a significant effect on total selfing ($F_{1, 76}$= 3.32, P= 0.072). All *N. plumbaginifolia* populations had significantly higher percentages of total selfing than *N. longiflora* populations (Fig. 6). Among *N. longiflora* populations, Mango showed the highest percentage of total self-pollination (80 ± 20 %), whereas Rio (21.53 ± 5.25) and Catolica (22.47 ± 14.83) showed the lowest one.

Seed set attributed to pollinators (Table 2) was significantly affected by population ($F_{5, 73}$= 78.2, P< 0.0001), but not by species ($F_{1, 73}$= 1.26, P= 0.266), sympatry ($F_{1, 76}$= 0.26, P= 0.608), nor the interaction species by sympatry ($F_{1, 76}$= 2.33, P= 0.131). Seed set attributed to pollinators showed the opposite pattern to that shown by total selfing (Fig. 6). *N. longiflora* in Catolica (77.53 ± 14.83 %) and Rio (78.47 ± 5.25 %) had significantly higher seed set attributed to pollinators than *N. longiflora* in Mango and either *N. plumbaginifolia* populations (Fig. 6).

Pollinator assisted selfing (Table 2) was significantly affected by site ($F_{4, 60}$= 5.12, P= 0.001) but not by species ($F_{1, 60}$= 0.16, P= 0.689), sympatry ($F_{1, 62}$= 2.3, P= 0.134), nor the interaction species by sympatry ($F_{1, 62}$= 1.72, P= 0.194). Both *N. longiflora* (100.0 ± 0.0 %) and *N. plumbaginifolia* (91.4 ± 0.0 %) in the Mango population had the highest

estimate of pollinator assisted selfing. Catolica (17.09 ± 12.69 %) and Rio (23.07 ± 5.39 %) had the lowest percentage of pollinator assisted selfing (Fig. 6).

Frequency of pollinators

A very low frequency of pollinators was observed on the flowers of both *Nicotiana* species, especially for *N. plumbaginifolia*. Only 116 visits were recorded during 69.2 h of observation, and only in the Rio and Mango populations.

The hypothesis that the number of pollinator visits to *N. longiflora* in the Mango and Rio populations was a function of population size and observation time was rejected (χ^2= 12.388, P< 0.001, df=1). In Mango, *N. longiflora* received less visits than expected, whereas in Rio the number of visits recorded was higher than expected (Fig. 7a). The null hypothesis that the number of pollinator visits in the sympatric Mango population was a function of the relative abundance of each *Nicotiana* species was also rejected (χ^2= 37.165, P< 0.0001, df=1). Pollinators had significant preferences; *N. longiflora* and *N. plumbaginifolia* received significantly higher and lower number of visits than expected, respectively (Fig. 7b).

Relationship between floral traits and selfing

Both corolla length (CL) ($F_{3,70}$=15.6, P<0.0001, r^2= 0.375) and pollen:ovule ratio (P:O) ($F_{3,70}$=11.32, P<0.0001, r^2= 0.298) had a significant relationship with total self-seed set via pollination treatments. In both cases the relationship was better explained with cubic models [% selfing= 274.319-10.884 CL + 0.163 $(CL)^2$ - 0.0008 $(CL)^3$; % selfing= -103.826 + 10.235 P:O - 0.161 $(P:O)^2$ + 0.0007 $(P:O)^3$; Fig. 8]. In both cases,

species are well differentiated, with all *N. plumbaginifolia* plants grouped together at high percentages of self-seed via pollination treatments (Fig. 8a,c), whereas *N. longiflora* is scattered throughout the whole range of percent self-seed set.

Anther-stigma distance prior to anthesis did not show a significant relationship with total self seed set ($F_{2,71}$=2.09, P=0.131, r^2= 0.029; Fig. 8b). *N. plumbaginifolia* and *N. longiflora* are not well differentiated, although *N. plumbaginifolia* had the high percentages of self-seed.

Discussion

Interpopulational variability in floral traits was found for *Nicotiana longiflora* but not for *N. plumbaginifolia*, which is consistent with the maintenance of variability in outcrossers or its loss in selfers (Jain, 1976; Lande and Schemske, 1985; Schemske and Lande, 1985; Dole, 1992,). However, sympatry effects were not detected in any of the floral traits. Pollination treatments strongly suggest that *N. plumbaginifolia* self-pollinates regularly, but pollinator-assisted selfing might also contribute. Pollinators had a strong preference for *N. longiflora* in the sympatric Mango population, which is consistent with the high levels of self seed set via pollination treatments for *N. plumbaginifolia* in that population. Corolla length and pollen:ovule ratio were significantly correlated with the percent of self-seed via pollination treatments, showing a clear separation between species, but not within *N. longiflora*.

Interpopulational variability in floral traits

All of the floral traits studied varied significantly among *Nicotiana longiflora* populations, as has been found in other outcrossing species (Armbruster, 1985; Dole, 1992). However, *N. plumbaginifolia*'s floral traits did not vary significantly among populations. It has the characteristic floral traits associated with selfing, such as reduced flower size, small anther-stigma distance and low pollen:ovule ratio (Cruden, 1977; Armbruster, 1985; Morgan and Barrett, 1989; Dole, 1992; Parker *et al*., 1995; Karron *et al*., 1997; Chang and Rausher, 1998; Sun, 1999; Fishman et al., 2002; Suso et al., 2003; Steven and Waller, 2004; van Kleunen and Ritland, 2004). It has been suggested that *N. plumbaginifolia* self-pollinates before anthesis, because anthers and stigma are either in direct contact with each other or anthers have already over passed the stigma prior to anthesis, thus potentially promoting self-pollination (Soule, 2007).

In contrast to the lack of variability in *N. plumbaginifolia*, all floral traits in *N. longiflora* varied significantly among populations, as expected for a presumed outcrosser. However, the Rio population showed a different pattern. It had the lowest values for ovule and pollen production as well as pollen:ovule ratio, but among the highest values for corolla length and anther-stigma distance. This result is surprising for two reasons. First, a positive relationship between secondary floral traits such as corolla length and ovule and pollen production is expected based on studies of mating system evolution (Cruden, 1977; Armbruster, 1985; Morgan and Barrett, 1989; Dole, 1992; Parker *et al*., 1995; Karron *et al*., 1997; Chang and Rausher, 1998; Sun, 1999; Fishman et al., 2002; Suso et al., 2003; Steven and Waller, 2004; van Kleunen and Ritland, 2004). Second, the Rio population is close to an ecological reserve, indicating a more preserved locality

(Table 1); it has a high frequency of pollinator visits (Fig.7); and seed-set attributed to pollinators is relatively high (Fig. 6), suggesting high levels of outcrossing and therefore also a high pollen:ovule ratio. However, it is possible that estimations of gamete number, pollen:ovule ratio, corolla length and anther-stigma distance will be different at different times within the flowering season and on different years.

According to Cruden's classification of mating systems (1977) both *Nicotiana* species are predicted to be between obligate and facultative autogamous. That is, both species seem to have a mixed mating system, as has been documented in many other species (Dole, 1992; Stewart *et al*., 1996; Affre and Thompson, 1997; Vogler and Kalisz, 2001; Steven and Waller, 2004). The characterization of *N. longiflora* as intermediate between obligate and facultative autogamous (following Cruden's 1977 classification) contrasts with *N. longiflora*'s floral morphology, nectar traits (Kaczorowski, 2005) and the results of pollination treatments and pollinator preferences found here, which suggest a strong trend towards outcrossing. Further studies quantifying outcrossing rates with molecular markers will be needed to truly determine the mating system of *N. longiflora*.

Sympatry effects on floral traits

Anther-stigma distance was the only trait measured that was significantly affected by sympatry. ASD was not significantly different between sympatric *N. plumbaginifolia* populations and both allopatric and sympatric *N. longiflora* populations. However, ASD was significantly smaller in allopatric *N. plumbaginifolia* populations than both allopatric and sympatric *N. longiflora* populations. This result suggests that in sympatric populations, ASD of *N. plumbaginifolia* tends towards an increase, thus, favoring

outcrossing (Dole, 1992; van Kleunen and Ritland, 2004); however, anther-stigma distance did not differ significantly between sympatric and allopatric *N. plumbaginifolia* populations. Armbruster (1985) and Carter and Murdy (1986) also found significant effects of sympatry on ASD and several other floral traits of their study species. However, their results varied between species and in some cases were dependent upon the identity of the other species co-occurring in the community. Therefore, it seems that sympatry effects cannot be generalized, since the responses are species-specific.

The lack of sympatry effects on the other floral traits could be due to lack of power of the analyses. First, because sympatric populations were not that common, the analyses included only two sympatric populations. Second, coefficients of determination of regression models for the estimation of gamete number only explained 61% and 68% of the variability in pollen and ovule production, respectively. Also, all the sampled populations were relatively close to each other, and if the contrasting floral traits seen in these *Nicotiana* species is the product of recent evolution it will be hard to see any sympatry effects unless populations farther from this interaction zone are also included. Therefore, an increase in the number of populations and in the species distribution range will be necessary to increase the power of the analysis and thus determine if sympatry has any effects on floral traits associated with mating systems.

Natural seed set, selfing and pollinator effectiveness

Pollination experiments showed variability in seed set produced by different treatments, supporting the hypothesis that different pollination dynamics have different effects on each population, as in a geographic mosaic. As predicted, seed set attributed to

pollinators was significantly higher for *N. longiflora* than *N. plumbaginifolia*, whereas self seed set was higher for *N. plumbaginifolia*. This suggests that *N. plumbaginifolia* has evolved into a mostly selfing species, possibly as a mechanism of reproductive assurance. The occurrence of self-pollination as a mechanism of reproductive assurance is strongly favored whenever it does not cause gamete discounting (i.e., an increase in selfing rate does not occur at the expense of gametes available for outcrossing; Holsinger *et al*., 1984; Chang and Rausher, 1998). In delayed selfing the opportunities for selfing do not interfere with the occurrence of outcrossing. However, assurance of reproduction through self-pollination will be also favored early in anthesis when pollinators are rare (Kalisz *et al*., 2004). The difference in seed set between the control and emasculated treatments has been used to estimate reproductive assurance (Stewart *et al*., 1996; Herlihy and Eckert, 2002). In *N. plumbaginifolia*, seed set from control flowers was much higher than that obtained through emasculation treatments (Fig. 5), so selfing is occurring. Therefore, this difference and the absence of floral visitors (Oran), or pollinator preference for *N. longiflora* over *N. plumbaginifolia* (Mango) support the occurrence of reproductive assurance. Selfing as a mechanism of reproductive assurance due to the lack of pollinators has been recorded in other small-flower species, such as *N. plumbaginifolia* (Gómez, 2002; Elle and Carney, 2003).

Floral visitors seem to be effective pollinators for *N. longiflora* (Figs. 5, 6), as predicted based on floral morphology. However, *N. longiflora* in the Mango population showed a high percentage of seed-set due to self-pollination. Observations of pollinators showed a clear preference for *N. longiflora* over *N. plumbaginifolia* (Fig. 7b); thus, it was expected to find high levels of seed-set attributed to pollinators instead of selfing. It is

possible that pollen from *N. plumbaginifolia* can be delivered to *N. longiflora* stigmas, causing seed abortion and thus preventing hybridization (Waser, 1978; Campbell and Motten, 1985). It is also possible that anther emasculation caused a decrease in flower attractiveness to pollinators, thus affecting seed-set attributed to pollinators. Alternatively, emasculation might have caused early senescence of flowers as has been observed in the greenhouse (*pers. obs.*) and therefore the flowers could not be visited by pollinators and set seeds via outcrossing.

Estimations of pollinator-assisted seed-set (Table 2) suggest that a great percentage of seeds set by *N. plumbaginifolia* are the consequence of foraging activities of insects. This result contrasts with the lack of pollinators to *N. plumbaginifolia* flowers, especially those in the Mango population, where pollinators clearly preferred *N. longiflora* over *N. plumbaginifolia*. It is possible that small moths, and/or beetles that were observed foraging on the flowers of *N. plumbaginifolia*, but apparently without contacting the reproductive parts (*pers. obs*.), are causing the high percentages of pollinator-assisted selfing, rather than the hawkmoths observed mostly on *N. longiflora* flowers. Alternatively, it is possible that pollinator-assisted selfing might be confounded with delayed selfing in *N. plumbaginifolia*. In a previous greenhouse study, Soule (2007) showed that in *N. plumbaginifolia* anther dehiscence and, potentially also pollination, occur prior to anthesis; suggesting that this species experiences prior selfing. However, in *Agalinis neoscotica*, self-pollen deposition occurs in bud but pollen does not germinate until after anthesis, thus allowing outcross before self- pollination (Stewart *et al.*, 1996). In other species, changes in the relative position of stigma and anthers throughout the lifespan of the flowers (Kalisz *et al*., 1999) or corolla abscission late in anthesis (Dole,

1990), allow contact between anthers and stigma and therefore promote self-pollination. Further studies will be necessary to test these hypotheses in *N. plumbaginifolia*.

To summarize, pollination treatments showed significant variability among populations, supporting the hypothesis that different populations experience different pollinator dynamics. The results presented here clearly show that *N. longiflora* has a strong tendency towards outcrossing whereas *N. plumbaginifolia* is a selfing species.

Relationship between mating system and floral traits

Floral morphology is strongly associated with mating system, such that plants with showy flowers, large anther-stigma distance and high pollen:ovule ratio are predicted to be outcrossers, whereas the opposite is true for selfers (Cruden, 1977; Armbruster, 1985; Morgan and Barrett, 1989; Dole, 1992; Parker *et al*., 1995; Karron *et al*., 1997; Chang and Rausher, 1998; Sun, 1999; Fishman et al., 2002; Suso et al., 2003; Steven and Waller, 2004; van Kleunen and Ritland, 2004). Results provide only partial support for that pattern. Contrary to what has been shown in other studies (Armbruster, 1985; Dole, 1992; Fishman *et al*., 2002; van Kleunen and Ritland, 2004; Soule, 2007) the relationship between anther-stigma distance and selfing was not significant. However, corolla length and pollen:ovule ratio turned out to be relatively better predictors of mating system estimated as self-seeds via pollination treatments. Although regression models for both corolla length and pollen:ovule ratio were significant, their determination coefficients were relatively low, explaining only 37.5 and 29.8% of the variability in percent self-seeds, respectively. Those factors include time during the flowering season in which experimental treatments were conducted, maternal plant vigor, climatic conditions, and

nature of the locality (forested vs. along highways). The lack of a relationship between anther-stigma distance and selfing might be explained by the fact that such floral trait was measured before anther dehiscence and flower anthesis. In other studies where significant relationships between mating system and anther-stigma distance were found, measurements where taken after flower anthesis (Karron *et al*., 1997; van Kleunen and Ritland, 2004; Soule, 2007). In a study of floral development in *N. longiflora* and *N. plumbaginifolia* Soule (2007) showed that anther-stigma distance changes before and after anthesis. In this way, anther-stigma distance increases in *N. longiflora* after anthesis, but it decreases in *N. plumbaginifolia*, in which anthers pass over the stigma (Soule, 2007). Therefore, by estimating anther-stigma distance before anther dehiscence and flower anthesis, further elongation of the stamens and/or styles might be missed; thus my results in this respect are not strictly comparable to those of Soule (2007), who found a correlation between anther-stigma distance of mature *N. longiflora* flowers and selfing rate. The discrepancy between Soule's (2007) results and mine can be also influenced by the environment, as his study was conducted in the greenhouse whereas mine was carried out in natural populations.

Overall, the results of the current study showed that floral traits in *N. plumbaginifolia* and *N. longiflora* are correlated with the ability to self-pollinate and consequently with the effectiveness of pollinators. Floral traits and therefore mating system as estimated via pollination treatments were relatively variable among populations, as was expected under the hypothesis that selective pressures that act, or have acted in the past, vary among populations. Seed-set in these species seems to be partially determined by the availability of pollinators and their preferences but not by

sympatry; however, further studies that explore interpopulational variability through time and space will shed more light in mating system evolution, particularly in the evolution of selfing in *Nicotiana.*

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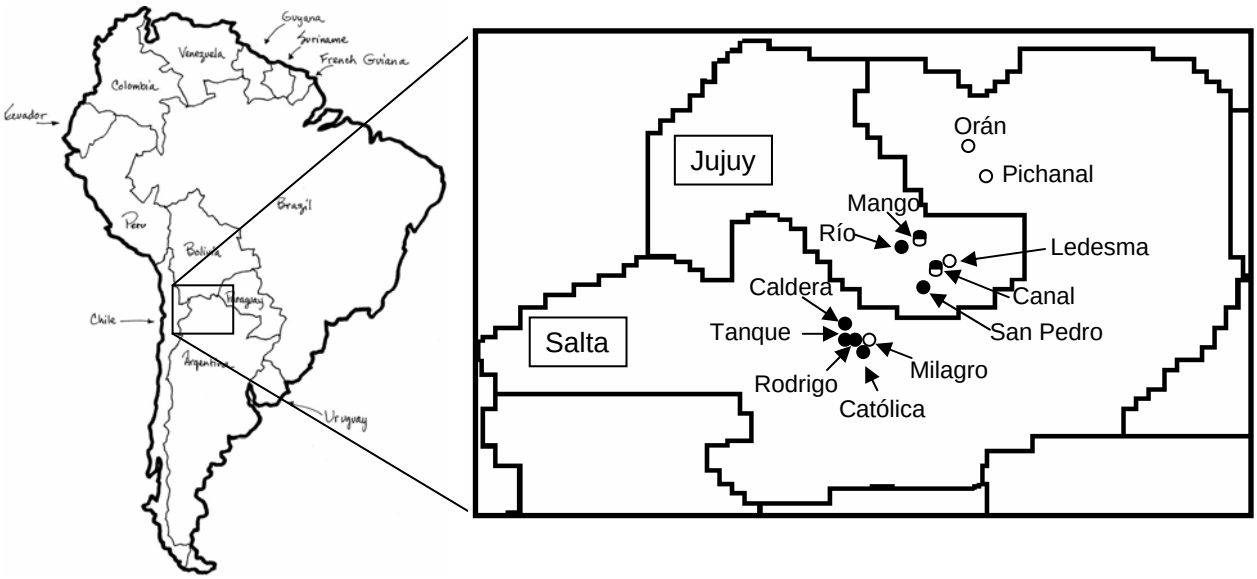

Figure 1. Geographic distribution of the *Nicotiana* populations studied in the Provinces of Salta and Jujuy, Argentina. Open, closed, and partially closed dots indicate pure *N. plumbaginifolia*, pure *N. longiflora* and sympatric populations, respectively.

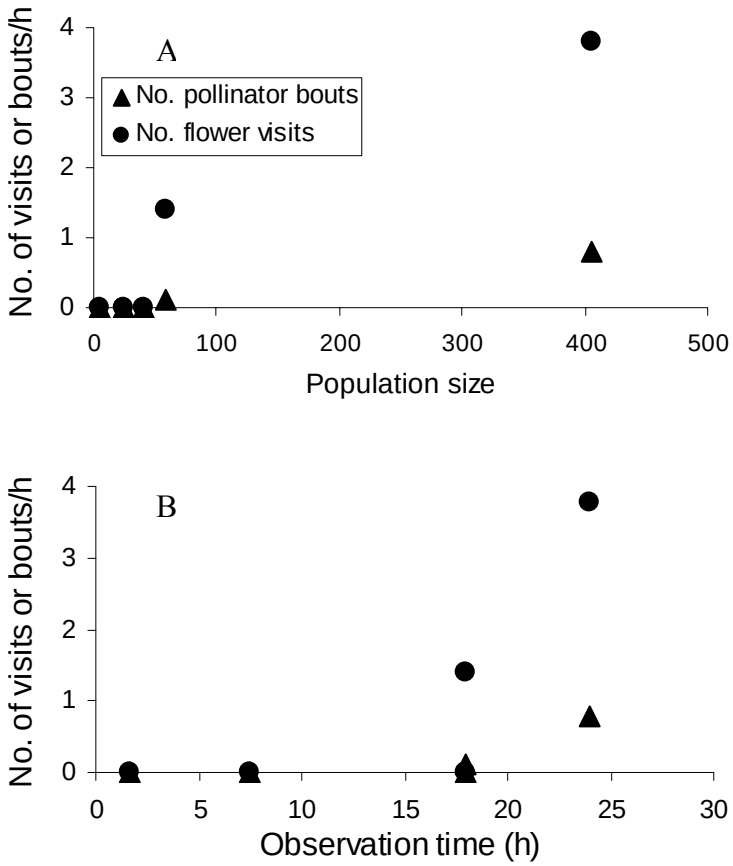

Figure 2. Number of pollinator visits (circles) and bouts (triangles) to populations of *N. longiflora* and *N. plumbaginifolia* as a function of A) observation time, and B) population size.

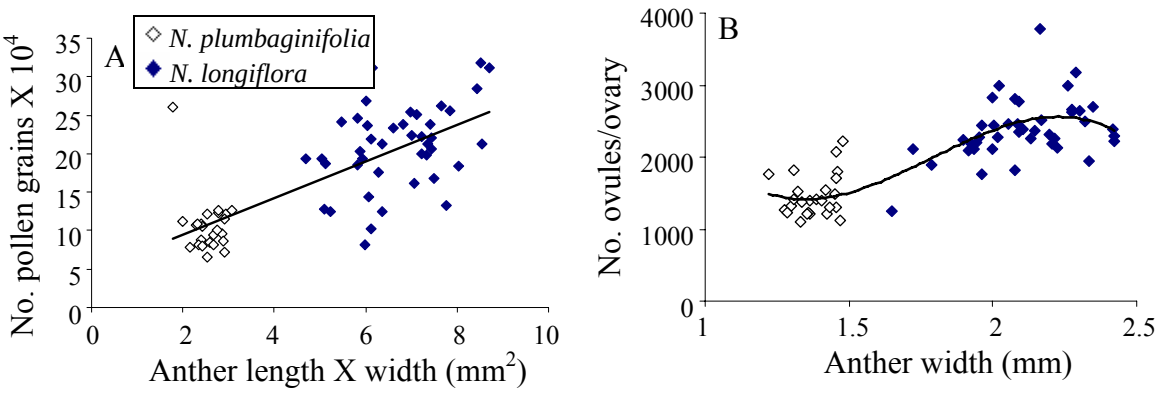

Figure 3. Best fit regression models to predict number of A) pollen grains, and B) ovules, per flower in natural populations of both *N. longiflora* and *N. plumbaginifolia*.

