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ISOLATION BARRIERS BETWEEN *PETUNIA AXILLARIS* AND *PETUNIA INTEGRIFOLIA* (SOLANACEAE)

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The isolation barriers restricting gene flow between populations or species are of crucial interest for understanding how biological species arise and how they are maintained. Few studies have examined the entire range of possible isolation barriers from geographic isolation to next generation hybrid viability. Here, we present a detailed analysis of isolation barriers between two flowering plant species of the genus *Petunia* (Solanaceae). *Petunia integrifolia* and *P. axillaris* feature divergent pollination syndromes but can produce fertile hybrids when crossed in the laboratory. Both *Petunia* species are primarily isolated in space but appear not to hybridize in sympatry. Our experiments demonstrate that pollinator isolation is very high but not strong enough to explain the absence of hybrids in nature. However, pollinator isolation in conjunction with male gametic isolation (i.e., pollen–pistil interaction) can explain the lack of natural hybridization, while postzygotic isolation barriers are low or nonexistent. Our study supports the notion that reproductive isolation in flowering plants is mainly caused by pre- rather than postzygotic isolation mechanisms.

KEY WORDS: **Hybridization, pollinators, postzygotic isolation, prezygotic isolation, *Petunia*, sympatric population.**

The analysis of mechanisms reducing gene flow between populations of sexually reproducing organisms is crucial for understanding how biological species arise and how they are maintained. The process of speciation usually involves the establishment of multiple isolation barriers that can be spatial and/or biological. Most biological isolation barriers have a genetic component and may be defined as "biological features of organisms that impede the exchange of genes with members of other populations" (Coyne and Orr 2004). Isolating barriers can be broadly classified as pre- or postzygotic according to their timing in life history. In addition, in seed plants the prezygotic phase can be subdivided into prepollination and postpollination. Early acting prepollination barriers in plants include spatial (geographic), temporal (time of flowering), and pollinator isolation (i.e., mechanical or physiological properties of flowers and pollinators affecting pollen transfer). Postpollination mechanisms involve pollen germination and growth (including pollen competition and pollen–pistil interactions), and the pollen's ability to fertilize the egg—these barriers can be also labeled as gametic. Possible postzygotic barriers comprise hybrid inviability (e.g., seed abortion or reduced germination rate of hybrids), reduced hybrid vigor (e.g., reduced growth), hybrid sterility (affecting the production and viability of one or both gamete types), and loss of fitness in subsequent generations (hybrid breakdown) (Dobzhansky 1937; Mayr 1942).

The different isolation barriers act in a linear order. Due to this, it is self-evident that early isolation barriers can contribute more to total isolation than late barriers. Despite the evolutionary importance of isolation processes few studies have so far attempted to quantify the effects of different isolation barriers on gene flow between plant species (reviewed by Rieseberg and

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Willis 2007; Lowry et al. 2008; Widmer et al. 2009). These studies suggest that prezygotic barriers are the most important for diploid speciation (Rieseberg and Willis 2007), although there could be a bias emphasizing prezygotic barriers (Widmer et al. 2009). It is of great interest to evolutionary biologists to know which of these barriers are most relevant for the process of speciation and the genetic isolation of species. Ecogeographic isolation has been suggested to be the pre-eminent mechanism driving speciation (Mayr 1942; Stebbins 1950). However, in most cases speciation (i.e., completion of genetic isolation) may be a long process that involves the emergence of multiple isolation barriers. The assessment of the relative contribution of different isolation barriers is therefore a first important step toward understanding how species evolve and how their integrity is maintained.