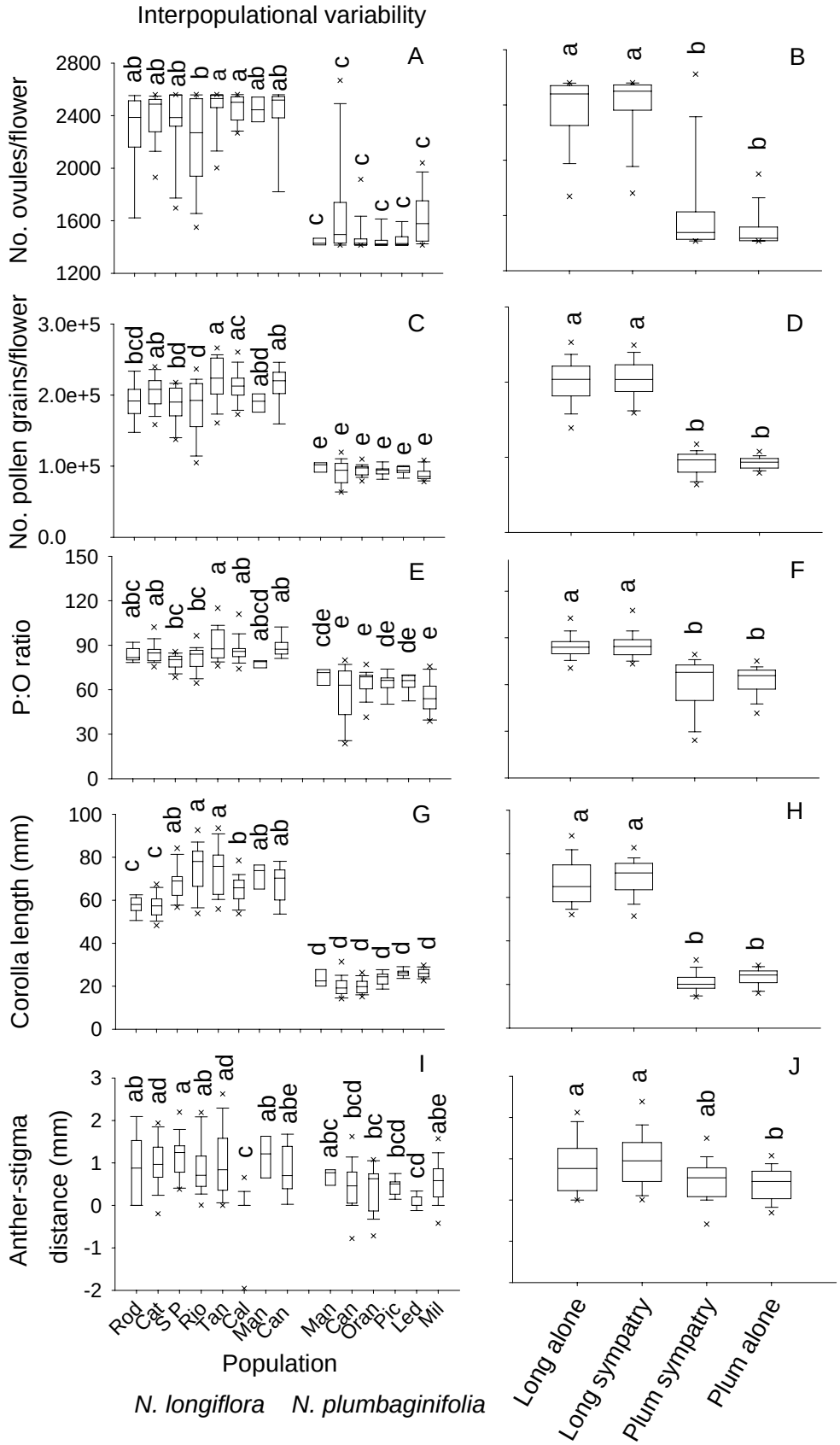
Interpopulational variability
No. ovules/flower
No. pollen grains/flower
P:O ratio
Corolla length (mm)
Anther-stigma distance (mm)
A
B
C
D
E
F
G
H
I
J
Rod
Cat
S P
Rio
Tan
Cal
Man
Can
Man
Can
Oran
Pic
Led
Mil
Population
N. longiflora
N. plumbaginifolia
Long alone
Long sympatry
Plum sympatry
Plum alone

Figure 4. Floral traits associated with mating systems plotted to show interpopulational variability (left column) and sympatry effects (right column) in *N. longiflora* and *N. plumbaginifolia*: A-B) number of ovules per flower; C-D) number of pollen grains per flower; E-F) pollen:ovule ratio per flower; G-H) corolla length, and I-J) anther-stigma distance. Pollen and ovule number were obtained with regression models developed from plants in natural populations. The boundaries of the boxes indicate the 25th and 75th percentile, the line within the box marks the median. Error bars indicate the 90th and 10th percentiles. "x" symbols below and above the boxes indicate the 5th and 95th percentiles (these are not estimated when sample size is below 9). Population names, except Rio and Oran were abbreviated as follows: Rod= Rodrigo, Cat= Catolica, S P= San Pedro, Tan= Tanque, Cal= Caldera, Man= Mango, Can= Canal, Pic= Pichanal, Led= Ledesma, Mil= Milagro. Long= *N. longiflora*, plum= *N. plumbaginifolia*. Different letters between populations indicate significant differences in pollen grains production (*post-hoc* Tukey test).

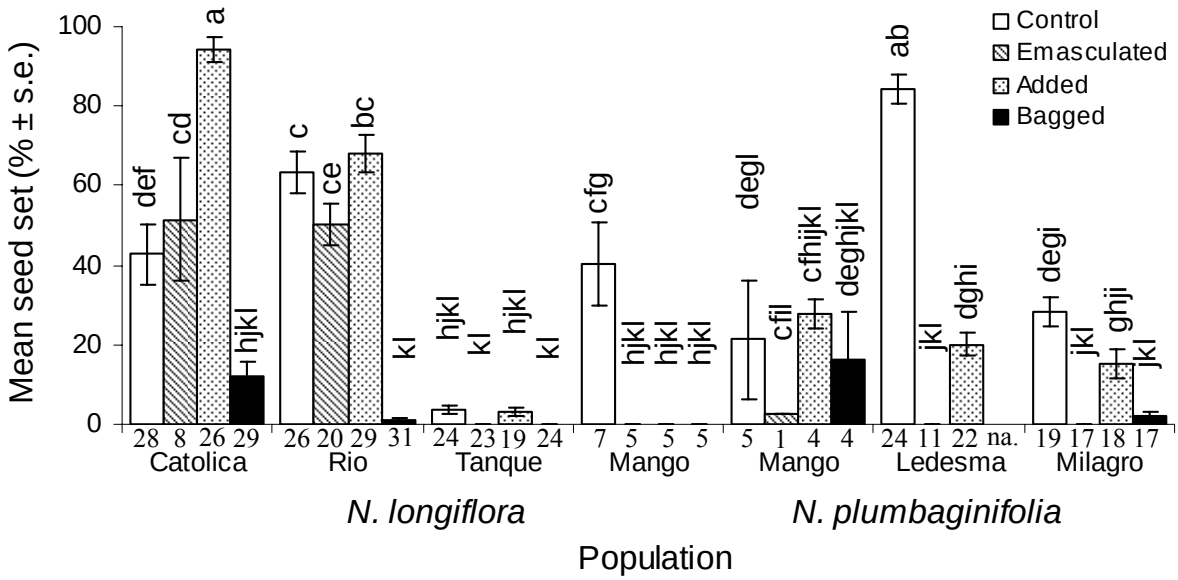

Figure 5. Variability in mean seed set obtained under different pollination treatments in various populations of *N. longiflora* and *N. plumbaginifolia*. Sample size (number of plants) is shown below each bar. Na= treatment not applied. Different letters indicate significant differences in seed set (*post-hoc* Tukey test).

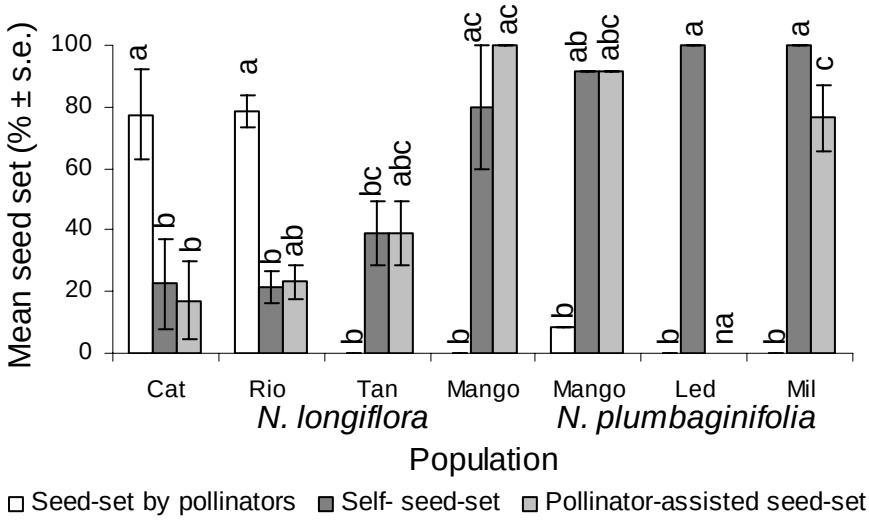

Figure 6. Percent seed set attributable to pollinators (open bars), total selfing (dark-grey bars) and pollinator-assisted selfing (clear-grey bars) estimated through pollination treatments in various populations of *N. longiflora* and *N. plumbaginifolia*. Na= treatment not applied. Different letters indicate significant differences among populations within seed-set estimate (*post-hoc* Tukey test).

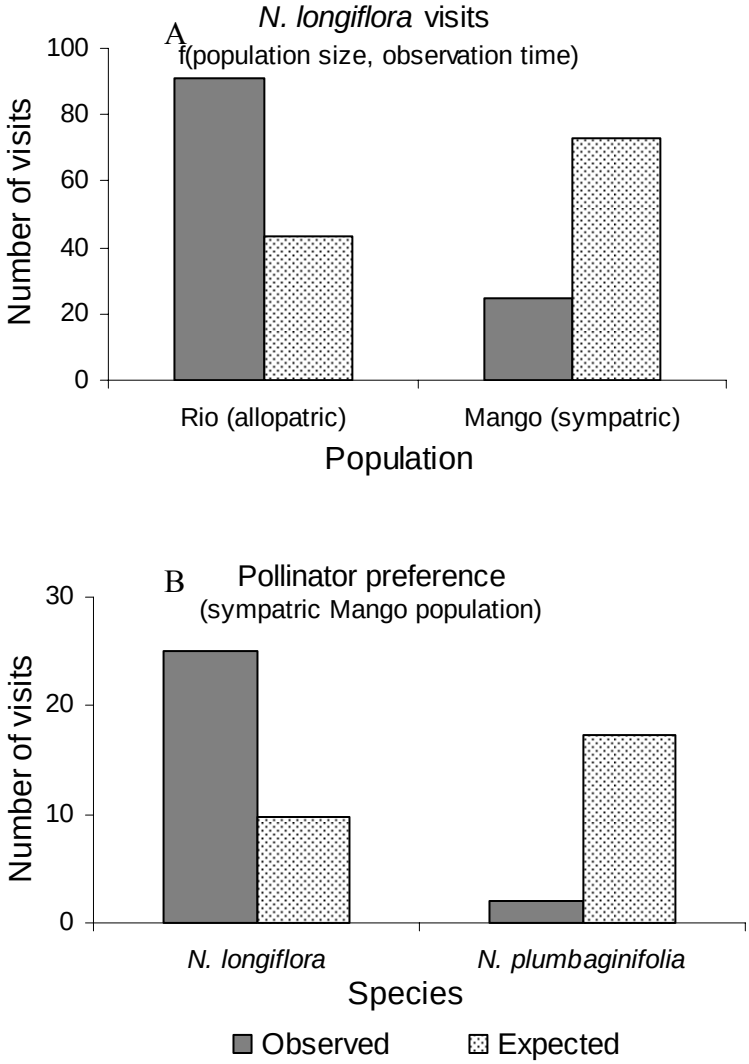

Figure 7. Number of pollinator visits to flowers of *Nicotiana* spp. comparing: A) number of visits to *N. longiflora* in allopatric (Rio) and sympatric (Mango) populations. Expected bars represent the number of visits as a function of population size and observation time. B) Number of visits to *N. longiflora* and *N. plumbaginifolia* in the sympatric Mango population. Expected bars indicate the number of visits as a function of the relative abundance of each species in the sympatric Mango population.

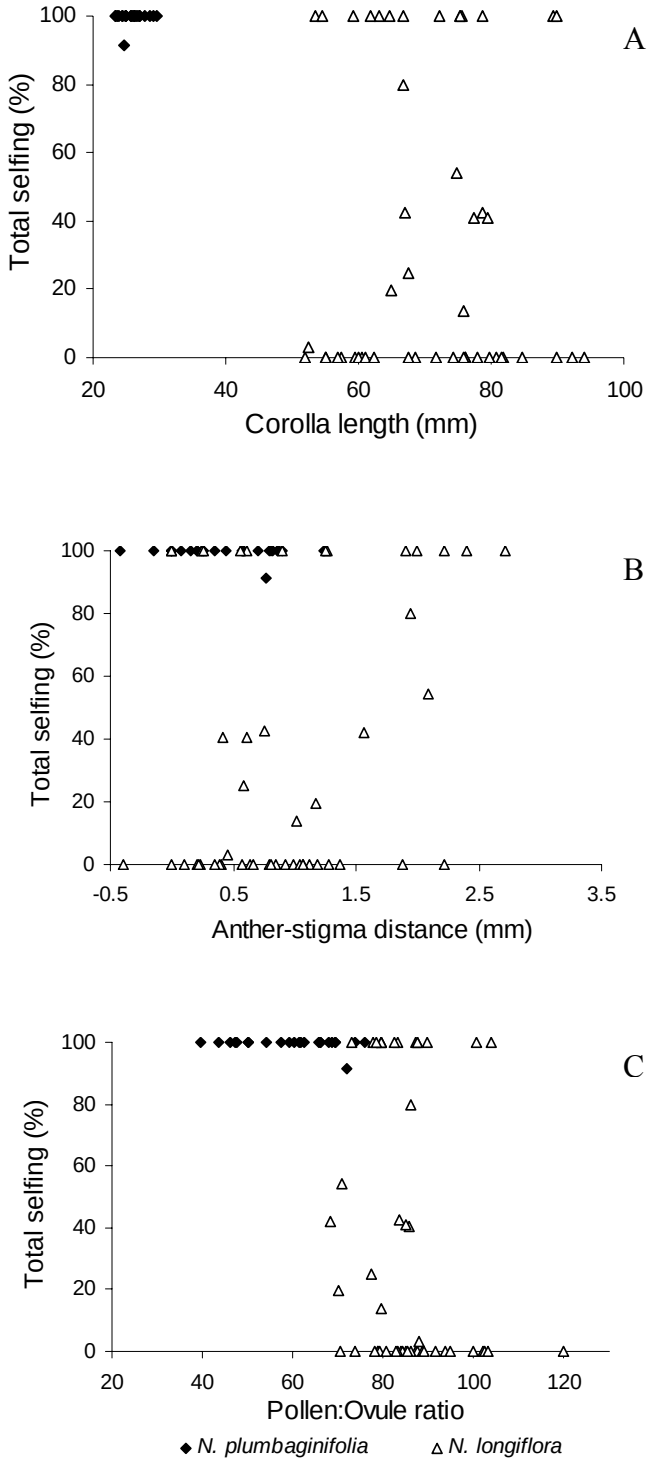

Figure 8. Association between percentage of self-seeds via pollination treatments and floral traits: A) corolla length, B) anther-stigma distance, and C) pollen:ovule ratio.

Table 1. General information for the populations of *N. longiflora* and *N. plumbaginifolia* sampled. Abbreviations for each population are in parenthesis. N= number of plants sampled in each population. Asterisks indicate those populations where observations of pollinators were also conducted.

Species	Name	Locality	Description	N	Altitude	Coordinates
N. longiflora	Caldera (Cal)	La Caldera, Salta	In town, rural road side	29	1405 m	S 24° 35' 52.3" W 65° 23' 08.6"
N. longiflora	Católica* (Cat)	Salta, Salta	In town, city road side	29	1223 m	S 24° 44' 29.2" W 65° 23' 58.6"
N. longiflora	Río*	Parque Nacional Calilegua, Jujuy	Forest edge	30	578 m	S 23° 45' 58.1" W 64° 50' 49.2"
N. longiflora	Rodrigo* (Rod)	Salta, Salta	In town, city road side	18	1233 m	S 24° 44' 37.26" W 65° 24' 45.59"
N. longiflora	San Pedro (S P)	San Pedro de Jujuy, Jujuy	In town, on highway	19	634 m	S 24° 13' 34" W 64° 52' 37"
N. longiflora	Tanque (Tan)	Salta, Salta	In town, city road side	25	1214 m	S 24° 45' 48" W 65° 24' 50.2"
N. longiflora *N. plumbaginifolia*	Canal (Can)	Ledesma, Jujuy	In town, on highway	14 19	492 m	S 23° 48' 36" W 64° 47' 46"
N. longiflora *N. plumbaginifolia*	Mango*	Parque Nacional Calilegua, Jujuy	Camping site	8 5	544 m	S 23° 46' 36.8" W 64° 49' 42.6"
N. plumbaginifolia	Ledesma (Led)	Libertador General San Martín, Jujuy	In town	11	486 m	S 23° 48' 09" W 64° 47' 16"
N. plumbaginifolia	Milagro (Mil)	Salta, Salta	In town	19	1224 m	S 24° 43' 20.77" W 65° 24' 25.14"
N. plumbaginifolia	Orán*	Orán, Salta	Park in town	21	358 m	S 23° 07' 41" W 64° 19' 50"
N. plumbaginifolia	Pichanal (Pic)	Pichanal, Salta	In town	15	307 m	S 23° 19' 10" W 64° 13' 25"

Table 2. Estimates derived from each pollination treatment or combination of treatments applied to natural populations of *N. longiflora* and *N. plumbaginifolia*.

Seed set estimate	**Treatment (or combination)**
Natural seed set	Unmanipulated; control
Seed set attributed to pollinators (outcross and/or geitonogamous)	Anther emasculation
Maximum seed set by outcrossing	Anther emasculation + outcross pollen
Spontaneous selfed seed set	Bagged
Pollinator assisted selfing	Total selfing (=unmanipulated - anther emasculation) - bagged
Total selfing (sponateous + pollinator assisted selfing)	Unmanipulated - Emasculation

Table 3. Results from the ANOVA analyses performed to test for the interpopulational variability in floral traits associated with plant mating systems in *Nicotiana longiflora* and *N. plumbaginifolia*.

Floral trait	**Species**	**Population**
Ovules	$F_{2,248}$=69.44; P<0.0001	$F_{11,248}$=62.34; P<0.0001
Pollen grains	$F_{2,248}$=9.11; P<0.0002	$F_{11,248}$=3.90; P<0.0001
Pollen:ovule ratio	$F_{2,248}$=47.27; P<0.0001	$F_{11,248}$=28.95; P<0.0001
Corolla length	$F_{2,248}$=264.62; P<0.0001	$F_{11,248}$=182.97; P<0.0001
Anther-stigma distance	$F_{2,248}$=2.89; P>0.05	$F_{11,248}$=9.57; P<0.0001

Table 4. Mean (± s.e.) number of pollen grains, ovules, pollen:ovule ratio, corolla length and anther-stigma distance estimated for various populations of *N. longiflora* and *N. plumbaginifolia*. N= number of plants sampled in each population. Also see Fig. 4.

Species	**Population**	**No. of pollen grains per flower (x10^5)**	**No. of ovules per ovary (x10^3)**	**Pollen : Ovule ratio**	**Corolla length (mm)**	**Anther-stigma distance (mm)**	**N**
N. longiflora	Caldera	2.13 ± 0.04	2.46 ± 0.019	86.59 ± 1.595	65.23 ± 1.19	-0.07 ± 0.11	29
N. longiflora	Católica	2.04 ± 0.04	2.39 ± 0.035	85.23 ± 1.304	57.34 ± 1.00	0.98 ± 0.10	29
N. longiflora	Río	1.82 ± 0.07	2.22 ± 0.06	81.23 ± 1.604	74.82 ± 2.00	0.90 ±0.11	30
N. longiflora	Rodrigo	1.90 ± 0.06	2.27 ± 0.07	83.91 ± 1.25	57.60 ± 0.94	0.92 ± 0.19	18
N. longiflora	San Pedro	1.86 ± 0.05	2.36 ± 0.06	79.05 ± 1.103	68.33 ± 1.59	1.15 ± 0.11	19
N. longiflora	Tanque	2.21 ± 0.06	2.46 ± 0.03	89.95 ± 2.161	74.75 ± 2.25	0.99 ± 0.16	25
N. longiflora	Canal	2.13 ± 0.07	2.40 ± 0.07	88.99 ± 1.985	67.67 ± 2.25	0.81 ± 0.15	14
N. longiflora	Mango	1.88 ± 0.05	2.41 ± 0.06	78.24 ± 1.256	71.52 ± 2.95	1.20 ± 0.25	8
N. longiflora	**Mean**	2.01 ± 0.02	2.37 ± 0.02	84.61 ± 0.65	66.99 ± 0.80	0.80 ± 0.06	
N. plumbaginifolia	Mango	0.99 ± 0.03	1.44 ± 0.01	68.88 ± 2.845	23.60 ± 2.06	0.68 ± 0.12	5
N. plumbaginifolia	Canal	0.91 ± 0.04	1.67 ± 0.09	58.27 ± 4.095	19.99 ± 0.98	0.44 ± 0.12	19
N. plumbaginifolia	Ledesma	0.94 ± 0.02	1.45 ± 0.02	65.15 ± 1.776	26.19 ± 0.52	0.07 ± 0.04	11
N. plumbaginifolia	Milagro	0.88 ± 0.02	1.62 ± 0.04	55.37 ± 2.486	26.05 ± 0.45	0.56 ± 0.11	19
N. plumbaginifolia	Orán	0.95 ± 0.02	1.47 ± 0.03	64.99 ± 1.825	19.79 ± 0.71	0.39 ± 0.11	21
N. plumbaginifolia	Pichanal	0.94 ± 0.02	1.45 ± 0.02	64.73 ± 1.88	23.49 ± 0.81	0.45 ± 0.05	15
N. plumbaginifolia	**Mean**	0.93 ± 0.01	1.54 ± 0.02	61.73 ± 1.23	22.76 ± 0.44	0.42 ± 0.05	

Table 5. Results from the ANOVA analyses performed to test for the effects of species, sympatry and the interaction species by sympatry on floral traits associated with plant mating systems in *Nicotiana longiflora* and *N. plumbaginifolia*.

Floral trait	**Species**	**Sympatry**	**Species x sympatry**
Ovules	$F_{1,258}$=430.08; P<0.0001	$F_{1,258}$=4.05; P=0.045	$F_{1,258}$=0.84; P>0.05
Pollen grains	$F_{1,258}$=650.18; P<0.0001	$F_{1,258}$=0.30; P>0.05	$F_{1,258}$=0.19; P>0.05
Pollen:ovule ratio	$F_{1,258}$=211.25; P<0.0001	$F_{1,258}$=0.13; P>0.05	$F_{1,258}$=0.49; P>0.05
Corolla length	$F_{1,258}$=979.28; P<0.0001	$F_{1,258}$=0.02; P>0.05	$F_{1,258}$=3.10; P>0.05
Anther-stigma distance	$F_{1,258}$=14.84; P<0.0001	$F_{1,258}$=1.48; P>0.05	$F_{1,258}$=0.16; P>0.05

Chapter 3. Interactions between *Nicotiana longiflora* and *N. plumbaginifolia*: effects on seed set, outcrossing rates and population structure

Abstract

Theoretical models have acknowledged the importance of species-species interactions mediated through pollinators, on the evolution of plant mating systems. However, there is a strong lack of empirical work in this field. Here I explore the effects of interactions between *Nicotiana longiflora* and *N. plumbaginifolia* on seed set and the genetic structure of mating within and among populations. These *Nicotiana* species are self-compatible with putatively contrasting mating systems. Allopatric and sympatric populations of both species can be found in Northern Argentina. In sympatric populations, the *Nicotiana* species have overlapping flowering seasons and share pollinators. Seed set was used as an estimator of female fitness. Outcrossing rates and population structure were estimated with codominant markers. The results show a negative effect of sympatry on seed set of *N. longiflora* but not on *N. plumbaginifolia*. The outcrossing *N. longiflora* had higher genetic diversity, outcrossing rates and heterozygosity, but lower fixation indices, biparental inbreeding and genetic differentiation than the selfer *N. plumbaginifolia*. Contrary to the expectations of a selfing species, *N. plumbaginifolia* in allopatry had high single-locus outcrossing rates. In sympatric populations, where more loci could be used to estimate outcrossing rates, *N. plumbaginifolia* showed outcrossing rates that were close to zero (0.001-0.046). Alleles typical of *N. longiflora* increased *N. plumbaginifolia*'s

genetic diversity in sympatric populations. Despite *N. plumbaginifolia's* tendency towards selfing, the results showed excess heterozygosity in mating structure, likely as a result of heterosis, asymmetrical hybridization, or segregation distortion. High genetic differentiation among *N. plumbaginifolia* populations is consistent with the expectations for selfing species. This study clearly shows that sympatry effects are species-specific and gene flow between species is asymmetrical.

Key words: mating system, competition, facilitation, pollinator-mediated interactions, *Nicotiana longiflora*, *Nicotiana plumbaginifolia*, sympatry.

Introduction

In natural populations, interactions between plants for floral visitors might be relatively common because different species of plants support, attract, and share pollinators. The output of interactions between species can be positive or negative in terms of both plant fitness (Rathcke, 1983) and mating system (Jain, 1976; Wyatt, 1983; Grant, 1994; Kay and Schemske, 2003). Additionally, movements of pollinators between species can lead to interspecific matings and thus affect the course of evolution if inter-species gene flow modifies the genetic variance within and among species.

Plant species might interact for pollinator visits through competition or facilitation (Rathcke, 1983; Waser, 1983). Competitive interactions occur when the presence of species A decreases pollinator visits to species B or when both species suffer a decrease in visits due to each other's presence (Brown and Kodric-Brown, 1979; Pleasants, 1980; Kephart, 1983; Rathcke, 1983; Rathcke, 1988a). This decrease in pollinator visits can

also have a negative effect on plant outcrossing rates, heterozygosity (Bell *et al*., 2005), and female and male fitness of at least one of the species, especially when pollinators are limited (Rathcke, 1983, 1988b; Kephart, 1983; Waser, 1983; Campbell, 1985; Feisinger and Tiebout III, 1991; Fishman and Wyatt, 1999; Moragues and Traveset, 2005). On the other hand, facilitation would occur when there is not pollen limitation and the presence of species A increases the visitation to species B at no cost to the species A (Rathcke, 1983; Feldman *et al*., 2004; Moeller, 2004; Moragues and Traveset, 2005). In this case, the increase in visitation rates promoted by the presence of a second species will also lead to an increase in fitness, outcrossing rates, and heterozygosity.

In sympatric populations of interfertile species, the evolutionary implications can be more drastic. If facilitative interactions between two interfertile species occur, outcrossing rates can increase due to interspecific pollinations; thus, hybridization can become relatively common unless isolation mechanisms are present. On the other hand, if the interaction between two interfertile species is competitive, a trend towards selfing and lower heterozygosity is expected (Bell *et al*., 2005).

Interactions between plants and pollinators are particularly interesting in the study of plant mating system evolution. For example, competitive interactions can cause a specialization for different pollinators or the independence of animal pollinators for reproduction (Waser, 1983). Moreover, if an obligate outcrossing species is strongly limited by pollen delivery, its female reproductive success will be seriously affected. In that case, selection will favor the evolution of self-fertilization in order both to assure reproduction and to minimize hybridization (Levin, 1971; Rathcke, 1988a,b; Fishman and Wyatt, 1999; Wendt *et al*., 2002; Goodwillie, 2001). For example, *Kalmia*

angustifolia (Rathcke, 1988b) and *Viscaria vulgaris* (Kwak and Jennersten, 1991) had an increase in self-fertilization when pollen was limited due to competition with coflowering species.

The importance of interactions mediated through pollinators on mating systems also has evolutionary implications because of pollinators' effects on mating structure. In populations where there is no pollinator limitation, outcrossing species have high gene flow within and among populations, and thus low differentiation among populations (Schoen, 1982; Holtsford and Ellstrand, 1989; van Treuren *et al*., 1993; Williams *et al*., 2001; Casiva *et al*., 2004; DeWoody *et al*., 2004; Iddrisu and Ritland, 2004; Ledig *et al*., 2005). Because these species have high outcrossing rates, low fixation indices and high heterozygosity levels are also common (Ellstrand *et al*., 1978 Ennos, 1981; Shea, 1987; van Treuren *et al*., 1993; Gaiotto *et al*., 1997; Kittelson and Maron, 2000; Lee *et al*., 2000; Williams *et al*., 2001; Rajora *et al*., 2002; Galloway *et al*., 2003; DeWoody *et al*., 2004; Iddrisu and Ritland, 2004; Travis *et al*., 2004; Ledig *et al*., 2005). By contrast, primarily selfing species have low outcrossing rates -therefore low heterozygosity and high fixation indices- and because gene flow between and among populations is strongly limited, high differentiation among populations is expected (Ennos, 1981; Holtsford and Ellstrand, 1989; Agren and Schemske, 1993; Penteado *et al*., 1996).

Although the importance of inter-specific interactions mediated through pollinators on the evolution of mating systems has been acknowledged in theoretical studies (Jain, 1976; Wyatt, 1983; Grant, 1994; Kay and Schemske, 2003), empirical work in natural populations is rare. To my knowledge, there is only one such study in which the effects of interactions mediated through pollinators on plant fitness and outcrossing

rates have been addressed through experimental work (Bell *et a*l., 2005). In that study, experimental gardens were used to determine how the presence of *Lobelia siphilitica* affects both plant fitness and outcrossing rates of *Mimulus ringens*. Two experimental arrays were composed of pure *M. ringens* and two others had both species as a replication of sympatric populations. In mixed-species arrays, *M. ringens* showed not only a decrease in seed production, but also lower outcrossing rates, probably as a consequence of the high percentage of between species pollinator movements.

Nicotiana longiflora and *N. plumbaginifolia* are sister species (Lim *et al*. 2006), whose geographic distributions overlap in northwest Argentina, so that allopatric and sympatric populations can be found. Both species are self-compatible and inter-crossable in the greenhouse (East, 1916; Goodspeed, 1954; Chapter 4). *Nicotiana longiflora*'s variability in pollen:ovule ratio, herkogamy and corolla length and its great dependence upon pollinators for set seed indicated it is an outcrosser species (Chapter 2). On the other hand, *N. plumbaginifolia* lacks variability in floral traits associated with mating system and does not depend upon pollinators for set seed, suggesting it is a highly selfing species (Chapter 2). Because in sympatric populations hawkmoths visit both species (Chapter 2), it is possible that interspecific pollinations might be occurring, therefore influencing outcrossing rates, and consequently, population structure and the possibilities for hybridization between the species. Given that *N. longiflora* is an outcrosser, high heterozygosity and low differentiation are expected. In a similar way, high inbreeding, low heterozygosity, and high differentiation among *N. plumbaginifolia* populations are expected. However, if interactions between the species are taking place, then different

patterns could be detected when comparing estimations from allopatric and sympatric populations.

The main goal of this study is to determine the effect of interspecific mating interactions, mediated through pollinators, on plant fitness (estimated as seed set) and outcrossing rates of both *N. longiflora* and *N. plumbaginifolia*. The particular goals are: i) to determine if sympatry affects fitness estimated as seed set, genetic diversity, outcrossing rates, heterozygosity and fixation indices of the study species, ii) to explore the possibilities for an increase in outcrossing rates as a consequence of interspecific fertilizations in sympatric populations, and iii) to characterize the population structure of allopatric and sympatric populations of both *Nicotiana* species through the estimation of F statistics.

Methods

Study species and populations

Nicotiana longiflora and *N. plumbaginifolia* (Solanaceae) are each other's closest relatives, forming a strongly supported two-species clade within section *Alatae* (Ippolito, 2000; Chase *et al*., 2003; Clarkson *et al*., 2004; Lim *et al.* 2006). These species are self-compatible and inter-crossable in the greenhouse (East, 1916; Goodspeed, 1954; Chapter 4). *Nicotiana longiflora* flowers are 40-120mm in corolla length, with 21.3 ± 0.3% solids in sucrose dominant nectar. On the other hand, *N. plumbaginifolia* has shorter flowers, ranging from 25 to 35 mm in length, with 19.3 ± 1.1% solids in sucrose-rich nectar (Goodspeed, 1954; Kaczorowski, 2005). In both species, nectar concentration fits within the range for hawkmoth pollinated species (Baker and Baker, 1982). However, nectar

production, the ratio sucrose to glucose+fructose and total energy are lower for *N. plumbaginifolia* (Kaczorowski, 2005). A study on floral organ development of these two species showed a negative anther-stigma distance (ASD) for mature flowers of *N. plumbaginifolia* and high levels of autogamy, whereas *N. longiflora* showed a small positive ASD and a rather low percentage of autogamy (Soule, 2007). These *Nicotiana* species co-occur in sympatry in northern-Argentina, from where *N. longiflora* distributes to the South and Southeast of the continent, whereas *N. plumbaginifolia* spreads out to the North (Goodspeed, 1954). The co-occurrence of the two species in sympatric populations, their overlap in flowering seasons and pollinator sharing suggest that interactions mediated through pollinators are common.

A total of twelve populations (five from each species in allopatry and two sympatric populations, Mango and Canal) were sampled for the estimation of seed set as a rough estimate of plant fitness (Fig. 1, Table 1). Sampling was conducted on October-November, 2005. All of the populations were located in northern Argentina, in the Provinces of Salta and Jujuy. Outcrossing rates and population structure were estimated for six populations, two from each species in allopatry (*N. longiflora*: Católica and Río; *N. plumbaginifolia*: Milagro and Ruta) and the two populations in which the species co-occur in sympatry (Fig. 1).

Seed set estimation

Percent seed set was estimated as number of seeds per ovule per flower; therefore I first estimated seed and ovule number. In order to estimate seed number, between three to five randomly selected indehiscent fruits per plant per population were collected, for a total of

28 to 88 fruits per population. Fruits were collected right before seed dispersal and stored in uncapped 1.5 ml microcentrifuge tubes to allow fruit dehiscence and prevent fungal growth. Dry fruits were brought back to the laboratory where seed number per fruit was estimated by first weighing 100 seeds per fruit and then extrapolating to the total number of seeds per fruit.

To determine number of ovules per flower, anther width of plants grown in the field was measured, as indicated by a previously developed regression model through which female gametes can be estimated by measuring other floral traits [#ovules/ovary= 17 720 + (-30 308) anther width + 18 046 (anther width)^2 + (-3364.62) (anther width)^3; Adj. R^2=0.68]. Therefore, the estimation of ovules per flower was conducted applying the regression model to measures of anther width. One to two indehiscent anthers per flower from two flowers per plant were measured in each plant sampled. Except in the Ruta population, in which the flowering season had already finished. Therefore, I estimated the number of ovules as the average of all other allopatric *N. plumbaginifolia* populations.

Percent seed set was estimated for each fruit and average seed set per plant was used for statistical analysis. To test for seed-set variability among populations, I used ANOVA with population nested within species as independent variables. A second ANOVA with species and sympatry as fixed effects was applied to test for sympatry effects. Analyses could not be combined because populations were nested within the species variable. *Post-hoc* Tukey analyses were performed to identify significant differences among groups. To improve normality of data, percentage seed set was transformed as arcsin $(x)^{0.5}$ (Zar, 1999).

Marker development

Codominant cleaved amplified polymorphic sequence (CAPS) markers were used to estimate outcrossing rates and population structure. Primer sequences were obtained from *Nicotiana alata* (NA; five sequences) and *N. plumbaginifolia* (NP, eight sequences) sequences in genbank and from *Arabidopsis thaliana* (eight sequences) conserved ortholog set (COS) sequences. Most markers (15 out of 21) were developed by amplifying and then sequencing potential marker loci from a few *N. longiflora* and *N. plumbaginifolia* plants. Then I determined restriction enzyme cut sites and enzymes using the DNASTAR program (Table 2). Six loci were assayed for polymorphism by conducting digestions of amplified products with a random set of 31 digestion enzymes (Table 2).

Only one polymorphic locus was detected in allopatric populations of *N. plumbaginifolia*, whereas nine polymorphic markers were found in *N. longiflora* (Table 2). However, three of them showed inconsistencies in banding patterns; therefore I used only six markers for the estimation of outcrossing rates and population structure in *N. longiflora* (Table 3).

DNA extraction, PCR and plant genotyping

From the twelve populations originally sampled, I chose seeds collected from six populations in 2005, two pure *N. longiflora*, two pure *N. plumbaginifolia* and two sympatric for the estimation of outcrossing rates and population structure. Seeds were pre-scarified with 70% bleach and rinsed twice with sterilized water. Afterwards, seeds were rinsed and planted with 500 µl of 750 ppm gibberelin solution in trays with pre-

mixed soil and covered with vermiculite. Plants were watered every other day and grown in the controlled environment of the greenhouse (14h days at 24ºC and 10 h nights at 13ºC at the University of Missouri-Columbia). Seeds from each maternal plant from each population were planted, for a total of 73 and 86 maternal families for *N. plumbaginifolia* and *N. longiflora*, respectively. However, not all of the seeds germinated, so the number of maternal families screened was decreased to 50 for *N. plumbaginifolia* and 66 for *N. longiflora*. At least six offspring per maternal family in *N. longiflora* were screened, except in a few cases [ranging from 1 (one family) to 17 (one family)]. Because *N. plumbaginifolia* was suspected to be a strong selfer, at least ten offspring per maternal family were genotyped for this species, except in a few cases [ranging from 3 (one family) to 13 (one family)]. However, in some families, offspring failed to grow enough to conduct DNA extractions; therefore, sample size decreased to a total of 584 offspring for *N. plumbaginifolia* (329 and 255 from allopatric and sympatric populations, respectively) and 456 for *N. longiflora* (326 and 130 from allopatric and sympatric populations, respectively; Table 4).

As soon as the seedlings had enough vegetative tissue (around a month after seed planting), DNA was extracted from leaves following the next protocol: tissue was ground in 300µl extraction buffer with β-mercaptoethanol, 300µl lysis buffer and 100µl sarkosyl; then incubated for 10 min at 65˚C; extracted twice with 24 chloroform:1 isoamyl alcohol; rinsed with 76% ethanol-10mM ammonium acetate and stored in 1/10 TE/RNase.

Six polymorphic markers were used to genotype all populations of *N. longiflora* and the sympatric populations of *N. plumbaginifolia*: i) self-incompatibility associated locus digested with Fok I (NA4); ii) cellulose synthase D-like protein mRNA digested

with Hha I (NA6); iii) Cu-Zn superoxide dismutase digested with Hha I (NP4); iv) mRNA glutathione S-transferase digested with Ahd I (NP6); v) chlorophyll a oxygenase digested with Nde I (COS-10), and vi) similar to tRNA(1-methyladenosine) methyltransferase digested with EcoR I (COS-16). Because markers COS-10 and COS-16 showed polymorphism in sympatric *N. plumbaginifolia* populations, I also screened allopatric populations of this species for these two markers in addition to NA6 which was the only polymorphic marker for this species detected originally.

PCR amplifications were conducted using 1:10 DNA dilutions. All NA and NP sequences were amplified per 39 cycles at 2.75 min at 94˚C; 1min at 60˚C, 1.5min at 72˚C. COS sequences were amplified per 39 cycles at 2.75 min at 94˚C; 1min at 55˚C, 1min at 72˚C. Amplified products were digested during two hours to overnight, ran on 1.5% agarose gels for 2.5 h and visualized with UV light.

Mating system

Multilocus (t_m) and single locus (t_s) outcrossing rates were estimated for all populations, using the program, MLTR version 3.0 written by Ritland (2002). Standard errors of mating system parameters were calculated from the standard deviation of 1000 bootstrap estimates. Families within populations were resampled and gamete frequencies were constrained to be equal among sexes. Because multilocus estimates of outcrossing are less sensitive to mating among relatives than single locus estimates, a positive difference between t_m and t_s provided an estimator of biparental inbreeding (Ritland, 2002). Expected and observed heterozygosity (H_o and H_e, respectively) and fixation indices [$F= 1-(H_o/ H_e)$] per loci were estimated in GENALEX 6.0 (Peakall and Smouse, 2006). H_e is

an estimate of the expected allelic diversity under the Hardy-Weinberg equilibrium. Expected fixation indices under equilibrium were estimated as F_{eq}= (1-t)/(1+t). F_{eq} was estimated per locus and per population. Also, observed gene frequencies were tested against the expected frequency under the Hardy-Weinberg equilibrium using GENALEX 6.0 (Peakall and Smouse, 2006).

Population structure

Estimations of F_{is} and F_{it}, the deviation of heterozygosity from Hardy-Weinberg expectations within single populations and in all the populations, respectively, as well as the estimation of genetic differentiation over populations, F_{st}, were obtained for each loci per species using the program GENALEX 6.0 (Peakall and Smouse, 2006). In order to test for significant differences in the three F-statistics between species *t*-tests were conducted. F_{is} and F_{it} vary from -1 to 1, indicating that heterozygotes are over-represented and deficient in the population, respectively. F_{st} only has positive values; the greater the value the more differentiated the populations are. The same program also provided a pairwise F_{st} matrix to estimate differentiation among all populations within and between species.

Results

Seed set

There was significant variability in percent seed set among populations ($F_{13, 280}$= 12.31, P< 0.0001). Both population ($F_{2, 280}$= 26.09, P< 0.0001) and species ($F_{11, 280}$= 10.79, P< 0.0001) had a significant effect on fitness estimated as seed set (Fig. 2). The Milagro

population had the highest percentage seed set, which was not significantly different from that in Catolica, Rio, Tanque, Oran, Pichanal, Ledesma, and *N. plumbaginifolia* seed set in Mango and Canal (Fig. 2). The lowest percentage seed set was recorded for *N. longiflora* in San Pedro and in both sympatric populations (Fig. 2a).

Pooling data from allopatric and sympatric populations within species to test for sympatry effects on seed set was also significant ($F_{3, 290}$= 19.67, P< 0.0001; Fig. 2b). Seed set was significantly affected by species ($F_{1, 290}$= 42.76, P< 0.0001), sympatry ($F_{1, 290}$= 22.06, P< 0.0001) and the interaction of species by sympatry ($F_{1, 290}$= 21.21, P< 0.0001). Mean seed set was significantly lower for *N. longiflora* in sympatric populations (27.44 ± 3.8 %) compared to *N. longiflora* in allopatry (62.93 ± 2.5 %) and *N. plumbaginifolia* in both sympatry (70.22 ± 3.4 %) and allopatry (71.22 ± 2.0; Fig. 2).

Genetic diversity and heterozygosity

Polymorphism was highly variable among loci within species. In *Nicotiana longiflora*, all six loci were polymorphic in the sympatric populations as well as in the allopatric population Rio (Table 4). However, in the population Catolica, only four loci were polymorphic (COS-10, COS-16, NP4 and NP6).

Overall, there were several loci that did not fit to the Hardy-Weinberg expectations. In the sympatric population Canal, all but the NP6 locus fitted the expectations of Hardy-Weinberg. In Mango, Rio, and Catolica, the allele frequencies of the NP4 and NP6 loci significantly departed from the Hardy-Weinberg equilibrium. In addition, COS-10 in Mango, COS-16 in Rio, and both COS-10 and COS-16 in Catolica showed a significant departure from Hardy-Weinberg (Table 4).

N. plumbaginifolia had fewer polymorphic loci. The allopatric Ruta and Milagro as well as the sympatric Canal populations, were polymorphic for only the NA6, COS-10 and NA6 loci, respectively (Table 4). The sympatric Mango population was polymorphic for three out of six loci (COS-10, COS-16, NA6). The NA6 locus, which was the only polymorphic locus for all *N. plumbaginifolia* populations, had a significant deficiency of heterozygotes compared to the expected frequencies under the Hardy-Weinberg equilibrium in Ruta, Canal and Mango populations (Table 4).

Heterozygosity was also variable among loci within populations, mainly in *N. longiflora* in which the observed frequency of heterozygotes was higher than the expected in most cases. In particular, a higher frequency of heterozygotes than expected was observed for the NP4 and NP6 loci in all populations (Table 4). On the contrary, in Mango, Rio and Catolica, the heterozygosity for the COS-16 locus was lower than expected.

In *N. plumbaginifolia* the differences between observed and expected frequency of heterozygotes were not as common. In Ruta, Canal and Mango, there was a deficiency of heterozygotes for the NA6 locus. The only other difference between expected and observed frequency of heterozygotes was found for the COS-10 locus in the population Mango, in which there was a slight overrepresentation of heterozygotes (Table 4).

Mating system

Outcrossing rate estimations for each locus were highly variable in both species. In *N. longiflora* there was a general trend towards high outcrossing rates for all loci in all populations, except for COS-16 and NP4 in the Catolica population which showed an

outcrossing rate close to zero (Table 4). Accordingly, there was a strong trend towards very low fixation indices, in most cases they were strongly negative and the few positive indices were very close to zero (Table 4). In the Catolica population, however, fixation indices per locus were as variable as outcrossing rates. Fixation indices varied from -1 to 1 and the most striking result was for the NP4 locus, in which outcrossing rates were close to zero and the fixation index was -1, indicating a strong preference for the formation of heterozygous genotypes, or in other words, outcross fertilization.

Overall, estimated fixation indices for both species were consistent with the expected fixation indices in equilibrium based on outcrossing rates (Table 4). However, the NP4 and NA6 loci in the Catolica population of *N. longiflora* and in the Ruta population of *N. plumbaginifolia* showed very strong departures from the expectations under equilibrium (NP4: F= -1.0, F_{eq}= 0.998; NA6: F= 1.0, F_{eq}=0.117).

On a per population basis, all *N. longiflora* sympatric and allopatric populations had high multilocus (1.164-1.2) and single locus (0.882-1.2) outcrossing rates (Table 5; Fig. 3). The mean multilocus estimations of outcrossing rate for both allopatric and sympatric populations were 1.18. Mean single-locus outcrossing rate estimations were 1.20 and 1.02 for sympatric and allopatric populations, respectively. In all populations, fixation indices were slightly below zero, which was consistent with the expectations for populations with high outcrossing rates; high outcrossing rates were also indicated by the expected fixation indices under equilibrium (Table 5). Biparental inbreeding estimates, calculated from the difference between t_m and t_s, were slightly negative for Canal and equal to zero for the Mango and Rio populations, suggesting that mating among relatives is avoided in these populations. However, the estimate of biparental inbreeding was

positive for Catolica, indicating that some mating among relatives occurs in this population (Table 5).