Our interest concerns the isolating barriers in the genus *Petunia*. Here, we assess the contribution of a wide range of barriers to the genetic isolation between two partially sympatric *Petunia* species in Uruguay. *Petunia axillaris* and *P. integrifolia* can be artificially cross-fertilized (Wijsman 1983; Watanabe et al. 1996; Tsukamoto et al. 1998; Ando et al. 2001)—in fact both species are regarded as the parents of the cultivar *P. hybrida.* The floral characters in *P. integrifolia* and *P. axillaris* suggest that each species conforms to a different pollination syndrome (Gübitz et al. 2009). *Petunia integrifolia* carries flowers that are small, purple, almost scentless, producing hexose-rich nectar (Stuurman et al. 2004; Hoballah et al. 2005; Galliot et al. 2006) and are pollinated by bees (Ando et al. 2001). In contrast, *P. axillaris* flowers are white, have a long corolla tube, produce a strong scent at night and the nectar is sucrose rich (Stuurman et al. 2004; Hoballah et al. 2005; Oyama-Okubo et al. 2005; Galliot et al. 2006) and are pollinated by nocturnal hawk moths and small bees (Ando et al. 2001; Hoballah et al. 2007). Where both species occur in sympatry in the wild, natural hybrids have never been found (Ando et al. 2001), suggesting that besides spatial isolation other strong isolation barriers must exist.

Petunia hybrida has been widely used as a model system for molecular genetics providing a range of genetic and molecular tools (Gerats and Vandenbussche 2005). This offers the prospect of an integrated ecological and molecular approach to the analysis of isolation mechanisms in this genus. Our previous work has identified major quantitative trait loci (QTL) for a number of traits relevant to pollinator preference (Stuurman et al. 2004; Galliot et al. 2006; Venail et al. 2010). A key gene that specifies the difference in corolla color between the two species is the transcription factor *AN2* (Quattrocchio et al. 1999). Comparative molecular analysis of *AN2* suggests, however, that the species diverged before functional diversification of *AN2*, i.e., that *AN2* loss of function mutations arose relatively late and multiple times independently, and hence were not the initial speciation event (Quattrocchio et al. 1999; Hoballah et al. 2007). This leads to the question what other mechanisms may be involved in reproductive isolation between these two *Petunia* species. Therefore, we assessed in a series of experiments the effects of spatial, pollinator, gametic and postzygotic isolation barriers.

Materials and Methods

STUDY SPECIES

Petunia axillaris and *P. integrifolia* (Solanaceae) are short-lived perennials that behave mostly as ephemeral annuals under natural conditions. Both species occur in similar habitats ranging from sandy soil and disturbed sites (e.g., along roads) to crevices in rocks. Both species are self-incompatible with exceptions in *P. axillaris.* The taxonomic work on the genus *Petunia* indicates that the two species *P. axillaris* and *P. integrifolia* are closely related (Ando et al. 1995a,b, 2001, 2005; Tsukamoto et al. 1998; Kulcheski et al. 2006). Crosses in the laboratory are straightforward and yield viable seeds.

Note that *P. axillaris* consists of two subspecies in Uruguay, *P. axillaris* ssp. *axillaris* and *P. axillaris* ssp. *parodii*. For the analysis of geographic distribution, both subspecies were included, because the two subspecies occur across Uruguay and most likely hybridize in the centre of the country (Wijsman 1982, 1983; Ando et al. 1994) resulting in an almost continuous range of *P. axillaris*. In the distribution range of *P. integrifolia* we found only self-incompatible populations of *P. axillaris* ssp. *axillaris.* Therefore all experiments were performed with this subspecies.

SPATIAL ISOLATION

We estimated spatial (geographic) isolation by comparing the number of allopatric and sympatric populations of *P. axillaris* and *P. integrifolia* found in Uruguay. We define "allopatric" conservatively as both species occurring more than 20 km apart. In the sympatric populations included in this study both species occur within a couple of meters of each other. The occurrence of the *Petunia* species in Uruguay was determined from our collection survey (November 2002, January–February 2004, January–February 2005), from the literature (Ando et al. 1994, 1995a, 2001), and from herbarium data from the University of Montevideo, Uruguay. Duplicates (multiple records of the same species at the same site) were excluded from the analysis. We computed geographic isolation as $RI_{spatial} = 1 -$ (number of sympatric populations/number of all populations).