In *N. plumbaginifolia*, oucrossing rates for both allopatric populations and both COS-10 and COS-16 in the sympatric Mango population were high (0.79-1.2). However, the NA6 locus in both of the sympatric populations had a low outcrossing rate (Canal: 0.001; Mango: 0.017) (Table 4). Accordingly, fixation indices were high for those loci with low outcrossing rates and low for the ones with high outcrossing rates (ranging from -0.122 for COS-10 in Milagro to 1 for NA6 in both Canal and Ruta). The relationship between outcrossing rate and fixation index for the only polymorphic marker in the Ruta population did not conform to the expectations under inbreeding equilibrium. In this population, the outcrossing rate is relatively high (0.79) but the fixation index is also high (1).

Comparisons at the population level showed that both allopatric populations have high single-locus estimation rates with an average of 0.956 (Table 5; Fig. 3). The mean fixation index for both allopatric populations was 0.439 (Table 5). Because estimations of outcrossing rates were conducted with data of only one polymorphic locus, biparental inbreeding was not estimated for the allopatric and the sympatric Canal populations of *N. plumbaginifolia* (Table 5). Multi- and single-locus outcrossing rates estimates for the sympatric *N. plumbaginifolia* populations were low with a mean of 0.021 and 0.023, respectively (Table 5; Fig. 3). In the Mango population and the mean for both sympatric populations, biparental inbreeding was negative. Fixation indices were positive in both sympatric populations (Canal: 1, Mango: 0.306), with a mean of 0.653 (Table 5).

Population structure

F-statistic estimations showed contrasting patterns between the *Nicotiana* species. In *N. longiflora* all loci but COS-16 and NA6 showed an excess of heterozygotes (average F_{is}= -0.217, average F_{it}= -0.103; Table 6). Accordingly, F_{st} estimations showed low differentiation among populations (average F_{st} = 0.108). Estimations of F_{is} for *N. plumbaginifolia* indicated an excess of heterozygotes for the COS-10 and COS-16 loci, but an excess of homozygotes for NA6, with an average over loci of 0.288 (Table 6). *N. plumbaginifolia*'s F_{it} estimations indicated a deficiency of heterozygotes for all loci but COS-16, with an average of 0.794. F_{st} estimations indicated a high differentiation among populations for all loci, with an overall mean over loci of 0.742 (Table 6).

Pairwise F_{st} estimations showed low genetic differentiation among all *N. longiflora* populations (range between 0.02-0.14) as well as for *N. plumbaginifolia* when comparing the sympatric populations (F_{st}= 0.01; Table 7). Intermediate F_{st} values were found for pairwise comparisons between the two *Nicotiana* species in the sympatric populations (0.21-0.27). Among allopatric populations, moderate to high differentiation was detected when comparing *N. longiflora* and *N. plumbaginifolia* (0.51-0.81, Table 7). Comparisons among *N. plumbaginifolia* populations revealed high differentiation (0.94).

Discussion

Seed set was strongly and significantly reduced in sympatric populations of *N. longiflora*. Allopatric and sympatric populations of *N. plumbaginifolia* did not differ in mean seed set. Genetic diversity, heterozygosity, fixation indices and population structure fit the expectations of species with contrasting mating systems as *N. longiflora* and *N.*

plumbaginifolia. The outcrosser *N. longiflora* had higher genetic diversity and heterozygosity, but lower fixation indices and genetic differentiation among populations than the selfer *N. plumbaginifolia*. *Nicotiana longiflora* also had high outcrossing rates; however, and contrary to the expectations, *N. plumbaginifolia* in allopatry had high outcrossing rates. As expected, biparental inbreeding estimates were low for *N. longiflora*. Overall, sympatry seems to have a negative effect on *N. longiflora*'s seed set, but not on all other parameters. On the other hand, sympatry did not affect *N. plumbaginifolia*'s seed set, but increased its polymorphism (at least for one sympatric population) and reduced its outcrossing rates. However, the single-locus outcrossing rates for allopatric *N. plumbaginifolia* populations may have been artificially high if segregation was affected by heterozygote advantage or segregation distortion.

Seed set

The results showed that seed set in *Nicotiana longiflora* is negatively affected by the presence of *N. plumbaginifolia* (Fig. 2), such as has been demonstrated for other plants sharing pollinators with other species when growing in sympatry (Kephart, 1983; Rathcke, 1988b; Fishman and Wyatt, 1999; Bell *et al*., 2005). Therefore, it seems that *N. plumbaginifolia* somehow interferes with the reproductive success of *N. longiflora*. Observations of pollinators in the sympatric Mango population (Chapter 2) demonstrated that hawkmoths visit both species. However, the frequency of visitation to *N. longiflora* is much higher than to *N. plumbaginifolia*. Other studies on sympatric populations have demonstrated that pollinators visit several species indiscriminately (Brown and Kodric-Brown, 1979; Pleasants, 1980; Schemske, 1981; Kephart, 1983; Ramsey *et al*., 2003; Bell

et al., 2005; Jacobi *et al*., 2005; Moragues and Traveset, 2005). Under this scenario, heterospecific pollinations might be common, causing pollen-pollen interactions that could lead to a decrease in seed-set (Brown and Kodric-Brown, 1979; Schemske, 1981; Kephart, 1983; Kohn and Waser, 1985). This could be an explanation for the decrease in seed-set experienced by *N. longiflora* in sympatric populations. Another possibility is that stigmas or styles get clogged as a consequence of the delivery of heterospecific pollen, thus reducing seed set (Cruzan, 1990; Erbar, 2003). Alternatively, it is possible that selective abortion by maternal plants occurs as a postzygotic barrier to hybridization (Charlesworth, 1988; Erbar, 2003). However, it is very unlikely in *N. longiflora* because this species strongly selects outcross intra-species pollen grains for ovule fertilization (Chapter 4).

Seed set in *N. plumbaginifolia* was not significantly affected by sympatry. Similar results have been found for other species sharing pollinators in sympatric populations (Fishman and Wyatt, 1999; Moragues and Traveset, 2005). The lack of sympatry effects on *N. plumbaginifolia*'s seed set suggests that selfing is an effective mechanism that allows the species to reproduce even when it is not visited by pollinators (Rathcke, 1988a, b). However, given that *N. plumbaginifolia* does not show any mechanisms to avoid inter-specific fertilizations (Chapter 4), it is possible that some percentage of seed set recorded here was produced via hybridization.

Genetic diversity, heterozygosity, mating system and population structure

As expected, *N. longiflora*, the outcrossing species had higher genetic diversity, outcrossing rate and heterozygosity and lower fixation indices and population

differentiation. Other studies with outcrossing species have found similar results (Ellstrand *et al*., 1978; Ennos, 1981; Shea, 1987; Holtsford and Ellstrand, 1989; van Treuren *et al*., 1993; Gaiotto *et al*., 1997; Kittelson and Maron, 2000; Lee *et al*., 2000; Williams *et al*., 2001; Rajora *et al*., 2002; Galloway *et al*., 2003; Casiva *et al*., 2004; DeWoody *et al*., 2004; Iddrisu and Ritland, 2004; Travis *et al*., 2004; Ledig *et al*., 2005;). Although most studies in outcrossing species have recorded high outcrossing rates and their corresponding low fixation indices, few have found outcrossing rates above 1. Glover and Barrett (1986) found an outcrossing rate between 0.9-1.01 in a large Brazilian population of *Eichornia paniculata*. Outcrossing rates above 1 indicate disassortative mating. This implies the existence of strong mechanisms that promote cross-fertilization, so that different genotypes mate at a higher frequency than expected (Ellstrand *et al*., 1978). Cryptic self-incompatibility and selective abortion of self-fertilized seeds are two possible mechanisms determining the high outcrossing rates in *N. longiflora*. Cryptic self-incompatibility has been demonstrated in many species as a mechanism to prevent self-fertilization (Bateman, 1956; Bowman, 1987; Casper *et al*., 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al*., 1990; Cruzan and Barrett, 1993; Rigney *et al*., 1993) and it has also been demonstrated in *N. longiflora* (Chapter 4). Although selective abortion of selfed seeds has not been shown in *N. longiflora*, it has been demonstrated as a common mechanism in other species (Charlesworth, 1988; Erbar, 2003).

Biparental inbreeding was not common in most *N. longiflora* populations studied, such as has been found for other outcrossing species (Glover and Barrett, 1986; Gaiotto *et al*., 1997; Costin *et al*., 2001; Collin and Shykoff, 2003; Casiva *et al*., 2004; Iddrisu and Ritland, 2004; Travis *et al*., 2004; Ledig *et al*., 2005). However, data indicated that it

is a relatively common phenomenon in the allopatric Catolica population. This is not surprising given that this is a relatively small population (27 individuals in 2005), located within the city of Salta, where pollinators are probably not very abundant. It is likely that the population is comprised of many siblings, and therefore mating among relatives is a relatively common scenario.

Results for *N. plumbaginifolia* were more variable. Contrary to the expectations of a selfing species, the allopatric populations of *N. plumbaginifolia* had high outcrossing rates. However, these results should be interpreted with caution, given that estimations of outcrossing rates for those three populations were conducted with only one polymorphic locus. Ritland and Jain (1981) state that for selfing species with low outcrossing rates, one polymorphic marker is enough for a relatively accurate estimation of outcrossing rates, but for species that predominantly outcross, accurate estimations require at least four or five polymorphic loci. In contrast to the high outcrossing rates in allopatric populations, sympatric populations had rather low outcrossing rates, which at least in the Mango population are better supported, given that three polymorphic markers were used for the estimations. Therefore, it seems that sympatry has a negative effect on the outcrossing rate of *N. plumbaginifolia*; however, further confirmation with more polymorphic markers is needed. Estimations of outcrossing rates and fixation indices recorded for the Mango population fit the expectations for a selfing species, as has been recorded for other species with this mating system (Ennos, 1981; Holtsford and Ellstrand, 1989; Agren and Schemske, 1993; Penteado *et al*., 1996).

Because there was only one polymorphic marker for the two allopatric and the sympatric Canal populations of *N. plumbaginifolia*, biparental inbreeding could not be

estimated. In the Mango population, however, biparental inbreeding was near zero (Table 5), suggesting that the low outcrossing rate in this population was due to selfing, not mating among relatives.

A surprising result was the lack of correspondence between the outcrossing rate estimation and the observed fixation index with the expected fixation index at inbreeding equilibrium for the NA6 and NP4 loci in the Ruta (t_s=0.79, F=1.0, F_{eq}= 0.117) and Catolica (t_s=0.001, F=-1.0, F_{eq}= 0.998) populations (Table 4). Similarly, Agren and Schemske (1993) also detected a discrepancy between the estimations of outcrossing rate and fixation index in *Begonia semiovata*. They attribute this discrepancy to three possible causes: selection for heterozygous genotypes, among-year variation of outcrossing rate, and a sub-estimation of the realized outcrossing rate. The pattern observed in Ruta might be a consequence of estimating those parameters with only one polymorphic locus; however, this cannot explain the pattern found in Catolica. Alternatively, it is possible that some other phenomena, such as linkage between loci are occurring (Ritland and Jain, 1981). In Catolica, it seems that there is a strong selection for heterozygous genotypes.

All Wright's *F*-statistics showed lack of heterozygosity for *N. plumbaginifolia* and excess of heterozygosity for *N. longiflora*, as would be expected for typical selfing and outcrossing species, respectively (Ellstrand *et al*., 1978; Ennos, 1981; Shea, 1987; Holtsford and Ellstrand, 1989; Agren and Schemske, 1993; van Treuren *et al*., 1993; Penteado *et al*., 1996; Gaiotto *et al*., 1997; Kittelson and Maron, 2000; Lee *et al*., 2000; Williams *et al*., 2001; Rajora *et al*., 2002; Galloway *et al*., 2003; DeWoody *et al*., 2004; Iddrisu and Ritland, 2004; Travis *et al*., 2004; Ledig *et al*., 2005;). This difference was significantly different between species for all three F estimates (F_{is}: t= -2.359, P<0.05;

F_{it}: t= -3.944, P<0.01; F_{st}: t= -3.907, P<0.01). Pairwise F_{st} estimations showed that the sympatric populations within species and the allopatric *N. longiflora* populations are not genetically differentiated. However, the allopatric *N. plumbaginifolia* populations are strongly differentiated, as would be expected for selfing species that hardly outcross within populations and even more rarely among populations (Holtsford and Ellstrand, 1989; Agren and Schemske, 1993). Outcrossing rate estimations for allopatric *N. plumbaginifolia* populations might be strongly biased as a consequence of using only one polymorphic marker, if there is a strong heterozygote advantage or other segregation distortion.

Low pairwise F_{st} between sympatric *N. plumbaginifolia* populations suggests that some gene flow had occurred in the past and could still be occurring in these populations. This might be caused by the presence of *N. longiflora*, which is visited by hawkmoths which, seemingly accidentally, also visit *N. plumbaginifolia* flowers (Chapter 2). In allopatric populations, which were located within towns, pollinators were scarce. Other studies comparing the availability of pollinators in contrasting environments have shown a negative effect of urbanization on the availability of potential pollinators (Zanette *et al*., 2005; Salinas-Peba and Parra-Tabla, 2007).

General conclusions

Overall, both *N. longiflora* and *N. plumbaginifolia* are affected by each other's presence; however, each species was affected in different traits. *Nicotiana longiflora*'s fitness, estimated as seed-set, was negatively affected by the presence of *N. plumbaginifolia*. On the other hand, the presence of *N. longiflora* affected the genetic

diversity of *N. plumbaginifolia*. An increase in polymorphism was observed in at least one of the sympatric populations of *N. plumbaginifolia*, probably as a result of heterospecific fertilizations that have incorporated *N. longiflora*'s alleles in the genome of the Mango population of *N. plumbaginifolia*. An increase in polymorphism as a consequence of intraspecific outcrossing is unlikely because *N. plumbaginifolia* strongly prevents this kind of cross (Chapter 4). Interspecific visits from hawkmoths observed in this population support the idea of hybridization as a contributor to the increase in genetic diversity in the Mango population of *N. plumbaginifolia*. In contrast to the increase in polymorphism, there is a significant decrease in outcrossing rate in sympatric *N. plumbaginifolia* populations (t-test= 5.7, $P<0.05$). This may be a consequence of an isolation mechanism between species, in which selfing assures reproduction and prevents hybridization, as has been suggested for other species sharing pollinators in which hybridization rates are rather low (Rieseberg *et al*., 1998; Ramsey *et al*., 2003). Alternatively, the difference in outcrossing rates between sympatric and allopatric populations of *N. plumbaginifolia* may be due to the fact that both allopatric populations had only one segregating locus to estimate t. Segregation at those loci may have been distorted due to selection for heterozygotes (NA6 in Ruta, COS-10 in Milagro).

In their study, Bell *et al*. (2005) found a negative effect of the presence of a second species on both fitness and outcrossing rates of *Mimulus ringens*. Unfortunately, they did not explore the effects of the interaction on the second species, *Lobelia siphilitica*. In that sense, my study is the first one showing not only the consequences of sympatry for both of the species interacting in natural populations, but also that the consequences of the interaction are species-specific. Undoubtedly, more empirical studies

addressing the importance of pollinators on plant mating system evolution and population differentiation are needed.

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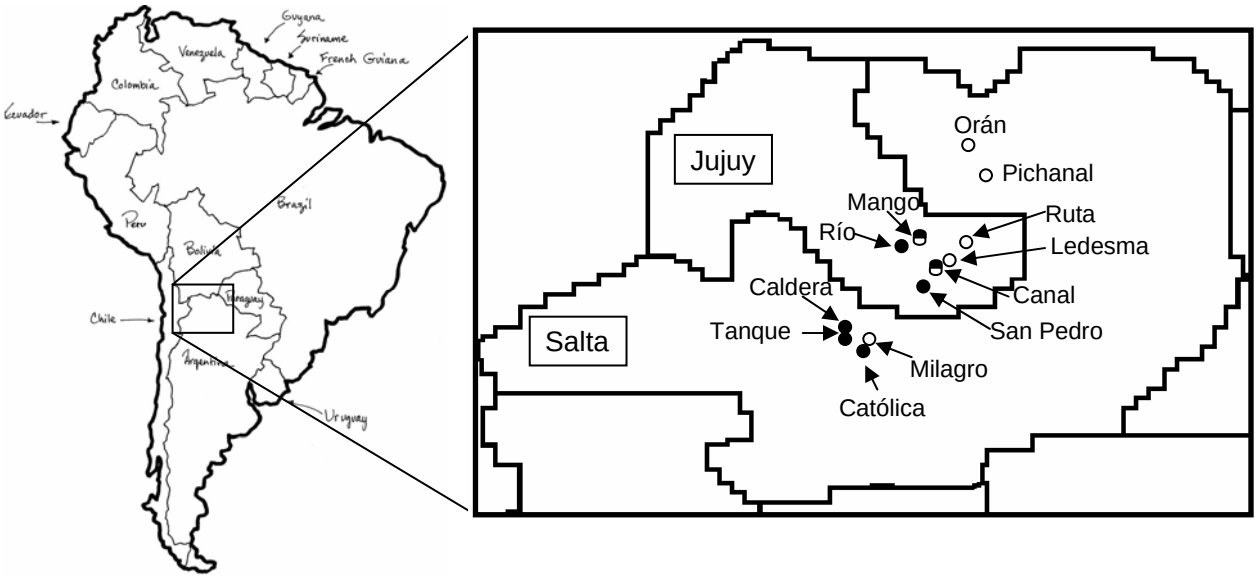

Figure 1. Geographic distribution of the *Nicotiana* populations sampled in the Provinces of Salta and Jujuy, Argentina for the estimation of sympatry effects on plant seed set. Open, closed, and partially closed dots indicate pure *N. plumbaginifolia*, pure *N. longiflora* and sympatric populations, respectively.

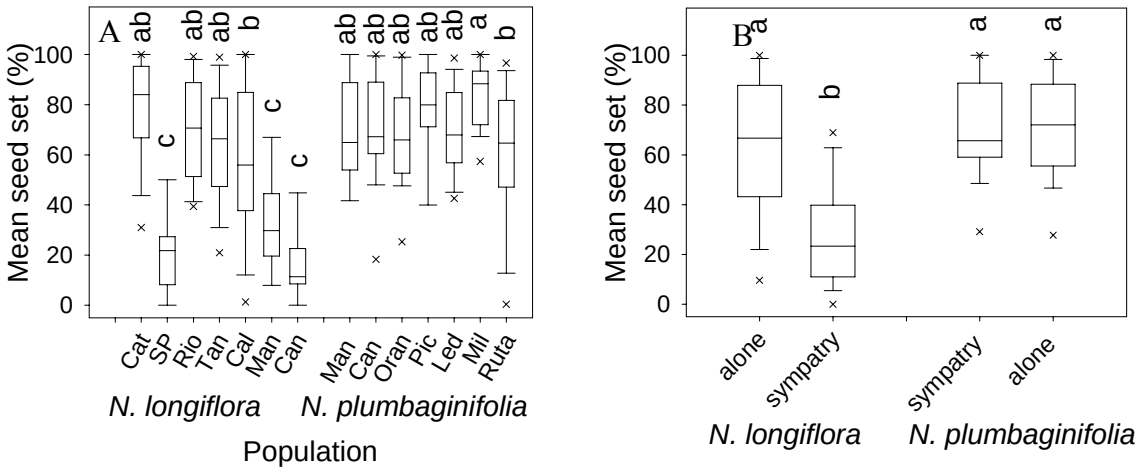

Figure 2. Seed-set (% ovules that set seed) variability among *Nicotiana longiflora* and *N. plumbaginifolia* populations (A) and, as a consequence of sympatry (B). The boundaries of the boxes indicate the 25th and 75th percentile, the line within the box marks the median. Error bars indicate the 90th and 10th percentiles. "x" symbols below and above the boxes indicate the 5th and 95th percentiles (these are not estimated when sample size is below 9). Population names, except Rio and Oran were abbreviated as follows: Rod= Rodrigo, Cat= Catolica, S P= San Pedro, Tan= Tanque, Cal= Caldera, Man= Mango, Can= Canal, Pic= Pichanal, Led= Ledesma, Mil= Milagro. Long= *N. longiflora*, plum= *N. plumbaginifolia*. Different letters between populations indicate significant differences in seed set production (*post-hoc* Tukey test).

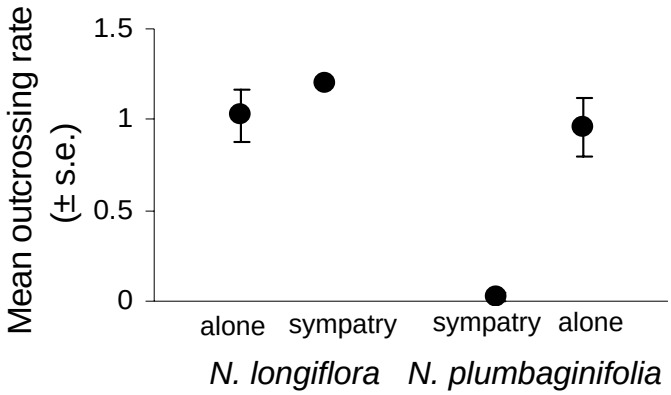

Figure 3. Mean outcrossing rate (± s.e.) in allopatric and sympatric populations of *N. longiflora* and *N. plumbaginifolia*.

Table 1. Location and description of the populations of *N. longiflora* and *N. plumbaginifolia* sampled. Abbreviations for each population name are in parenthesis. N= number of plants sampled in each population for the estimation of sympatry effects on seed set. Asterisks indicate those populations where outcrossing rates and genetic structure were also estimated.

Species	Name	Locality	Description	N	Altitude	Coordinates
N. longiflora	Caldera (Cal)	La Caldera, Salta	In town, rural road side	22	1405 m	S 24° 35' 52.3" W 65° 23' 08.6"
N. longiflora	Católica* (Cat)	Salta, Salta	In town, city road side	27	1223 m	S 24° 44' 29.2" W 65° 23' 58.6"
N. longiflora	Río*	Parque Nacional Calilegua, Jujuy	Forest edge	34	578 m	S 23° 45' 58.1" W 64° 50' 49.2"
N. longiflora	San Pedro (SP)	San Pedro de Jujuy, Jujuy	In town, on highway	15	634 m	S 24° 13' 34" W 64° 52' 37"
N. longiflora	Tanque (Tan)	Salta, Salta	In town, city road side	25	1214 m	S 24° 45' 48" W 65° 24' 50.2"
N. longiflora *N. plumbaginifolia*	Canal (Can) *	Ledesma, Jujuy	In town, on highway	9 20	492 m	S 23° 48' 36" W 64° 47' 46"
N. longiflora *N. plumbaginifolia*	Mango*	Parque Nacional Calilegua, Jujuy	Camping site	17 14	544 m	S 23° 46' 36.8" W 64° 49' 42.6"
N. plumbaginifolia	Ledesma (Led)	Libertador General San Martín, Jujuy	In town	24	486 m	S 23° 48' 09" W 64° 47' 16"
N. plumbaginifolia	Milagro (Mil) *	Salta, Salta	In town	19	1224 m	S 24° 43' 20.77" W 65° 24' 25.14"
N. plumbaginifolia	Orán	Orán, Salta	Park in town	30	358 m	S 23° 07' 41" W 64° 19' 50"
N. plumbaginifolia	Pichanal (Pic)	Pichanal, Salta	In town	15	307 m	S 23° 19' 10" W 64° 13' 25"
N. plumbaginifolia	Ruta *	Out of Libertador General San Martin town	On highway	23	498 m	S 23° 41' 23" W 64° 47' 29"

Table 2. Loci assayed to find polymorphic markers to be used on the estimation of outcrossing rates of *N. longiflora* and *N. plumbaginifolia*. NA and NP markers were developed from *N. alata* and *N. plumbaginifolia* sequences published in Genbank, whereas COS markers were developed from published conserved ortholog set sequences from *Arabidopsis thaliana*. Methods used to screen for polymorphism (sequencing and restriction enzyme assays) for each locus are indicated. Polymorphic loci for *N. longiflora* are denoted with an asterisk. *N. plumbaginifolia* was polymorphic only for the NA6 locus.

Locus abbreviation	**Accession number**	**Screening method**
NA1*	S48582	Sequencing
NA4*	NAU88587	Sequencing
NA6*	AF304375	Sequencing
NA8	AF298594	Sequencing
NA10*	AY159325	Sequencing
NP1	AH002583S1, S2	Restriction enzymes
NP2	TOBATPASA	Sequencing
NP3	TOBCABA	Sequencing
NP4*	TOBCCZSODM	Sequencing
NP5*	TOBRBCS8B	Restriction enzymes
NP6*	NPGSTMR	Sequencing
NP11	AH008040	Restriction enzymes
NP12	Y14676	Restriction enzymes
COS-1	AT1G13380	Sequencing
COS-3	AT1G14310	Restriction enzymes
COS-5	AT1G20050	Sequencing
COS-10*	AT1G44446	Sequencing
COS-11	AT4G34700	Sequencing
COS-16*	AT2G45730	Sequencing
COS-18	AT1G77470	Restriction enzymes
COS-20	AT1G55870	Sequencing

Table 3. Single nucleotide polymorphic markers used for the estimation of outcrossing rates and genetic structure of *N. longiflora* and *N. plumbaginifolia*. NA and NP markers were developed from sequences published in genbank. COS markers were obtained from *Arabidopsis thaliana* conserved ortholog set sequences.

Locus abbreviation	**Accession number**	**Locus name**
NA4	NAU88587	also necessaary for SI, Bam pers comm., mRNA-c
NA6	AF304375	Cellulose synthase D-like protein mRNA, pollen tubes
NP4	TOBCCZSODM	Cu-Zn superoxide dismutase
NP6	NPGSTMR	mRNA for glutathione S-transferase,
COS-10	AT1G44446	Encodes chlorophyllide *a* oxygenase
COS-16	AT2G45730	Eukaryotic initiation factor 3 gamma subunit family protein; similar to tRNA methyltransferase

Table 4. Gene frequencies, observed and expected heterozygosity (Ho and He, respectively), fixation index (F), outcrossing rate (t ± sd) and Hardy-Weinberg (HW) tests for the gene frequencies of the six loci used to characterize the mating system of *N. longiflora* and *N. plumbaginifolia*. n.a. indicates that estimations were not conducted because of lack of polymorphism.

Species	Population	COS-10	COS-16	NA4	NA6	NP4	NP6
N. longiflora	**Rio**						
Allopatry	Allele 1	0.147	0.232	0.003	0.026	0.383	0.519
	Allele 2	0.853	0.768	0.997	0.974	0.617	0.481
No. families= 30	H_o	0.272	0.296	0.006	0.051	0.744	0.844
No. offspring= 180		0.251	0.356	0.006	0.050	0.473	0.499
	F	-0.084	0.169	-0.003	-0.026	-0.575	-0.691
	F_{eq}	-0.043	0.049	-0.09	-0.09	-0.09	-0.09
	t	1.09 ± 0.109	0.906 ± 0.258	1.2 ± 0.58	1.2 ± 0.144	1.2 ± 0.0	1.2 ± 0.0
	HW	>0.05	0.024	>0.05	>0.05	<<0.000	<<0.000
N. longiflora	**Catolica**						
Allopatry	Allele 1	0.226	0.103	0.000	0.000	0.500	0.336
	Allele 2	0.774	0.897	1.000	1.000	0.500	0.664
No. families= 21	H_o	0.459	0.0	0.0	0.0	1.0	0.671
No. offspring= 146	H_e	0.354	0.184	0.0	0.0	0.5	0.446
	F	-0.298	1.000	n.a.	n.a.	-1.000	-0.505
	F_{eq}	-0.09	0.998	n.a.	n.a.	0.998	-0.09
	t	1.2 ± 0.0	0.001 ± 0.0	n.a.	n.a.	0.001 ± 0.0	1.2 ± 0.0
	HW	<<0.000	<<0.000	n.a.	n.a.	<<0.000	<<0.000
N. longiflora	**Canal**						
Sympatry	Allele 1	0.167	0.636	0.030	0.394	0.167	0.667
	Allele 2	0.833	0.364	0.970	0.606	0.833	0.333
No. families= 3	H_o	0.333	0.485	0.061	0.364	0.303	0.667
No. offspring= 33	H_e	0.278	0.463	0.059	0.478	0.257	0.444
	F	-0.200	-0.048	-0.031	0.238	-0.179	-0.500
	F_{eq}	-0.09	-0.09	-0.09	-0.02	-0.09	-0.09
	t	1.2 ± 0.0	1.2 ± 0.04	1.2 ± 0.541	1.042 ± 0.134	1.2 ± 0.0	1.2 ± 0.0
	HW	>0.05	>0.05	>0.05	>0.05	>0.05	0.004
N. longiflora	**Mango**						
Sympatry	Allele 1	0.170	0.541	0.134	0.082	0.438	0.608
	Allele 2	0.830	0.459	0.866	0.918	0.562	0.392
No. families= 10	H_o	0.340	0.485	0.268	0.165	0.876	0.753
No. offspring= 97	H_e	0.282	0.497	0.232	0.151	0.492	0.474
	F	-0.205	0.024	-0.155	-0.090	-0.780	-0.587
	F_{eq}	-0.09	0.063	-0.09	-0.09	-0.09	-0.09
	t	1.2 ± 0.0	0.881 ± 0.213	1.2 ± 0.033	1.2 ± 0.023	1.2 ± 0.0	1.2 ± 0.006
	HW	0.044	>0.05	>0.05	>0.05	<<0.000	<<0.000
N. plumbaginifolia	**Canal**						
Sympatry	Allele 1	1.000	1.000	0.000	0.241	0.000	1.000
	Allele 2	0.000	0.000	1.000	0.759	1.000	0.000
No. families= 17	H_o	0.0	0.0	0.0	0.0	0.0	0.0
No. offspring= 174	H_e	0.0	0.0	0.0	0.366	0.0	0.0
	F	n.a.	n.a.	n.a.	1.000	n.a.	n.a.
	F_{eq}	n.a.	n.a.	n.a.	0.998	n.a.	n.a.
	t	n.a.	n.a.	n.a.	0.001 ± 0.058	n.a.	n.a.
	HW	n.a.	n.a.	n.a.	<<0.000	n.a.	n.a.
N. plumbaginifolia	**Mango**						
Sympatry	Allele 1	0.981	0.993	0.000	0.131	0.000	1.000
	Allele 2	0.019	0.007	1.000	0.869	1.000	0.000
No. families= 8	H_o	0.039	0.015	0.0	0.013	0.0	0.0
No. offspring= 81	H_e	0.038	0.015	0.0	0.228	0.0	0.0
	F	-0.020	-0.008	n.a.	0.945	n.a.	n.a.
	F_{eq}	-0.09	-0.09	n.a.	0.966	n.a.	n.a.
	t	1.2 ± 0.348	1.2 ± 0.569	n.a.	0.017 ± 0.158	n.a.	n.a.
	HW	>0.05	>0.05	n.a.	<<0.000	n.a.	n.a.
N. plumbaginifolia	**Ruta**						
Allopatry	Allele 1	1.000	1.000	n.a.	0.986	n.a.	n.a.
	Allele 2	0.000	0.000	n.a.	0.014	n.a.	n.a.
No. families= 17	H_o	0.0	0.0	n.a.	0.0	n.a.	n.a.
No. offspring= 143	H_e	0.0	0.0	n.a.	0.028	n.a.	n.a.
	F	n.a.	n.a.	n.a.	1.000	n.a.	n.a.
	F_{eq}	n.a.	n.a.	n.a.	0.117	n.a.	n.a.
	t	n.a.	n.a.	n.a.	0.79±0.253	n.a.	n.a.
	HW	n.a.	n.a.	n.a.	<<0.000	n.a.	n.a.
N. plumbaginifolia	**Milagro**						
Allopatry	Allele 1	0.858	1.000	n.a.	0.000	n.a.	n.a.
	Allele 2	0.142	0.000	n.a.	1.000	n.a.	n.a.
No. families= 18	H_o	0.274	0.0	n.a.	0.0	n.a.	n.a.
No. offspring= 186	H_e	0.244	0.0	n.a.	0.0	n.a.	n.a.
	F	-0.122	n.a.	n.a.	n.a.	n.a.	n.a.
	F_{eq}	-0.056	n.a.	n.a.	n.a.	n.a.	n.a.
	t	1.118 ± 0.094	n.a.	n.a.	n.a.	n.a.	n.a.
	HW	>0.05	n.a.	n.a.	n.a.	n.a.	n.a.

Table 5. Multilocus outcrossing rate (t_m), single locus outcrossing rate (t_s), biparental inbreeding (t_m-t_s) and fixation index (F) estimates (± s.e.) for each population of *N. longiflora* and *N. plumbaginifolia* studied. Means of each estimate per species in allopatry vs. sympatry are also provided. n.a.= not estimated given that only one locus was polymorphic.

Species	**Population**	**t_m**	**t_s**	**t_m-t_s**	**F**	**F_{eq}**
N. longiflora	Rio	1.164 ± 0.038	1.161 ± 0.028	0.003 ± 0.039	-0.202 ± 0.129	-0.074
Allopatry	Catolica	1.2 ± 0.0	0.882 ± 0.138	0.318 ± 0.138	-0.201 ± 0.369	0.063
	Mean	1.182 ± 0.018	1.021 ± 0.139	0.160 ± 0.157	-0.2015 ± 0.176	-0.011
N. longiflora	Canal	1.167 ± 0.123	1.2 ± 0.076	-0.033 ± 0.055	-0.120 ± 0.091	-0.09
sympatry	Mango	1.2 ± 0.006	1.2 ± 0.013	0.0 ± 0.011	-0.299 ± 0.117	-0.09
	Mean	1.183 ± 0.016	1.2 ± 0.0	-0.016 ± 0.016	-0.2095 ± 0.082	-0.09
N. plumbaginifolia	Canal	n.a.	0.001 ± 0.058	n.a.	1.0 ± 0.0	0.998
Sympatry	Mango	0.042 ± 0.209	0.046 ± 0.208	-0.004 ± 0.006	0.306 ± 0.261	0.912
	Mean	n.a.	0.023 ± 0.022	n.a.	0.471 ± 0.285	0.954
N. plumbaginifolia	Ruta	n.a.	0.794 ± 0.253	n.a.	1.0 ± 0	0.115
Allopatry	Milagro	n.a.	1.118 ± 0.094	n.a.	-0.122 ± 0	-0.056
	Mean	n.a.	0.956 ± 0.162	n.a.	0.439 ± 0.561	0.022

Table 6. Estimates of genetic differentiation of individuals within populations (F_{is}), in relation to the total sample of populations (F_{it}), and differentiation among populations relative to the total sample (F_{st}) for the six loci used to characterize the population structure of *N. longiflora* and *N. plumbaginifolia*. Means of each estimate across all loci are also provided. n.a.= not apply because loci were not polymorphic.

	N. longiflora			***N. plumbaginifolia***		
Locus	**F_{is}**	**F_{it}**	**F_{st}**	**F_{is}**	**F_{it}**	**F_{st}**
COS-10	-0.206	-0.198	0.006	-0.108	0.771	0.794
COS-16	0.156	0.327	0.203	-0.008	-0.002	0.006
NA4	-0.127	-0.044	0.074	n.a.	1.0	1.0
NA6	0.146	0.340	0.227	0.980	0.993	0.653
NP4	-0.698	-0.571	0.075	n.a.	1.0	1.0
NP6	-0.574	-0.474	0.064	n.a.	1.0	1.0
Mean	-0.217	-0.103	0.108	0.288	0.794	0.742

Table 7. Pairwise estimates of subpopulation differentiation relative to the total population (F_{st}) within and between *Nicotiana longiflora* and *N. plumbaginifolia* populations.

			N. longiflora				***N. plumbaginifolia***			
			Allopatry		**Sympatry**		**Sympatry**		**Allopatry**	
			Rio	Catolica	Canal	Mango	Canal	Mango	Ruta	Milagro
N. longiflora	**Allopatry**	Rio								
		Catolica	0.017							
	Sympatry	Canal	0.079	0.137						
		Mango	0.033	0.070	0.047					
N. plumbaginifolia	**Sympatry**	Canal	0.337	0.482	0.210	0.274				
		Mango	0.318	0.458	0.212	0.259	0.011			
	Allopatry	Ruta	0.788	0.811	0.649	0.688	0.896	0.626		
		Milagro	0.513	0.656	0.502	0.441	0.778	0.631	0.944	

Chapter 4. Post-pollination mechanisms in *Nicotiana longiflora* and *N. plumbaginifolia*: pollen tube growth rate, offspring paternity and hybridization

Abstract

In natural populations where interfertile species coexist, conspecific and heterospecific pollen can be delivered on the stigmas. Thus, post-pollination mechanisms might determine the paternity success of different pollen donors within species as well as the chances for hybridization between species. *Nicotiana longiflora* and *N. plumbaginifolia* occur in sympatry in Northwest Argentina, where they have overlapping flowering seasons and share floral visitors. First, I explored outcrossed vs. selfed pollinations for pollen tube growth rates (PTGR) in single-donor pollinations. Second, I performed self vs. outcross competitive pollinations and determined offspring sired by each type of pollen donor. Offspring paternity was assigned using single nucleotide polymorphism (SNP) genetic markers. Third, I investigated the possibilities for hybridization between species by performing two- (outcross conspecific vs. heterospecific) and three-pollen donor (self vs. outcross vs. heterospecific) crosses. In *N. longiflora,* both PTGR and siring success favored outcross pollen over self pollen, leading to cryptic self-incompatibility. *Nicotiana longiflora* also showed a strong preference to set conspecific outcrossed seeds over both selfed and hybrid seeds. In *N. plumbaginifolia,* selfed and outcrossed PTGR were not significantly different, but paternity in mixed pollinations showed more offspring set from self than outcross pollen donors. Therefore, the bias

toward setting selfed seeds happened after pollen tubes had reached the base of the style. *Nicotiana plumbaginifolia* showed a surprising and strong preference to set hybrid seeds when outcross conspecific pollen was applied with heterospecific pollen. In three-donor pollinations, *N. plumbaginifolia* set the fewest seeds with intraspecific outcross pollen. Self pollen and heterospecific pollen sired roughly the same number of progeny. Results suggest that in natural sympatric populations, interspecific crosses would likely lead to unidirectional hybridization with *N. plumbaginifolia* as the seed parent.

Key words: competitive pollinations, cryptic self-incompatibility, hybridization, *Nicotiana longiflora*, *Nicotiana plumbaginifolia*, paternity, pollen tube growth rate, post-pollination mechanisms, sympatry.

Introduction

In plants, post-pollination mechanisms act as selective agents determining the siring success of pollen donors (Snow and Spira, 1991, 1996; Montalvo, 1992; Walsh and Charlesworth, 1992; Rigney *et al*., 1993; Carney *et al*., 1994; Eckert and Allen, 1997; Kruszewski and Galloway, 2006). Therefore, offspring paternity not necessarily reflects the composition of pollen loads delivered on stigmas (Montalvo, 1992; Rigney *et al*., 1993; Carney *et al*., 1994; Jones, 1994; Snow and Spira, 1996; Kruszewski and Galloway, 2006). Self-incompatibility reactions are a common post-pollination mechanism present in angiosperms. In Solanaceae, incompatibility occurs when incompatible pollen is recognized by SRNases and thus pollen tube growth is arrested (McClure *et al*., 2000; de Nettancourt, 2001; Cruz-Garcia *et al*., 2003; Franklin-Tong and

Franklin, 2003; McClure, 2004; Cruz-Garcia *et al*., 2005; Hancock *et al*., 2005). If pollen loads delivered to the stigmas include not only conspecific but also heterospecific pollen grains, incompatibility reactions not only will favor outcross pollen but also will prevent hybridization. However, it has been shown that self-compatible plants can be fertilized by pollen from self-incompatible species, but crosses in the opposite direction fail to set seeds (Liedl *et al*., 1996; Murfett *et al*., 1996; McClure *et al*., 2000; de Nettancourt, 2001; Hancock *et al*., 2003; Hayes *et al*., 2005 . This phenomenon, known as unilateral incompatibility, has been previously recorded in *N. plumbaginifolia* through single donor pollinations (Murfett *et al*., 1996; McClure *et al*., 2000; Lee *et al*., 2008)

In natural populations, mixtures of self and outcross pollen can be delivered on the stigmas of self-compatible species. The abilities of pollen from each source to germinate and grow down the style would greatly determine siring success for self and outcross donors. For instance, many studies have shown that outcross pollen tubes have an advantage over self pollen tubes when growing in the style (Bateman, 1956, Bowman, 1987; Casper *et al*., 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al*., 1990; Cruzan and Barrett, 1993; Rigney *et al*., 1993; Travers and Mazer, 2000; Kruszewski and Galloway, 2006). Such advantage seems to be determined by incompatibility reactions in which tube growth of incompatible pollen grains is arrested (McClure *et al*., 2000; de Nettancourt, 2001; Cruz-Garcia *et al*., 2003; Franklin-Tong and Franklin, 2003; McClure, 2004; Cruz-Garcia *et al*., 2005; Hancock *et al*., 2005). Therefore, outcross pollen tubes would fertilize more ovules and have a higher reproductive success than self pollen tubes (Bateman, 1956, Mulcahy, 1979; Lassere *et al*., 1996). This phenomenon has been known as cryptic self-incompatibility since Bateman's (1956) publication.

Because after fertilization there might be maternal selection or hybrid vigor of embryos, it is hard to separate between prezygotic and postzygotic selection, and therefore to differentiate between post-pollination and post-fertilization selection. In order to determine an advantage of outcross- over self- pollen based on differential pollen tube growth rate, it is necessary to demonstrate that differential siring success among pollen types is caused by differences in pollen tube growth rate and not due to selective seed abortion (Bateman, 1956; Walsh and Charlesworth, 1992). Therefore, by determining both pollen tube growth rate and offspring paternity, it may be possible to separate post-pollination from post-zygotic selection (Bateman, 1956; Walsh and Charlesworth, 1992; Eckert and Allen, 1997). If fertilization is random, then the siring success of self versus outcross donors can be predicted by the proportion of self versus outcross pollen deposited on the stigma (Jones, 1994). However, processes occurring within the ovary remain masked even with the estimation of both pollen tube growth rates and offspring paternity. Therefore, distinguishing between pre- and post-zygotic processes occurring in the style is not trivial.

Pollen tube growth rate is an intrinsic characteristic of each species that influences the competitive abilities of pollen tubes when growing in the style (Snow and Spira, 1996). For example, it is often the case that pollen tube growth rate is correlated with pollen size and/or style length (Aizen *et al.* 1990; Williams and Rouse, 1990; Diaz and Macnair, 1999; Lee *et al*., 2008).

In natural populations of inter-crossable sympatric species with overlapping flowering seasons and pollinator sharing, post-pollination processes are particularly important. Post-pollination selective mechanisms can act as isolating barriers between

species and therefore determine the evolutionary trajectory for each species (Carney *et al*., 1994). Although interspecific pollinations might determine the degree of hybridization and gene exchange among taxa, few studies have been carried out to study their frequency and consequences (Carney *et al*., 1994; Emms *et al*., 1996; Diaz and Macnair, 1999). In particular, there is a lack of literature investigating the importance of post-pollination mechanisms as isolation barriers between naturally intercrossable species with contrasting floral morphology and mating systems, such as *N. longiflora* and *N. plumbaginifolia*. When interspecific pollen mixtures are delivered on stigmas, it is expected that intraspecific pollen tubes would be favored for fertilization to minimize hybridization, assuming hybrids are less fit than progeny from conspecific crosses (Smith 1968; Carney *et al*., 1994; Emms *et al*., 1996). However, if pollen tube growth is correlated with style length (Diaz and Macnair, 1999; Lee *et al*., 2008), and the sympatric species have contrasting style lengths, then pollen tubes from the long-style species are expected to have an advantage in fertilizing ovules of a short-style species. Therefore, whenever pollen tubes from long-style species fertilize ovules of short-style species, asymmetric hybridization will be common (Emms *et al*., 1996). Results from Lee *et al*., (2008) support this trend. In single donor hand pollinations, these authors found that *N. plumbaginifolia* flowers pollinated with conspecific pollen sired only 15% more seeds than flowers pollinated with *N. longiflora* pollen. On the other hand, *N. longiflora* showed a 59% reduction in seed set when pollinated with *N. plumbaginifolia* pollen, compared to conspecific crosses. Although Lee *et al.* (2008) did not estimate pollen tube growth rate in crosses between short-style by long-style species, seed set results from that study showed that any pistil-length mismatch, that is, long X short or short X long,

reduced seed set, and thus hybridization, in *Nicotiana* sect. *Alatae***.** However, if short styles are not able to support greater tube rates than those carried by their own pollen as it has been recorded for *Mimulus nasutus*, then hybridization will be prevented (Diaz and Macnair, 1999).

Nicotiana longiflora and *N. plumbaginifolia* are self-compatible, sister species that co-occur in sympatry in northern Argentina (Goodspeed, 1954; Lee *et al*., 2008). Because these species share pollinators and have overlapping flowering seasons (Chapter 2), it is possible that the delivery of pollen from different sources (self, conspecific outcross, and interspecific) might be happening. *Nicotiana longiflora* has a long style (73.905 mm ± s.e. 5.195) and is a strong outcrosser, whereas *N. plumbaginifolia* is a short-style species (25.043 mm ± 1.264) with high levels of self-fertilization (Chapter 3). Therefore, if intraspecific pollen interactions occur within these species, strong cryptic SI will be expected for *N. longiflora* but not for *N. plumbaginifolia*, in which the high level of selfing will select pollen that performs better in particular stylar environments (Cruzan, 1990). In the case of mixed pollinations in which not only self- and outcross-, but also interspecific- pollen is delivered on the stigma, pollen from the long-styled *N. longiflora* might have an advantage over *N. plumbaginifolia*'s pollen.