TESTING FOR HYBRIDIZATION IN SYMPATRIC POPULATIONS

F1 hybrids of *P. axillaris* and *P. integrifolia* are morphologically intermediate between both parental species, with light pink

flowers, and can thus be easily distinguished from their parents. We tested for the occurrence of hybrids in a bulk collection of naturally formed seeds from two sympatric populations, Las Cañas (33°09′ S/58°21′ W, 16 m above sea level) and in Nuevo Berlin (32°59′S/58°03′W, 14 m above sea level). Seeds were germinated under greenhouse conditions and transplanted to larger pots (transplanting survival was high: 96.7% of the *P. axillaris* from Nuevo Berlin, 90.9% of *P. axillaris* from Las Cañas, 94.3% of *P. integrifolia* from Las Cañas and 79% of *P. integrifolia* from Nuevo Berlin).

Flowers of a total of 1914 *P. axillaris* and 290 *P. integrifolia* plants were scored for the occurrence of hybrids.

POLLINATOR CONSTANCY

We tested pollinator fidelity and constancy in an artificial mixed population in Las Cañas, Uruguay. Experiments were conducted with *P. integrifolia* and *P. axillaris* seeds collected from a sympatric population in Las Cañas (see above). Pollinator fidelity was observed in *P. integrifolia* and *P. axillaris* plants (with the same number of flowers, grown from seeds collected previously from this site) arranged in a semi-randomized 4 × 4 plot. Observations were carried out in six sessions during the day (from 1000 h to 1400 h) and five sessions at night (from 2000 h to 2200 h) in February 2007. During the night sessions only two hawkmoth visits were observed—one to a single and one to two different *P. axillaris* plants. Therefore we could not calculate nocturnal pollinator isolation (but see also above).

We computed diurnal pollinator isolation as $RI_{pollinator}$ = 1 – (number of pollinators visiting flowers of both *Petunia* species/(number of pollinators visiting flowers of both *Petunia* species + visiting flowers of more plants of the same *Petunia* species).

To assess the importance of diurnal and nocturnal pollinators, we tested whether flowers covered during the day or at night produced the same seed weight. For this, we analyzed a dataset obtained from pollinator exclusion experiments described previously (Hoballah et al. 2007).

SEEDS USED FOR GREENHOUSE EXPERIMENTS

Seeds collected in Las Cañas, Uruguay in November 2002 from a sympatric population of *P. axillaris* and *P. integrifolia* were used for carrying out tests on gametic isolation and postzygotic isolation in hybrids. Plants were grown in a greenhouse at the Institute of Plant Sciences, University of Bern, Switzerland (13:11 L: D cycle, 18–25 C) in pots (11 cm height, 12.5 cm diam.) in commercial soil (70% Klasman substrate, 15% Seramis clay granules, 15% quartz sand). The plants received standard fertilization (N16%; P6%; K26%; Mg2%; traces elements including Fe) weekly.

NONCOMPETITIVE POSTPOLLINATION ISOLATION

To test for noncompetitive gametic and early postzygotic isolation (noncompetitive postpollination), we compared seed capsule set (CR) and capsule weight (CW) derived from hetero- and homospecific pollinations. We used CW as a proxy for seed set as it is strongly correlated with seed weight (for *P. axillaris* linear regression, $r^2 = 0.933$, $n = 45$ and for *P. integrifolia* $r^2 = 0.966$, $n = 37$) and with seed number (for *P. axillaris* linear regression, $r^2 = 0.814$, $n = 45$ and for *P. integrifolia* $r^2 = 0.859$, $n = 37$). These experiments were carried out with five *P. axillaris* and four *P. integrifolia* plants in the greenhouse. We calculated the indexes as follows: $RI_{non-comp\ postpoll\ CR} = 1$ – (heterospecfic capsule rate/homospecific capsule rate) and $RI_{non-comp\ postpoll\ CW} = 1$ – (heterospecfic CW /homospecific CW).