The main objective of this study is to determine the siring success of different pollen donors (self, conspecific outcross and interspecific) of *N. longiflora* and *N. plumbaginifolia* when delivered on conspecific and heterospecific stigmas. The specific goals are to quantify: i) differences in pollen tube growth rate between self- and outcross-pollen for each species; ii) intraspecific bias in siring success of outcross vs. self pollen donors, and iii) probability of hybridization when two (outcross- vs. heterospecific-

pollen) and three (self- vs. outcross- vs. heterospecific- pollen) sources of pollen interact in the styles of each species.

Methods

Study species

Nicotiana longiflora and *N. plumbaginifolia* (Solanaceae) are each other's closest relatives (chromosome *n*= 10), forming a strongly supported clade within section *Alatae* (Ippolito, 2000; Chase *et al*., 2003; Clarkson *et al*., 2004; Lim *et al*. 2006). These species are self-compatible and inter-crossable in the greenhouse (East, 1916; Goodspeed, 1954; Lee *et al.,* 2008). *Nicotiana longiflora* has floral traits suggesting allogamy. Flowers are 40-120mm in corolla length, with 21.3 ± 0.3% solids in sucrose dominant nectar (Goodspeed, 1954; Kaczorowski, 2005). This species has been confirmed to be a strong outcrosser (Chapter 3). On the other hand, *N. plumbaginifolia* has shorter flowers, ranging from 25 to 35 mm in length, with 19.3 ± 1.1% solids in sucrose-rich nectar (Goodspeed, 1954; Kaczorowski, 2005) and outcrossing rates suggest a mixed mating system (Chapter 3). In both species, nectar concentration fits within the range for hawkmoth pollinated species (Baker and Baker, 1982). However, nectar production and the ratio of sucrose to glucose+fructose are lower for *N. plumbaginifolia* (Kaczorowski, 2005). A study on floral organ development of these two species showed a negative anther-stigma distance (ASD) for newly-opened *N. plumbaginifolia* flowers and nearly 100% of flowers set seed autogamously, whereas *N. longiflora* showed slightly positive ASD and about a 20% rate of autogamy in the glasshouse (Soule, 2007). These species occur in sympatry in northern Argentina (Fig. 1), from there, *N. longiflora* is distributed

to the south and southeast of the continent, whereas *N. plumbaginifolia's* range extends to the North (Goodspeed, 1954). The co-occurrence of the two species in sympatric populations and the overlap in their flowering seasons was shown to have a negative effect on *N. longiflora* seed set and *N. plumbaginifolia* outcrossing rates (Chapter 3).

Pollen tube growth rate

The rate of growth of pollen tubes was determined for each species through single donor hand-pollinations conducted in the greenhouse. Four flowers per plant were emasculated within 24 h before anthesis and anther dehiscence and hand-pollinated on the first day of anthesis. Pollinations were done by rubbing two to three recently dehiscent anthers on the stigmas of plants grown in the greenhouse. One of two sources of pollen was applied: i) self-pollen, collected from other flowers on the same plant, and ii) outcross-pollen, collected from 2-3 other plants within the same population. Each kind of pollen was applied on four flowers per plant. Self- and outcross- pollinated flowers were collected at 12, 24, 36, and 48 h from the time of pollination. Styles were fixed for at least one hour in 1:3 acetic acid: ethanol, cleared and softened by autoclaving for 15 min in 10% (w/v) sodium sulfite solution, stained for at least 12 h in 0.1 M tripotassium phosphate, 0.1% (w/v) aniline blue solution and squashed (Kho and Baer, 1968). Pollen-tube length was measured under a Leica MZFLIII stereoscope fitted with a UV filter, provided by the Electron Microscopy core facility at the University of Missouri-Columbia. Pollen tube length was measured from the tip of the stigma to the point in which less than 10 pollen tubes were visible.

Pollen tube growth treatments were applied to 80 flowers distributed in 40 plants per species. Because flower availability was limited in *N. longiflora*, some plants had two sets of treatments; thus, pollen tube growth rate was estimated on 20 *N. longiflora* and 40 *N. plumbaginifolia* plants. Flowers were chosen according to the availability of new flowers, prior to anthesis and anther dehiscence.

Statistical analyses - Pollen tube growth

Pollen tube-growth per unit of time was analyzed in SAS 9.1 with an analysis of covariance using species, time of styles collection (12, 24, 36 and 48 h after pollination) and treatment (self- vs. outcross pollen) as fixed effects and style length as covariate. *Post-hoc* Tukey tests were conducted to determine differences among groups.

ANOVA tests with time of style collection and treatment as fixed effects were applied to each species to determine significant differences in distance traveled by pollen tubes. Tukey tests were conducted to determine differences among significant changes in style distance traveled by pollen tubes over time.

Mixed pollinations and paternity determination

Plant material

All crosses for the evaluation of paternity from mixed pollinations were conducted on plants grown in the controlled environment of the greenhouse (14h days at 24ºC and 10 h nights at 13ºC at the University of Missouri-Columbia). Naturally pollinated seeds were collected in 2005 from two natural populations (Mango and Canal), in the Province of Jujuy in northern Argentina (Fig. 1, Table 1), where both *Nicotiana* species co-occur in

sympatry. Seeds were pre-rinsed with 70% bleach and rinsed twice with sterilized water. Afterwards, seeds were rinsed and planted with 500 µl of 750 ppm gibberelin solution in trays with pre-mixed soil and covered with vermiculite.

Pollen quantification

In order to conduct assays of pollen competition to explore the chances of hybridization between the *Nicotiana* species, I first quantified the average number of pollen grains per anther per species. Between six to eight plants per population and species were chosen for crosses. From each plant, between four and five mature but indehiscent anthers from at least two flowers per plant were collected for pollen quantification (*N. longiflora*: 30 and 39 anthers for Mango and Canal, respectively; *N. plumbaginifolia*: 34 and 32 anthers for Mango and Canal, respectively). Anthers were left to dehisce and dry in uncapped 1.5 ml microcentrifuge tubes stored in a dust-free space. Pollen number was estimated with an Elzone 280 PC particle counter, previously calibrated with 40µm beads, which are in the range of pollen size for both *Nicotiana* species (15-45µm). 1.5 ml of 2% saline solution were added to each tube, anthers were dissected with forceps and sonicated for 4min. Tubes were then rinsed into a 20 ml sample vial which was left from eight hours to overnight to set. Pollen estimations were done after sonicating each 20 ml vial for 1 min. Five estimations were made for each tube, the highest and lowest scores were discarded and the three other measurements were used to get the average number of pollen per anther. The average number of pollen per anther indicated that the ratio of *N. longiflora* (29322.49 ± s.e. 2035.97; 95% C.I: 25332.05-33312.93; N= 69 anthers from 13 plants) to *N. plumbaginifolia* (7738.59 ± 543.37, 95% C.I.: 6673.61-8803.57; N= 66 anthers from

14 plants) pollen per anther is 3.8:1. Therefore, I used four *N. plumbaginifolia* anthers and one *N. longiflora* anther as an attempt to apply approximately a 1:1 ratio pollen number in interspecific crosses (described below).

Crosses

Two donor crosses were applied on plants from both Canal and Mango populations, whereas three donor crosses were only conducted on plants from the Mango population. To quantify the paternity results from mixed pollinations plants that were homozygous for alternative alleles were chosen. For three donor crosses two markers were used. One marker was used to distinguish between self and outcross fertilizations within species (Cos 16 for *N. longiflora* and NA6 for *N. plumbaginifolia*) and the other marker was useful to distinguish between intra- and inter-specific fertilizations. Reciprocal crosses were conducted, so that all six plants acted as mothers and pollen donors for a total of 18 crosses per population (Fig. 2). In those cases in which the plants originally selected for crosses did not have dehiscent anthers, other plants with the same genotype were used. Pollen from recently dehiscent anthers from each parent was mixed thoroughly in 0.5 ml microcentrifuge tubes and applied with flat toothpicks on stigmas of flowers emasculated within 24h before anthesis. In self vs. outcross pollen crosses the same number of anthers from each donor were used (2-3 anthers per parent); whereas in interspecific crosses four *N. plumbaginifolia* anthers per each *N. longiflora* anther were used to approximate a 1:1 ratio. Pollinations were made within 24 h after anthesis. I applied abundant pollen to all stigmas to assure pollen-pollen competitive interactions and no pollen limitation to set seeds. Mature fruits were collected before seed dispersal (two and three weeks for *N.*

plumbaginifolia and *N. longiflora*, respectively) and used the seeds within to determine seedling paternity.

Offspring genotyping

Seeds from each kind of cross were planted and grown in the greenhouse as previously described. Ten seedlings per cross, per species, per population were grown for genotyping, for a total of 180 seedlings. Because some of the crosses did not set any seeds and in some cases seeds did not germinate or seedlings died before being large enough to extract DNA, the number of seedlings genotyped was reduced to 124 *N. longiflora* and 62 *N. plumbaginifolia* maternal parents for self- vs. outcross- pollen crosses; 286 *N. longiflora* and 230 *N. plumbaginifolia* for outcross- vs. interspecific-crosses and 124 *N. longiflora* and 62 *N. plumbaginifolia* for three donor pollinations. This reduction in sample sizes hardly has an effect on the power of the results because pooled data over five to eight maternal plants per cross type (Table 3), per population or per species (Figs. 5-9 and Appendices) were used in all analyses.

DNA was extracted from leaves as soon as seedlings had enough vegetative tissue (around 1 month old), following the next protocol: 300µl extraction buffer (.35M Sorbitol, 0.1M Tris pH 7.5, 5mM EDTA) with β-mercaptoethanol, 300µl lysis buffer and 100µl sarkosyl; incubation for 10 min at 65˚C; extracted twice with 24 chloroform:1 isoamyl alcohol; rinsed with 76% EtOH-10mM $NH_4C_2H_3O_2$ and stored in 1/10 TE buffer with RNase. Seedlings were then genotyped using the codominant CAPS markers tRNA methyltransferase (locus abbreviation, Cos-16) and cellulose synthase D-like protein mRNA (locus abbreviation, NA6). Both of these markers are polymorphic for *N.*

longiflora, whereas NA6 is the only polymorphic marker known for both *N. plumbaginifolia* sympatric populations (Fig. 3; Table 2).

DNA was amplified for Cos 16 (39 cycles with 2.75 min at 94˚C; 1min at 55˚C, 1min at 72˚C) and NA6 (39 cycles with 2.75 min at 94˚C; 1min at 60˚C, 1.5min at 72˚C) and digested for two hours to overnight with EcoR I and Hha I, respectively. Digested products were run on 1.5% agarose gels at 70 volts for 2.5 h and visualized with UV light.

Statistical analyses – Seed set data

Data were analyzed with several χ^2 tests (Fig. 4). First, I tested if offspring from the self- vs. outcross-; outcross- vs. interspecific-; and self- vs. outcross- vs. interspecific- pollen is significantly different from the expected ratios of 1:1 or 1:1:1, respectively, based on the pollen mixtures applied. Because pollen mixtures might not be exactly in a 1:1 ratio, a set of tests with expectations 0.6 : 0.4 and 0.7 : 0.3 were also performed on data of two-donor crosses. Second, for two- and three-donor crosses 2 X 2 (pollen source vs. species and pollen source vs. population) and 2 X 3 (pollen source vs. species, and pollen source vs. population) contingency tables, respectively, were used. In this way, I tested if the results of competitive interactions among different kinds of pollen were similar across species and across populations within species. Because several tests were applied to the same set of data, significance was considered at P=0.01 in order to avoid Type I error.

Results

Pollen tube growth rate in selfed vs outcrossed single donor pollinations

Analysis of covariance showed a significant effect on PTGR of pollination treatment ($F_{1, 143}$=11.04, P< 0.05), time of style collection ($F_{3, 143}$=15.84, P< 0.05), and the interaction of pollen treatment X species ($F_{1, 143}$= 7.98, P< 0.05; Fig. 5, Table 3). Pollen tube growth rate was not significantly affected by species ($F_{1, 143}$= 0.5, P= 0.48) or the interactions of species X time ($F_{3, 143}$=0.44, P=0.72), time X treatment ($F_{3, 143}$=0.73, P= 0.54) and species X time X treatment ($F_{3, 143}$=0.5, P= 0.68, Table 3). Overall, PTGR was significantly lower at 12 h than at any other time of style collection (Fig. 5, Table 4). In *N. longiflora*, outcross pollen had significantly higher PTGR than self pollen as well as *N. plumbaginifolia*'s pollen. In *N. longiflora*, outcross-pollen showed a significantly higher growth rate than self-pollen (Fig. 5, Tables 3, 4). In *N. plumbaginifolia*, pollen tube growth rate did not differ between self- and outcross-pollen (Fig. 5, Tables 3, 4). Mean pollen tube growth rate for *N. plumbaginifolia* was 0.55 ± 0.02 mm/h (self: 0.54 ± 0.03, outcross: 0.56 ± 0.03), whereas for *N. longiflora* the mean pollen tube growth rate was 0.54 ± 0.06 mm/h (self: 0.39 ± 0.06, outcross: 0.70 ± 0.08, Table 4).

Distance traveled by pollen tubes of *N. longiflora* was significantly affected by self vs. outcross treatment ($F_{1, 72}$= 8.27, P< 0.01) and time of style collection ($F_{3, 72}$=11.81, P< 0.0001) but not by the interaction time X treatment ($F_{3, 72}$=1.05, P> 0.05; Table 5, Fig. 6). Distance traveled by pollen tubes was significantly shorter at 12 h after pollination (Fig. 6). Overall, outcross pollen tubes traveled faster than self pollen tubes (Tables 5, 6; Fig. 6).

In *N. plumbaginifolia* the distance traveled by pollen tubes was significantly affected by time of style collection ($F_{3, 72}$=261.0, P< 0.0001), but not by treatment ($F_{1, 72}$=0.78, P> 0.05) or the interaction of time X treatment ($F_{3, 72}$=0.31, P> 0.05; Table 5, Fig. 6). The longest distance traveled by pollen tubes was after 36 and 48 h of style collection. The shortest distance traveled by pollen tubes was at 12 h, which was significantly shorter than the distance traveled at 24 h (Fig. 6; Tables 5, 6).

Offspring paternity from self and outcross competitive pollinations

The observed proportions of selfed vs. outcrossed seeds in mixed pollinations were significantly different from null expectations and were significantly different between the sister species, *N. longiflora* and *N. plumbaginifolia*. The outcrosser, *N. longiflora*, set more outcrossed seed than expected, based on the ratio of 1:1 selfed:outcrossed pollen applied to stigmas (χ^2= 248.4, df=1, P<<0.001). The selfing species, *N. plumbaginifolia*, showed a smaller bias, but in the opposite direction, tending to set more selfed seed (χ^2= 16.1, df=1, P<<0.001; Fig. 7a; App. 1). Because I did not count pollen applied to stigmas directly, relying instead on average pollen number per anther per species, I also tested the sensitivity of this test to sampling variation in pollen ratios applied to stigmas. Even if the pollen ratios applied had been consistently off by more than 20%, the seed set bias in *N.longiflora* remained significant (χ^2= 86.56, df=1, P<<0.001; Fig. 7a, App. 1). In the selfer *N. plumbaginifolia*, the bias toward setting selfed seed was not significant if self to outcross pollen ratios had consistently been 10% off (6:4 expectations: χ^2= 0.4, df=1, P>0.01). So the most conservative interpretation is that in *N.plumbaginifolia*, selfed

pollen may have an advantage over outcrossed in setting seeds, but the selfing advantage is small.

Testing for heterogeneity between species was also significant (χ^2= 214.6, df=1, P<<0.001; Fig. 7a; App. 1). The frequency of self- and outcross- progeny did not differ significantly between populations of either *N. longiflora* (χ^2= 0.196, df=1, P>0.01) or *N. plumbaginifolia* (χ^2= 0.055, df=1, P>0.01; Fig. 7b; App. 1).

In *N. longiflora* the number of outcross-offspring (306 out of 327) was almost 15 times greater than self-offspring (21 out of 327), whereas in *N. plumbaginifolia* outcross- (110 out of 288) and self-offspring (178 out of 288) was only slightly biased from a 1:1 ratio (Fig. 7a). The strong preference of *N. longiflora* for outcross-pollen was very constant among six maternal plants in two populations (Table 7). However, in *N. plumbaginifolia* seed set biases varied among maternal plants and between populations. In the Canal population five of eight seed parents set more selfed seed by at least 2:1. In the Mango population the selfed seed bias was only strong in two seed parents, while two other parents set approximately equal proportion of selfed and outcrossed seed (Table 7).

Offspring paternity from outcross vs. interspecific pollinations

Results for outcross vs. interspecific offspring paternity from mixed pollinations, showed a significant departure from the 1:1 ratio expected based on pollen mixtures for both *N. longiflora* (χ^2= 258.7, df=1, P<<0.001) and *N. plumbaginifolia* (χ^2= 167.0, df=1, P<<0.001; Fig. 8a, App. 2). The frequency of offspring from each kind of pollen was also significant for both species when testing the observed frequencies against an expected 0.6 : 0.4 ratio (*N. longiflora*: χ^2= 168.0, df=1, P<<0.001; *N. plumbaginifolia*: χ^2= 101.9, df=1,

P<<0.01) and 0.7 : 0.3 ratio (*N. longiflora*: χ^2= 103.4, df=1, *P*<<0.001; *N. plumbaginifolia*: χ^2= 56.0, df=1, *P*<<0.001; Fig. 8a; App. 2).

Nicotiana longiflora and *N. plumbaginifolia* showed contrasting patterns in the number of outcross vs. hybrid offspring between species (heterogeneity χ^2= 423.7, df=1, *P*<<0.001; Fig. 8a; App. 2). Most *N. longiflora*'s seeds were sired by conspecific outcross pollen, whereas seeds in *N. plumbaginifolia* were more successfully sired by interspecific pollen (Fig. 8a; App. 2). Within species, both populations were consistent in their paternity bias (within *N. longiflora*, χ^2= 4.35, df=1 *P*>0.01 and within *N. plumbaginifolia* χ^2= 5.04, df=1 *P*>0.01, Fig. 8b; App. 2).

In *N.longiflora*, conspecific outcross offspring (279 out of 286) was around 40 times higher than interspecific offspring (7 out of 286, Table 7). On the other hand, *N. plumbaginifolia* showed the opposite pattern, with most offspring being produced via interspecific fertilization (213 out of 230, Table 7). The strong preference of *N. longiflora* and *N. plumbaginifolia* for conspecific outcross vs. interspecific pollen, respectively, was consistent among maternal plants and across populations (Table 7).

Three-donor pollinations

Testing the observed frequency of self-, outcross-, and hybrid offspring from each species against a 1:1:1 ratio was significant for both *N. longiflora* (χ^2= 61.5, df=2, *P*<<0.001) and *N. plumbaginifolia* (χ^2= 22.9, df=2, *P*<<0.001; Fig. 9, App. 3). Heterogeneity in the frequency of offspring sired from each kind of pollen was also significant between species (χ^2= 67.86, df=2, *P*<<0.001; Fig. 9, App. 3).

In *N. longiflora* most offspring were produced via conspecific outcross fertilizations (81 out of 124), whereas in *N. plumbaginifolia* most offspring were produced by self or interspecific pollen (28 and 31 out of 62, respectively, Fig. 9). Seed from outcross, conspecific donors were underrepresented in *N. plumbaginifolia*. Preferences for outcross- and interspecific- pollen in *N. longiflora* and *N. plumbaginifolia*, respectively, were strongly heterogeneous among maternal plants (Table 7).

Discussion

The sister species *N. longiflora* and *N. plumbaginifolia* coexisting in sympatry in northern Argentina have overlapping flowering seasons and share pollinators (Chapter 2). Hawkmoths, the main floral visitors to these species have a strong preference for *N. longiflora* flowers; however, occasional visits to *N. plumbaginifolia* have been also recorded. Once *N. plumbaginifolia* flowers are visited, pollinators immediately stop visiting more flowers from this species and restart visiting *N. longiflora* flowers (Chapter 2). Because of these foraging patterns, hawkmoths visiting *N. plumbaginifolia* flowers probably deliver a higher proportion of *N. longiflora*'s pollen compared to those of conspecific *N. plumbaginifolia* plants. Foraging patterns of pollinators might lead to post-pollination intra- and also inter-specific interactions between these species.

In this chapter I have demonstrated that post-pollination selective mechanisms occur in both *Nicotiana* species. *N. longiflora*'s outcross pollen showed a strong advantage over self pollen which was predicted by the species' differential PTGR. In two- and three-donor crosses with heterospecific pollen, *N. longiflora* outcross pollen

was also more successful at siring seeds. These results suggest that *N. longiflora* experiences incompatibility reactions that prevent both self-fertilization and hybridization. Self-incompatibility mechanisms have been intensely studied in the *Nicotiana* genus (McClure *et al*., 2000; de Nettancourt, 2001; Cruz-Garcia *et al*., 2003; Franklin-Tong and Franklin, 2003; McClure, 2004; Cruz-Garcia *et al*., 2005; Hancock *et al*., 2005). On the other hand, although differential pollen tube growth rate was not detected in *N. plumbaginifolia*, this species experiences strong post-pollination mechanisms when conspecific and heterospecific pollen mixtures grow in the style. Interspecific pollen was more successful at siring seeds when competing with conspecific outcross pollen. In three-donor crosses, self and interspecific pollen sired similar number of seeds, thus having a greater reproductive success than conspecific outcross pollen (Fig. 9). The similar success of selfed vs. interspecific pollen donors in three-donor crosses was due to only one maternal family, which set 19:0:5 selfed:outcrossed:heterospecific seeds. These results suggest that when *N. longiflora* pollen is delivered on *N. plumbaginifolia* stigmas, hybridization can occur, such as has been predicted by the unilateral incompatibility rule as well as has been described in other studies with *N. plumbaginifolia* using single donor crosses (Murfett *et al*., 1996; McClure *et al*., 2000; Hancock *et al*., 2003; Lee *et al*., 2008). The post-pollination selection detected in both species seem to be acting at different times and through different mechanisms in each species, i.e., through greater pollen tube growth rate of outcross over self pollen within *N. longiflora* and seemingly through selective abortion of conspecific seeds in *N. plumbaginifolia* (Fig. 5).

Because this preference for interspecific pollen in *N. plumbaginifolia* was detected with one marker locus (NA6) the effect could be due to overdominance for fitness at the marker or a linked locus. Alternatively, it could be due to a chromosomal phenomenon, such that only certain chromosomal combinations in the hybrid cross can result in viable embryos. However, this can not explain the low proportion of conspecific offspring. Further, the interspecific preference in *N. plumbaginifolia* was observed in both two- and three-donor crosses (Figs. 8 and 9). Performing interspecific single donor crosses within section *Alatae*, Lee *et al*. (2008) found that seed set in *N. plumbaginifolia* stays the same no matter which species was the pollen donor. On the other hand, interspecific pollinations had a negative effect on seed set of *N. longiflora*.