IN-VITRO POLLEN GERMINATION

Pollen germination of the two parental species was assayed to test for a noncompetitive male gametic prezygotic isolation barrier. Five *P. axillaris* and five *P. integrifolia* plants were used for this experiment. Pollen of freshly dehisced anthers from five plants per species was collected in the morning. The pollen grains were suspended in a germination medium (GM), (Gass et al. 2005) pollen germination rates were recorded after 2 h and 24 h of incubation. Counting was performed by placing 10 μl GM with pollen and 20 μl water on a glass plate. Between 50 and 200 pollen grains were assayed for their state of germination. When a grain showed a pollen tube longer than the diameter of the grain itself, it was counted as "germinated." Pollen germination rate was compared among species with a nonparametric Kruskal–Wallis test followed by the Mann–Whitney test.

SEMI-VIVO POLLEN TUBE GROWTH

To assess the male noncompetitive gametic isolation barrier, we tested whether pollen tubes grow faster in homo- or heterospecific styles in a second semi-in-vivo experiment (Gass et al. 2005). Closed flowers of both species were emasculated and were pollinated when the flowers had opened, with pollen from either species. We incubated the flower at 37°C at 1600 h. One hour later, we cut the style 10 mm below the stigma with a sharp razor blade. The cut styles were then placed in a 200 μl PCR well plate with wells filled with GM up to 5 mm below the stigma. The styles were then incubated for 15 h at 37°C. The length of the longest pollen tube (PTL) growing out of the cut end of the stigma was recorded. The isolation index was calculated as $RI_{non-comp\ postpoll\ PTL} = 1$ – (mean length heterospecific pollen tubes/mean length homospecific pollen tubes).

POLLEN COMPETITION

A competitive form of gametic isolation can arise when gametes of both species are transferred onto a stigma. Pollen of one species

may germinate faster, grow better, or fertilize the ovule more efficiently. However, it is difficult to directly assay components of competitive gametic isolation (pollen germination, pollen growth, fertilization) or even to clearly separate them from postzygotic components (e.g., seed abortion). Here, we assayed the competitive component by measuring seed set resulting from different pollination treatments. Emasculated flowers were pollinated with either: (1) homospecific pollen from a different individual, (2) heterospecific pollen, (3) first homospecific pollen and then heterospecific pollen, (4) first heterospecific pollen and then homospecific pollen, (5) pollen of the same flower (self-pollination), and (6) no pollen (negative control). We make the assumption that covering the style with pollen is sufficient to avoid pollen limitation. These experiments were carried out with the same plants used for the noncompetitive postpollination isolation experiment. The CW data was compared with analysis of variance (ANOVA). We calculated the *RI* index based on CW as: $RI_{comp\,postpoll\,CW} = 1 -$ (CW mixed pollinations/CW of homospecific pollinations). Based on capsule formation rate (CR), the index of competitive postpollination isolation was calculated as follows: $RI_{comp\,postpoll\,RC} = 1 -$ (mixed pollination capsule formation rate/homospecific pollination capsule formation rate). Note that *RI* indexes were calculated using the treatment (4) as mixed pollinations. Measuring total seed production after mixed pollinations does not tell whether the seeds formed are hybrids or not. In principle, ovules might have been fertilized exclusively by homospecific pollen. To test this, seeds were germinated and the floral phenotypes screened for hybrids.

F1 HYBRID SEED GERMINATION AND TIME TO FLOWERING

To compare seed germination rate of the wild species and the F1 hybrids 20 seeds per capsule (number of replications depending on number of capsules obtained in the previous experiment, see Table 2) were sown in pots. The number of germinated seeds was checked three weeks after sowing. We calculated an index for postzygotic isolation based on seed germination as follows: $RI_{seed-ger\,F1} = 1 -$ (seed germination heterospecific crosses/seed germination homospecific crosses). Five seedlings per replication were allowed to flower. We checked for the first flowers every two days, recorded and eliminated the flowering plants from the tray.