Pollen tube growth rate and self vs. outcross paternity success

In self-compatible species, the siring advantage of outcross over self pollen caused by differential pollen tube growth rate (i.e. cryptic self-incompatibility) is a mechanism that prevents self-pollinations through the competitive ability of outcross pollen over self-pollen in pollen mixtures (Bateman, 1956; Bowman, 1987; Casper *et al*., 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al*., 1990; Cruzan and Barrett, 1993; Rigney *et al*., 1993; Travers and Mazer, 2000; Kruszewski and Galloway, 2006). Both *Nicotiana longiflora* and *N. plumbaginifolia* are self-compatible; however, results demonstrated that only *N. longiflora* outcross pollen has greater pollen tube growth rate than self pollen, thus conferring outcross pollen with a strong significant fertilization advantage. Other studies on self-compatible species with floral traits typical of outcrossers have also shown

greater pollen tube growth rate and siring success of outcross pollen (Bowman, 1987; Cruzan and Barrett, 1993; Jones, 1994).

In *N. longiflora*, the results showed that outcross pollen grows almost twice as fast as self-pollen (Fig. 5a, Table 6). However, the difference in seeds sired by outcross pollen was almost 15 times greater than self pollen. This suggests that other mechanisms besides differential pollen tube growth rate between self and outcross pollen are also favoring outcross pollen to sire more seeds.

Multiple mechanisms determine the success of pollen from the time they are deposited on the stigmas to fertilization and post-fertilization success. Pre-zygotic mechanisms providing an advantage to outcross pollen for siring seeds include germination rate, differential pollen tube growth rate, tube attrition, and substances that inhibit or facilitate pollen tube growth (Bowman, 1987; Casper *et al*., 1988; Hessing, 1989; Weller and Ornduff, 1989; Aizen *et al*., 1990; Cruzan, 1990; Walsh and Charlesworth, 1992; Cruzan and Barrett, 1993; Jones, 1994; Erbar, 2003; Kruszewski and Galloway, 2006). Pre-zygotic processes taking place within the ovary that are determinant for fertilization also play a role in determining paternity success; however, little is known about them. Once fertilization occurs, post-zygotic selection can take place through mechanisms primarily associated with inbreeding depression such as seed abortion, fruit abortion, and seed filling biased toward outcross pollen donors (Charlesworth, 1988; Weller and Ornduff, 1991; Montalvo, 1992; Eckert and Allen, 1997; Erbar, 2003). It is probable that both differential pollen tube growth rate between self and outcross pollen and selective abortion of seeds are favoring the high reproductive success of outcross pollen in *N. longiflora*. This is not surprising given the importance of

pollinators for plant reproductive success, high outcrossing rates and low fixation indices previously recorded in *N. longiflora* (Chapters 2, 4).

On the other hand, pollen tube growth rate of outcross and self pollen in *N. plumbaginifolia* was not significantly different (Fig. 5), indicating that both sources of pollen have similar probabilities for ovule fertilization. This was also confirmed with paternity determination of self vs. outcross mixed pollinations. Seeds sired from selfed vs. outcrossed pollen were just slightly different from each other under random mating (expected 1:1 ratio), but not from an expected ratio 0.6: 0.4 (Fig. 7a; App. 1). *Nicotiana plumbaginifolia*'s floral morphology (short corolla tubes, and reduced herkogamy) and mating system is that of a strong selfer (Chapters 2 and 3); hence, selection for competitive pollen is not expected to be common, and therefore outcross and self pollen have the same competitive ability, as estimated by pollen tube growth rate. Lack of differences in pollen tube growth rate between outcross and self pollen has also been found in other species (Casper, 1985; Bertin and Sullivan, 1988; Fenster and Sork, 1988; Weller and Ornduff, 1991; Travers and Mazer, 2000), including species exhibiting a mixed mating system (Montalvo, 1992; Baker and Shore, 1995), such as *N. plumbaginifolia*.

***Paternity success on two- and three- donor crosses in* N. longiflora**

Nicotiana longiflora's conspecific outcross pollen was significantly more successful at siring seeds than self or interspecific pollen donors. This result might be first, a consequence of pollen tube growth rate being greater for outcross pollen than self pollen. The low success of interspecific pollen at siring seeds could also be related to

differential pollen tube growth rates. Although mean pollen tube growth rates between *N. longiflora* and *N. plumbaginifolia* were not significantly different from each other, the mean pollen tube growth rate of *N. longiflora*'s outcross pollen (0.69 ± 0.08 mm/h) was the greatest, compared with self- pollen (0.39 ± 0.06 mm/h) or *N. plumbaginifolia*'s pollen (0.55 ± 0.02 mm/h). In single pollen donor crosses, Lee *et al*. (2008) found that only pollen from species with similar style length was able to fertilize *N. longiflora*'s ovules. In single- and two- donor assays in other species, Emms *et al*. (1996) and Carney *et al*. (1994), also found a strong discrimination against heterospecific pollen. Pollinator observations in the sympatric Mango population showed that the frequency of interspecific movements between the *Nicotiana* species is rather low (3.7% visits), therefore, the 1:1 pollen proportions used in the current study probably have an excess of heterospecific pollen compared with what occurs in natural populations. Even though the pollen mixtures were not an exact representation of those that are probably found in sympatric natural populations, my study is unique being, to my knowledge, the first one conducting three-donor pollinations on single stigmas to test the paternity success of pollen from selfed, outcrossed and interspecific sources.

***Paternity success on two- and three- donor crosses in* N. plumbaginifolia**

When *N. plumbaginifolia* acted as the maternal parent, hybridization was strongly favored, so that when *N. longiflora*'s pollen is delivered, it fertilizes approximately the same number of ovules as self pollen, with very few offspring being sired by intraspecific outcross pollen (Figs. 8, 9). Three phenomena, not mutually exclusive from each other, might be in play here. First, the high number of ovules fertilized by interspecific pollen

can be a consequence of the greater pollen tube growth rate of *N. longiflora* as compared with that of *N. plumbaginifolia*. While I did not estimate PTGR in interspecific crosses, several studies using interspecific crosses have shown that the success in fertilizing ovules is predicted by the style lengths of the species being crossed (e.g., Aizen *et al*., 1990; Williams and Rouse, 1990; Diaz and Macnair, 1999), including my study species (Lee et al. 2008). Therefore it is not surprising that *N. longiflora*'s pollen fertilized a high number of *N. plumbaginifolia*'s ovules. However, results from Chapter 3 showed that *N. plumbaginifolia* is experiencing higher self-fertilization in sympatric populations, perhaps as a mechanism of isolation between the species.

Second, if ovule fertilization was only determined by pollen tube growth rate, then one would expect very few offspring sired by self pollen. However, the results showed similar offspring sired by self and interspecific pollen. It is possible that the great number of self-offspring was a consequence of mentor effects, such that self pollen tube growth and fertilization success will be facilitated by the presence of interspecific pollen growing in the style. Mentor effects occur when mixtures of pollen from different donors are deposited on the stigmas, and one donor type influences the growth of different pollen types (Cruzan, 1989, 1990; De Nettancourt, 2001). Alternatively, it is possible that seeds sired by interspecific pollen experience hybrid vigor and therefore, might be selected over seeds sired by the other sources of pollen. For instance, hybrid vigor in several aspects of fitness has been recorded for crosses between *Iris fulva* and *I. hexagona* (Burke *et al*., 1998), *Populus fremontii* and *P. angustifolia* (Schweitzer *et al*., 2002), *Iris brevicaulis* and *I. fulva* (Johnston *et al*., 2003) and *Oryza rufipogon* and *O. sativa* (Song *et al*., 2004).

Third, the selective disadvantage of outcross compared to self and interspecific pollen in fertilizing ovules might be caused by a lack of affinity of the stylar environment for outcross pollen. It has been suggested that in species with a long history of selfing, pollen has been selected to grow in particular stylar environments, thus conferring it with a slight competitive ability over outcross-pollen (Cruzan, 1990). Studies on interspecific pollen-pollen interactions have suggested the existence of allelopathic effects of heterospecific pollen on the performance of pollen growing in styles of its own species, such that germination, tube growth and seeds sired are negatively affected (Kanchan and Jayachandra, 1980; Thomson *et al*., 1981) Alternatively, the stylar tissue could have negative interactions with outcross- pollen (Cruzan, 1989). Further studies are certainly needed in order to clarify pollen-pollen and pollen-stylar tissue interactions occurring in three-donor pollinations. For example, studies focused on within-ovary phenomena together with differentially stained pollen tubes, will shed some light on pre- and post-fertilization processes determining paternity success of different pollen donors.

Implications

In this paper I have shown that the sister species *N. longiflora* and *N. plumbaginifolia* have different post-pollination mechanisms that favor particular sources of pollen when delivered on the stigmas in mixed pollinations. In *N. longiflora* pre- and post-zygotic mechanisms favoring outcross over other sources of pollen seem to be acting. Paternity determination of offspring obtained via self vs. outcross mixed pollinations showed a strong advantage of outcross pollen at siring seeds, probably as a consequence of the greater pollen tube growth rate of outcross over self pollen. It is possible that post-zygotic

abortion of inbred seeds also contribute to the high difference in reproductive success between self and outcross pollen. On the other hand, *N. plumbaginifolia* does not seem to experience pre-zygotic selective mechanisms mediated through differential pollen tube growth rate that favor the reproductive success of outcross over self pollen.

Results from crosses with mixed conspecific and heterospecific pollen demonstrated that hybridization between the *Nicotiana* species is relatively common, especially in *N. plumbaginifolia* (Figs. 8, 9; Table 3; App. 2, 3). In sympatric, natural populations, *N. longiflora* and *N. plumbaginifolia* have overlapping flowering seasons and share pollinators (Chapter 2). Results from artificial crosses might have strong implications in a more realistic scenario because of the gene flow that probably is taking place in natural populations (Chapter 3).

First, hybrids produced by pollinating *N. plumbaginifolia* flowers with *N. longiflora* pollen probably have intermediate morphology between the parental species and thus still be attractive to pollinators. Intermediate floral morphology between hybrids and paternal parents has been documented for other *Nicotiana* within section *Alatae* (Ippolito *et al*., 2004) as well as in other species (Sersic *et al*., 2001; Archibald *et al*., 2004). Because of the intermediate morphology between hybrids and parental species, pollinators might visit both F1 hybrids and *N. longiflora* flowers without discrimination. Other authors have recorded pollinator movements between parental species and hybrids (Wendt *et al.*, 2001; Ippolito *et al*., 2004). Under this scenario, gene flow promoted by pollinators will introduce more *N. plumbaginifolia* genes into *N. longiflora* than the reverse direction, due to the biased paternity results found here. Similarly, Martin and Willis (2007) found asymmetric hybridization whenever *Mimulus guttatus* pollen was

delivered on *M. nasutus*. They suggest that asymmetric introgression is also occurring, with backcrosses more frequent to the outcross species *Mimulus guttatus*. Broyles (2002) also found asymmetric introgression between two *Asclepias* species, suggesting that backcrosses with the parental species are possible because pollinators do not discriminate between hybrids and the parental species.

Second, the delivery of mixed pollen loads on the stigmatic surface of plants could lead to gametophytic selection (Marshall and Ellstrand, 1985). Pollen with the abilities to germinate, grow and fertilize faster will be favored by selection. Thus, in *N. longiflora* conspecific outcross pollen will be favored no matter what other kinds of pollen are present. On the other hand, in *N. plumbaginifolia* it seems that conspecific outcross pollen will be selected against, and that may explain why this kind of pollen was less successful at siring offspring.

In natural populations, ratios of pollen from different sources are probably different from the 1:1 ratios that were used in the mixed pollinations in this study. Despite the ratio of pollen used in each cross, it is clear that by sharing pollinators (Chapter 2), *N. plumbaginifolia* and *N. longiflora* might receive interspecific pollen on their stigmas, and thus hybridization is a possibility.

Although in natural populations, hybridization between *N. longiflora* and *N. plumbaginifolia* seems to be rare (Chapter 3), the results presented in this chapter show that hybridization is a real possibility. However, not knowing how long there has been gene flow between *N. longiflora* and *N. plumbaginifolia* prevents us from determining how much introgression has occurred or how much of the original genome from each species is still conserved. New studies using a higher number of polymorphic markers on

populations outside the sympatric zone will be of great relevance to follow up on these questions.

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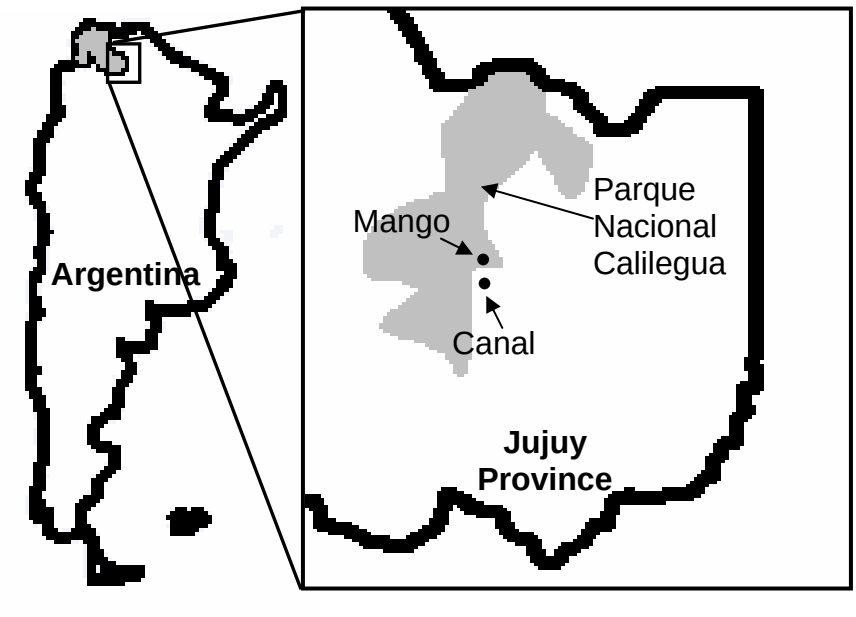


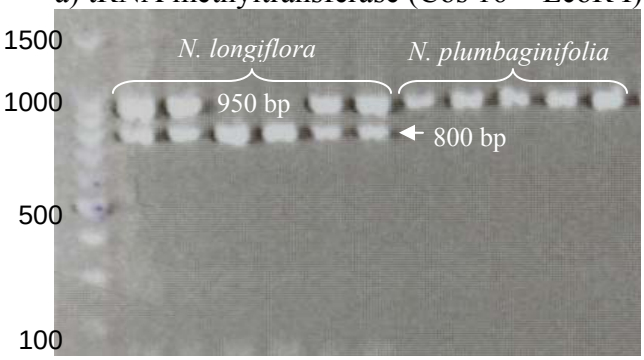
Figure 1. Location of the two sympatric populations (Mango and Canal) of *Nicotiana longiflora* and *N. plumbaginifolia* from where seeds were collected to conduct pollen competition assays.

Maternal plant	**Pollen donors**		
	4	5	6
1	1+4	1+5	1+6
2	2+4	2+5	2+6
3	3+4	3+5	3+6

Maternal Plant	**Pollen donors**		
	1	2	3
4	4+1	4+2	4+3
5	5+1	5+2	5+3
6	6+1	6+2	6+3

Figure 2. General design for crosses to test for pollen tube interactions in *N. longiflora* and *N. plumbaginifolia* via paternity determination. Reciprocal crosses are shown on the right. Each cell shows the combinations of pollen used for two-donor hand pollinations.

a) tRNA methyltransferase (Cos 16 + EcoR I)



b) Cellulose synthase D-like protein mRNA (NA6 + Hha I)

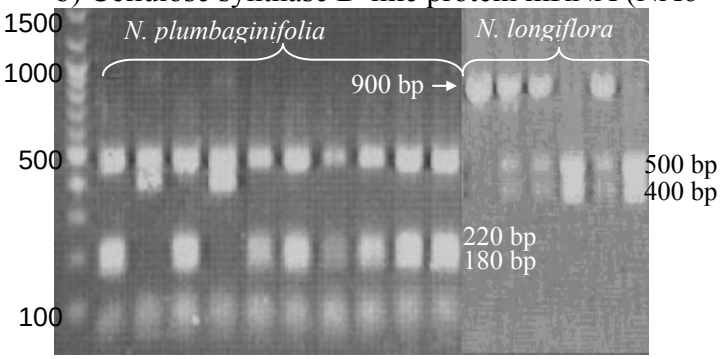


Figure 3. Agarose gels showing markers used for paternity determination after two- and three- donor hand pollinations in *Nicotiana longiflora* and *N. plumbaginifolia*. a) tRNA methyltransferase marker (Cos 16) digested with EcoR I in *N. longiflora* and *N. plumbaginifolia* plants. Single bands at 800 and 950 bp indicate alternative homozygotes, whereas two bands around 950 bp and 800 bp indicate heterozygotes (indicated with arrows). B) Cellulose synthase D-like protein mRNA (NA6) digested with Hha I. In *N. plumbaginifolia* bands around 500 bp are not polymorphic. Homozygous have alternative bands at around 400 or 220+180 bp, whereas heterozygotes have three bands at 400 + 220 + 180 bp (not shown). In *N. longiflora*, homozygous have alternative bands at 900 and 500+400 bp, whereas heterozygotes have three bands at 900+500+400 bp. In both cases 100 bp ladder was used.

a) Analysis per species

Two-donor crosses

	Observed frequency
Self	
Outcross	

Three-donor crosses

	Observed frequency
Self	
Outcross	
Interspecies	

b) Heterogeneity among species

Two donor crosses

	Self	Outcross
N. longiflora		
N. plumbaginifolia		

Three donor crosses

	Self	Outcross	Interspecific
N. longiflora			
N. plumbaginifolia			

c) Heterogeneity among populations

Two donor crosses

	Self	Outcross
Canal		
Mango		

Three donor crosses

	Self	Outcross	Interspecific
Canal			
Mango			

Figure 4. Summary of χ^2 tests applied to paternity data from offspring obtained through two- and three- donor crosses. A) Analyses to test for the performance of different kinds of pollen within species. B and C) Analyses to test for heterogeneity among species and populations, respectively. Results are shown in Figs. 6-8 and App. 1-3.

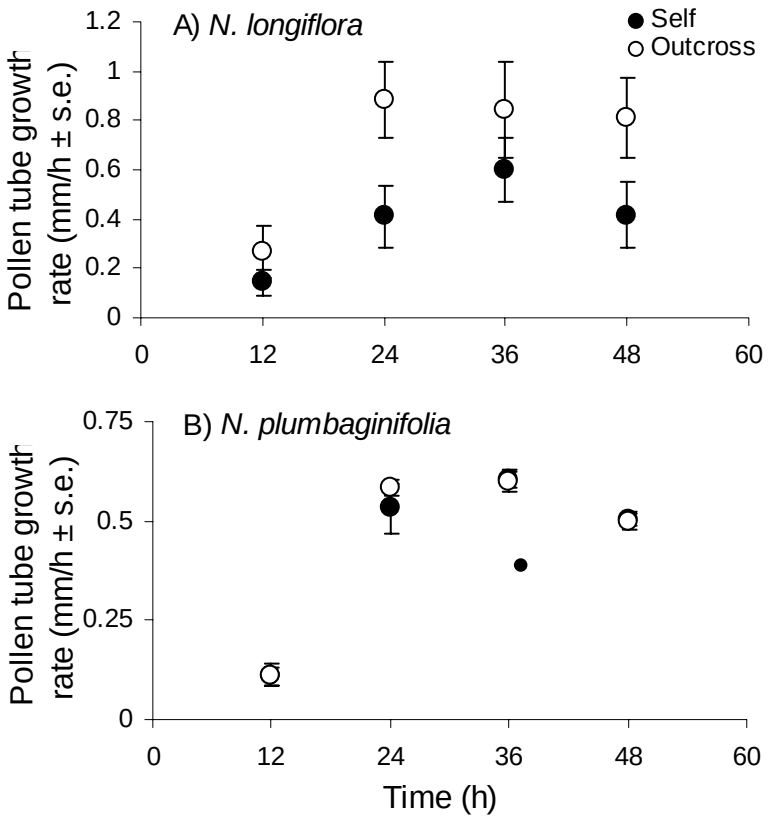


Figure 5. Changes in pollen tube growth rate (± s.e.) of self- (closed circles) and outcross- (open circles) pollen tubes through time. A) *N. longiflora* and B) *N. plumbaginifolia*. *Post hoc* Tukey tests showed that self-pollen tube growth rate was significantly lower than outcross pollen in *N. longiflora*. Pollen tube growth rate at 12 h was significantly lower than the other style collection times.

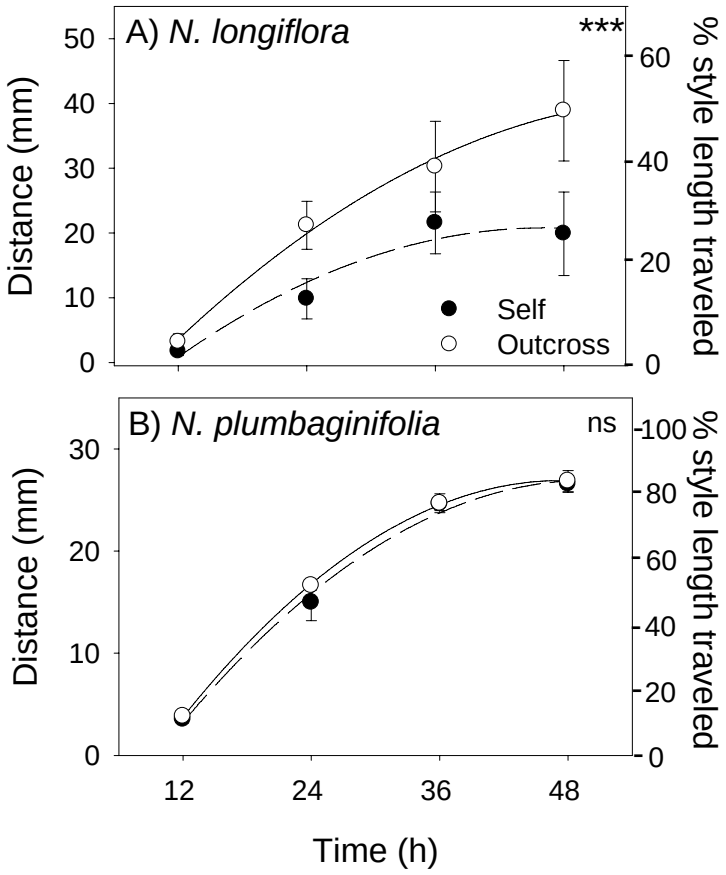


Figure 6. Mean distance (± s.e.) and percentage style length (± s.e.) traveled by outcross (open circles) and self (closed circles) pollen tubes growing in conspecific styles collected every 12h after pollination in A) *N. longiflora* and, B) *N. plumbaginifolia*.

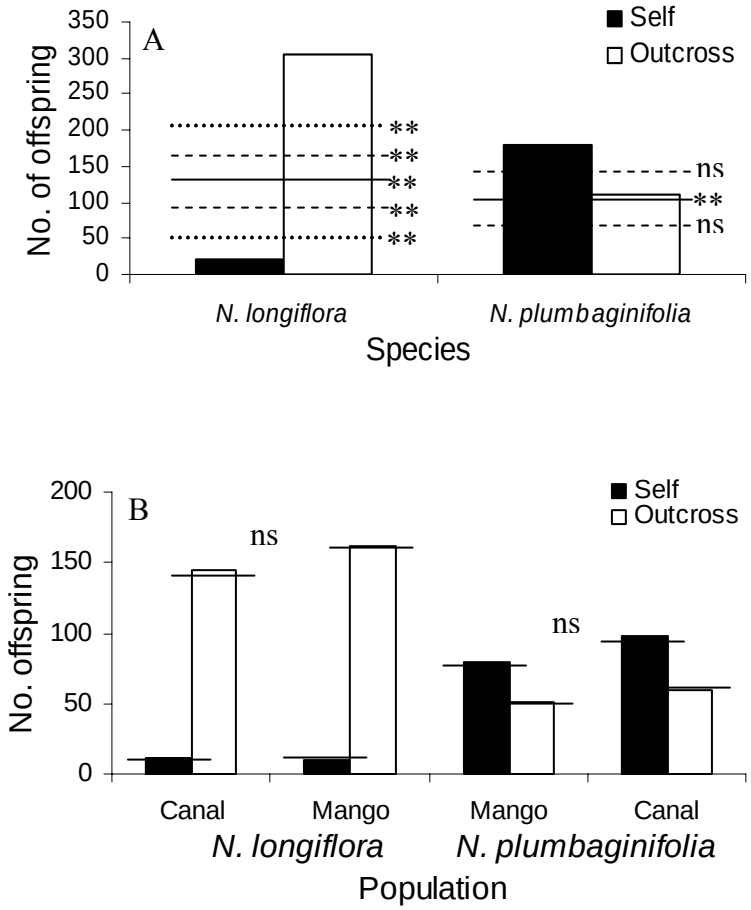
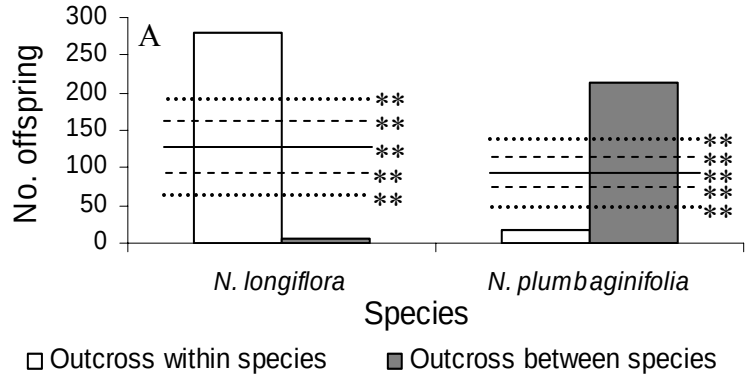


Figure 7. Offspring obtained through two-donor mixed pollinations using self- and outcross-pollen in *N. longiflora* and *N. plumbaginifolia*. A) Pooled data per species showing the expected frequencies under random mating (1:1 ratio; solid line) and under the biased expectations of 0.6:0.4 (dashed lines) and 0.7:0.3 (dotted lines). B) Heterogeneity among populations. Expected values are shown for each bar (see appendix for expected values based on contingency tables). Asterisks indicate significant differences between observed and expected frequencies. ns= expected and observed frequencies are not significantly different from each other in paternity tests (A) and heterogeneity analyses between populations (B).



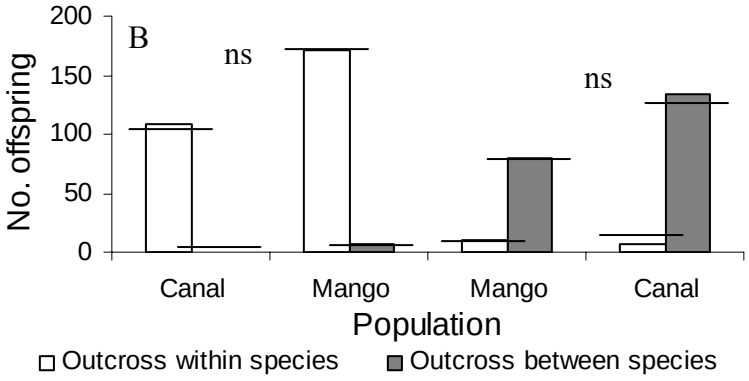


Figure 8. Offspring obtained through two-donor mixed pollinations using intra- and inter-specific pollen in *N. longiflora* and *N. plumbaginifolia*. A) Pooled data per species showing the expected frequencies under random mating (1:1 ratio; solid line) and under the biased expectations of 0.6:0.4 (dashed lines) and 0.7:0.3 (dotted lines). B) Heterogeneity among populations. Expected values are shown for each bar (see appendix for expected values based on contingency tables). Asterisks indicate significant differences between observed and expected frequencies. ns= expected and observed frequencies are not significantly different from each other as shown by heterogeneity tests for each species.

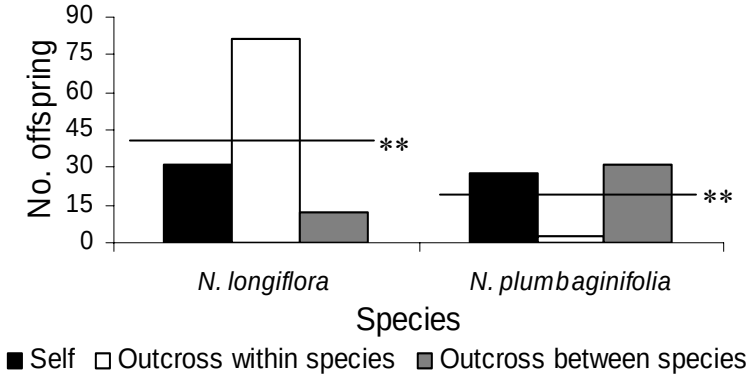


Figure 9. Offspring obtained through three-donor mixed pollinations using self- outcross- and interspecific- pollen in *N. longiflora* and *N. plumbaginifolia*. Solid lines indicate the expected frequencies under random mating (1:1:1). Asterisks indicate significant differences between observed and expected frequencies.

Table 1. General information of the sympatric populations from which *Nicotiana longiflora* and *Nicotiana plumbaginifolia* seeds were collected and grown in the greenhouse.

Population	Locality	Description	Altitude	Coordinates
Canal	Ledesma, Jujuy	In town, on highway	492 m	S 23° 48' 36" W 64° 47' 46"
Mango	Parque Nacional Calilegua, Jujuy	Camping site	544 m	S 23° 46' 36.8" W 64° 49' 42.6"

Table 2. Electrophoresis band sizes for the genotypes of the two loci used to determine paternity after mixed hand pollinations on *N. longiflora* and *N. plumbaginifolia.* Cos 16= tRNA methyltransferase digested with EcoRI; NA6= cellulose synthase D-like protein mRNA digested with HhaI. Notice that Cos 16 is monomorphic for *N. plumbaginifolia*.

Species	**Locus**	**Genotypes (bp)**		
		Homozygote 1	**Homozygote 2**	**Heterozygote**
N. longiflora	Cos 16	950	800 + 150	950 + 800 + 150
	NA6	900	500 + 400	900 + 500 + 400
N. plumbaginifolia	Cos 16	950	950	950
	NA6	400	220 + 180	400 + 220 + 180

Table 3. Analysis of covariance to test for differences in self and outcross pollen tube growth rate between *Nicotiana longiflora* and *N. plumbaginifolia* at different times of style collection. Style length was included as covariate.

Source	**df**	**Mean squares**	***F***	***P***
Style length	1	0.050	0.49	0.484
Species	1	0.051	0.50	0.482
Style collection time (SCT)	3	1.619	15.84	< 0.0001
Pollination treatment (PT)	1	1.128	11.04	0.0011
Species x SCT	3	0.045	0.44	0.725
Species x PT	1	0.815	7.98	0.005
SCT x PT	3	0.074	0.73	0.537
Species x SCT x PT	3	0.051	0.50	0.681

Table 4. Mean tube growth rate (mm/h ± s.e.) of self and outcross pollen of *Nicotiana longiflora* and *N. plumbaginifolia* at different times from hand-pollinations.

Style collection time (h)	***Nicotiana longiflora***		***Nicotiana plumbaginifolia***	
	Self	**Outcross**	**Self**	**Outcross**
12	0.142 ± 0.051	0.265 ± 0.104	0.111 ± 0.028	0.109 ± 0.024
24	0.410 ± 0.129	0.883 ± 0.154	0.532 ± 0.065	0.583 ± 0.021
36	0.599 ± 0.132	0.840 ± 0.194	0.604 ± 0.018	0.601 ± 0.027
48	0.414 ± 0.134	0.810 ± 0.161	0.503 ± 0.017	0.501 ± 0.023
Overall	0.391 ± 0.062	0.699 ± 0.085	0.438 ± 0.035	0.448 ± 0.034

Table 5. Analysis of variance to test for differences in distance traveled by pollen tubes at different times of style collection.

Source	**df**	**Mean squares**	***F***	***P***
		Nicotiana longiflora		
Style collection time (SCT)	3	2933.030	11.81	< 0.0001
Pollination treatment (PT)	1	2052.844	8.27	0.005
SCT x PT	3	261.597	1.05	0.374
		Nicotiana plumbaginifolia		
Style collection time (SCT)	3	2197.918	261.00	< 0.0001
Pollination treatment (PT)	1	6.589	0.78	0.379
SCT x PT	3	2.618	0.31	0.817

Table 6. Mean distance (mm ± s.e.) traveled by self and outcross pollen tubes of *Nicotiana longiflora* and *N. plumbaginifolia* at different times from hand-pollinations.

Style collection time (h)	***Nicotiana longiflora***		***Nicotiana plumbaginifolia***	
	Self	**Outcross**	**Self**	**Outcross**
12	1.705 ± 0.614	3.183 ± 1.253	3.526 ± 0.370	13.824 ± 0.370
24	9.833 ± 3.095	21.188 ± 3.701	14.982 ± 1.792	16.631 ± 0.472
36	21.555 ± 4.765	30.254 ± 6.997	24.608 ± 0.646	24.683 ± 0.928
48	19.891 ± 6.449	38.884 ± 7.754	26.572 ± 0.831	26.846 ± 1.028
Overall	13.246 ± 2.437	23.377 ± 3.415	17.422 ± 1.549	17.996 ± 1.490

Table 7. Paternity determination of offspring obtained through two- and three- donor hand pollinations in *Nicotiana longiflora* and *N. plumbaginifolia*. Results from each maternal parent are pooled from crosses with three different parent donors. In the Canal *N. plumbaginifolia* population, eight maternal parents were used because two of the originally selected plants stopped producing flowers. Empty cells indicate fruit abortion.

Species	Population	Maternal Plant	Two-donor		Two-donor		Three-donor crosses		
			Self	Outcross	Outcross	Interspecies	Self	Outcross	Interspecies
N. longiflora	Mango	1	5	25	30	0	22	8	0
		2	0	28	29	1	0	11	7
		3	0	28	29	1	4	24	0
		4	2	24	27	1	5	9	4
		5	0	29	30	0	0	6	0
		6	3	27	26	4	0	23	1
	Canal	1	2	27	20	0			
		2	0	19	26	0			
		3	0	24	6	0			
		4	7	23	26	0			
		5	2	26	30	0			
		6	0	26					
	Total		21	306	279	7	31	81	12
N. plumbaginifolia	Mango	1	1	1	7	23	19	0	5
		2	3	7	2	19	3	1	6
		3	13	17	0	13	4	0	10
		4	24	4	0	20	1	1	7
		5	27	3	2	4	1	1	3
		6	12	19					
	Canal	1	6	3	0	25			
		2	2	18	0	26			
		3	0	9	2	21			
		4	23	7	0	23			
		5	26	4	3	21			
		6	14	6	1	18			
		7	5	5					
		8	22	7					
	Total		178	110	17	213	28	3	31

Appendix 1. χ^2 tests performed to determine offspring paternity, in two-donor (self vs. outcross) pollinations in *Nicotiana longiflora* (A) and *N. plumbaginifolia* (C). To determine offspring paternity within species, three tests were conducted: under a 1:1 pollen mixtures, and biased expectations under 0.6:0.4 and 0.7:0.3 ratios (expected 0.7:0.3 ratio was not tested in *N. plumbaginifolia* because the observed frequencies were already equal to the expected 0.6:0.4 rate tested). Contingency tests for heterogeneity between populations within species (B, D) and heterogeneity between species (E) were also conducted. Because multiple tests were conducted with the same data, significance was considered at P=0.01.

	Self- pollinated		**Outcross- pollinated**		**Σ (obs - exp)^2/Exp**
	Observed	**Expected**	**Observed**	**Expected**	
N. longiflora					
A) Paternity tests					
1:1 expectation	21	163.5	306	163.5	χ^2= 248.394, P<< 0.001
0.6:0.4 expectation	21	130.8	306	196.2	χ^2= 153.62, P<< 0.001
0.7:0.3 expectation	21	98.1	306	228.9	χ^2= 86.564, P<< 0.001
B) Heterogeneity between populations					
Canal	11	10.018	145	145.982	χ^2= 0.196, P> 0.01
Mango	10	10.98	161	160.018	
N. plumbaginifolia					
C) Paternity tests					
1:1 expectation	178	144	110	144	χ^2= 16.056, P< 0.001
0.6:0.4 expectation	178	172.8	110	115.2	χ^2= 0.391, P> 0.01
D) Heterogeneity between populations					
Canal	98	97.035	59	59.965	χ^2= 0.055, P> 0.01
Mango	80	80.965	51	50.035	
E) Heterogeneity between species					
N. longiflora	21	105.81	306	221.19	χ^2= 214.601, P<< 0.001
N. plumbaginifolia	178	93.19	110	194.81	

Appendix 2. χ^2 tests performed to determine offspring paternity, in two-donor (outcross vs. interspecies) pollinations in *Nicotiana longiflora* (A) and *N. plumbaginifolia* (C). To determine offspring paternity within species, three tests were conducted: under 1:1 pollen mixtures, and biased expectations under 0.6:0.4 and 0.7:0.3 ratios. Contingency tests for heterogeneity between populations within species (B, D) and heterogeneity between species (E) were also conducted. Because multiple tests were conducted with the same data, significance was considered at P=0.01.

	Outcross- pollinated		**Interspecies- pollinated**		**Σ (obs - exp)^2/Exp**
	Observed	**Expected**	**Observed**	**Expected**	
N. longiflora					
A) Paternity tests					
1:1 expectation	279	143	7	143	χ^2= 258.686, P<< 0.001
0.6:0.4 expectation	279	171.6	7	114.4	χ^2= 168.047, P<< 0.001
0.7:0.3 expectation	279	200.2	7	85.8	χ^2= 103.387, P<< 0.001
B) Heterogeneity between populations					
Canal	108	105.357	0	2.643	χ^2= 4.353, P> 0.01
Mango	171	173.643	7	4.357	
N. plumbaginifolia					
C) Paternity tests					
1:1 expectation	17	115	213	115	χ^2= 167.026, P<< 0.001
0.6:0.4 expectation	17	92	213	138	χ^2= 101.902, P<< 0.001
0.7:0.3 expectation	17	69	213	161	χ^2= 55.983, P<< 0.001
D) Heterogeneity between populations					
Canal	6	10.348	134	129.652	χ^2= 5.042, P> 0.01
Mango	11	6.652	79	83.348	
E) Heterogeneity between species					
N. longiflora	279	164.06	7	121.94	χ^2= 423.725, P<< 0.001
N. plumbaginifolia	17	131.94	213	98.06	

Appendix 3. χ^2 tests performed to determine offspring paternity in three-donor (self vs. outcross vs. interspecies) pollinations in *Nicotiana longiflora* (A) and *N. plumbaginifolia* (B) under 1:1:1 pollen mixtures. A contingency test for heterogeneity between species was also conducted (C). Because multiple tests were conducted with the same data, significance was considered at P=0.01.

	Self- pollinated		**Outcross- pollinated**		**Interspecies-pollinated**		**Σ(obs-exp)^2/Exp**
	Observed	**Expected**	**Observed**	**Expected**	**Observed**	**Expected**	
N. longiflora							
A) Paternity test 1:1:1 expectation	31	41.33	81	41.33	12	41.33	χ^2= 61.473 P<< 0.001
N. plumbaginifolia							
B) Paternity test 1:1:1 expectation	28	20.67	3	20.67	31	20.67	χ^2= 22.866 P<< 0.001
C) Heterogeneity between species							
N. longiflora	31	39.33	81	56	12	28.67	χ^2= 67.858
N. plumbaginifolia	28	19.67	3	28	31	14.33	P<< 0.001

Chapter 5. Conclusions and future perspectives

The research presented here was motivated by the possibility of ecological factors as shaping forces of plant mating systems. In particular, I explored the importance of interactions between *Nicotiana longiflora* and *N. plumbaginifolia* as influences on their mating systems. In order to determine how mating systems are affected by interactions between these species, I first determined if in fact plant mating systems could vary among populations as a possible indicator of different ecological forces acting at different levels in the various populations. Then, I determined the output of both direct (pollen-pollen interactions when growing in the style) and indirect (mediated through pollinator preferences) interaction, by estimating plant fitness, outcrossing rates, population differentiation and offspring paternity.

Overall, results showed that mating systems (i.e., outcrossing rates) vary among populations (Chapter 3). Interpopulational variability in mating systems was demonstrated by using both pollen:ovule ratio (Chapter 2) and outcrossing rate estimates (Chapter 3). Although both methods estimate mating system variability among populations, detailed scrutiny shows surprising differences in the outcomes of the two methods. Following Cruden's (1977) classification of mating systems based on pollen:ovule ratios, mating systems for both *Nicotiana* species should range from facultative to obligate autogamous. By contrast, a potentially more accurate method, based on offspring paternity, demonstrated that *N. longiflora* is a strong outcrosser, whereas *N. plumbaginifolia* varies between strong selfer (in sympatry) to a mixed mating

system (in allopatry). Moreover, this difference in mating system between sympatric and allopatric populations found via outcrossing rates, was not shown by using pollen:ovule ratio estimates. These contradictory results demonstrate that estimations of mating system through pollen:ovule ratio following the classification of Cruden (1977) may not always be accurate.

Theoretical studies on the evolution of plant mating systems have acknowledged the importance of ecological factors as selective agents shaping mating systems (Jain, 1976; Wyatt, 1983; Grant, 1994; Kay and Schemske, 2003). Interpopulational variability in mating systems in the *Nicotiana* species (Chapters 2 and 3), together with observations of inter-species pollinator movements in the sympatric Mango population (Chapter 2), suggested that ecological interactions via both pollinator preferences and pollen-pollen interactions might be occurring. In exploring this possibility, results from Chapters 3 and 4 demonstrate that both kind of interactions influence mating system in *N. longiflora* and *N. plumbaginifolia*.

Indirect interactions caused a decrease in *N. longiflora* seed set (as an estimator of fitness) and *N. plumbaginifolia* outcrossing rates (Chapter 3), suggesting the existence of a competitive interaction between the species. Direct pollen-pollen interactions showed that *N. longiflora* has a strong advantage of outcross pollen over either self- (i.e. there is cryptic self-incompatibility) or heterospecific- pollen (i.e. isolation mechanism between species to prevent hybridization) on siring seeds (Chapter 4). On the other hand, *N. plumbaginifolia* showed a strong discrimination against outcross pollen, favoring either self- or heterospecific-pollen, which suggests that hybridization with this species as the maternal parent is a possibility. However, results from both indirect and direct

interactions and pollinator observations provide evidence that even though these *Nicotiana* species might receive infrequent heterospecific pollen, both species have strong barriers preventing hybridization. *Nicotiana longiflora* is only fertilized by its own outcross pollen (Chapter 4), and *N. plumbaginifolia* tends towards an increase in self-fertilization when in sympatry (Chapter 3). If hybridization occurs, it might be happening at very low rates, such as has been recorded in other pairs of sympatric species (Rieseberg *et al*., 1998; Ramsey *et al*., 2003).

Although hybridization between *N. longiflora* and *N. plumbaginifolia* might not be very common, it can have strong implications for the evolutionary pathways of these species. Whenever these *Nicotiana* species intercross with each other, hybrids have a *N. longiflora*-like phenotype (*pers. obs.*). This, together with the fact that pollinators have a strong preference for *N. longiflora* over *N. plumbaginifolia* flowers (Chapter 2), suggests that *N. plumbaginifolia* genes are being introgressed into *N. longiflora*'s genome.

Although this study demonstrated that *N. longiflora* and *N. plumbaginifolia* interact with each other, it also brought new questions that remain to be explored in the future. For example, the populations sampled for this research were located relatively close to each other and to the hybrid zone; therefore, it would be of great interest to explore whether the results obtained for allopatric populations in this study will hold true for populations sampled far from the sympatric zone. In such studies, it will be interesting to look at ouctrossing rates in allopatric *N. plumbaginifolia* and the advantage of outcross over self pollen in *N. longiflora*.

In reference to sympatric populations in the hybrid zone, it will be of great ecological and evolutionary interest to determine actual hybridization rates and to

determine if, in effect, introgression is occurring, and if so, at which rate. Also, if introgression is occurring in these populations, how much of the genomes have been involved? How much of the original genome from each species still remains? In my research, I found two mechanisms that seem to be acting as isolation barriers to prevent hybridization: the strong preference of *N. longiflora* for its own outcross pollen and the trend of *N. plumbaginifolia* towards an increase in self-fertilization in sympatric populations. However, it is unknown if there are other prezygotic and/or postzygotic mechanisms preventing hybridization and if so, what their relative roles are.

At a broader level, it will be of great interest to determine how the different aspects evaluated in the current research vary on a time scale, not only among years, but also at different times during the flowering season of each species. Are outcrossing rates and possibilities for hybridization density-dependent? In sympatric populations, do pollinators visit *N. plumbaginifolia*'s flowers more frequently when *N. longiflora* has not yet started flowering?

Overall, this research strongly supports that interactions between *N. longiflora* and *N. plumbaginifolia* are occurring in sympatric natural populations at the present time. However, the effect of such interactions is manifested in different traits (seed set for *N. longiflora*, and outcrossing rates for *N. plumbaginifolia*). This study constitutes an important contribution to the evaluation of ecological interactions as forces shaping mating systems. To my knowledge there is only one other study that determined the effect of ecological interactions on outcrossing rates. Bell *et al*. (2005) evaluated the effect of the sympatric *Lobelia siphilitica* on outcrossing rates and fitness of *Mimulus ringens*; however they did not study the effect on both species. Therefore, my research

represents the first documentation of the effects of interspecific interactions on fitness and realized mating systems in both of the species interacting. This study is broader by not only looking at outcrossing rates but also evaluating interpopulational variability and sympatry effects on floral traits associated with mating systems, particularly pollen:ovule ratio, as well as offspring paternity from mixed hand-pollinations. Therefore, this study evaluates both pre- and post-pollination mechanisms that might function as isolation barriers that prevent hybridization between two sister species with contrasting mating systems.

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