POLLINATOR ISOLATION OF F1 HYBRIDS

We tested whether F1 hybrids receive as many pollinator visits as their parents in the wild. These experiments were carried out in Puerto Viejo (32°38′S 58° 8′W) and Minas (34°21′S 55°08′W) in Uruguay during January and February 2005. F1 hybrids (*P. axillaris* × *P. integrifolia* from Las Cañas) were germinated and grown in pots at INASE (Pando, Uruguay) and then transported to natural sites with *P. integrifolia* (Puerto Viejo) or *P. axillaris* (Minas). The F1 hybrid plants were placed in direct proximity to wild plants. Pollinators feeding on the wild and hybrid flowers were observed from 0800 h to 1200 h and from 2100 h to 2300 h. Fitness (seed set) was assayed, as in the pollinator fidelity experiment for wild species, by confining plants under tents during the night (from 2000 h to 0800 h) or during the day (from 0800 h to 2000 h).

F1 HYBRID POLLEN GERMINATION AND POLLEN TUBE GROWTH

Postzygotic isolation due to differences in pollen germination of the hybrids (*P. axillaris* × *P. integrifolia*, 5 hybrid plants used) was tested in parallel to that of the parents (see sections in-vitro pollen germination and semi-vivo pollen tube growth above). We calculated an index for postzygotic isolation based on the pollen germination rate and pollen tube length of F1 as follows: $RI_{pollen-ger\,F1} =$ 1 – (% pollen germination hybrid /% pollen germination parent) and $RI_{non-comp\,postpoll\,PTL\,F1} = 1 -$ (mean length pollen tube hybrid/mean length pollen tube parent).

HYBRID GAMETIC FITNESS

We tested for hybrid sterility in the same experimental set-up as in the pollen competition experiments (see section noncompetitive postpollination isolation above). Male hybrid fitness was assayed by pollinating the two wild species with F1 (*P. axillaris* × *P. integrifolia*) pollen in the greenhouse. We used five *P. axillaris* plants, five *P. integrifolia* plants, and five hybrids. The *RI* for male hybrid fitness were calculated as follows: $RI_{male-postpoll\,CR\,F1} =$ 1 – (capsule rate of parent plants pollinated with F1 pollen/capsule rate of homospecific pollinations) and $RI_{male-postpoll\,CW\,F1} = 1 -$ (CW of parent plants pollinated with F1 pollen/CW of homospecific pollinations). Female hybrid fitness was tested by pollinating the F1 hybrids with pollen of the wild species in the greenhouse. We used five *P. axillaris* plants, six *P. integrifolia* plants, and 15 hybrids. The rate of capsule formation and the CW was assessed for both wild species and hybrids. The *RIs* for female hybrid fitness were calculated using the means of capsule rate and CW of both previous homospecific pollination experiments (See Tables 1 and 3) because female hybrid fitness experiment was not carried out at the same period of the year. The index was then calculated as follows: $RI_{female-postpoll\,CR\,F1} = 1 -$ (capsule rate of hybrids pollinated with pollen of the parent/capsule rate of homospecific pollinations) and $RI_{female-postpoll\,CW\,F1} = 1 -$ (CW of hybrids pollinated with pollen of the parent/CW of homospecific pollinations). An ANOVA was carried out to compare seed set and CW between wild species and hybrids.

HYBRID BREAKDOWN

We tested for hybrid breakdown by analyzing germination rate of seeds produced in the hybrid gametic fitness experiment above and

Table 1. Reciprocal cross-compatibility between *P. axillaris* and *P. integrifolia*. Differences between treatments comparing the number of crosses and number of capsules of the homospecfic crosses with the other treatments were analyzed in a 2×2 contingency test (Fischer's exact test). Only the purely heterospecific crosses produced significantly fewer capsules ($P<0.001$, indicated by $**$). ANOVA for capsule weight for *P. axillaris* group was $P=0.0001$ and $F=9.271$ and *P. integrifolia* group was $P=0.037$ and $F=3.041$. Letters at end of lines represent the results of the post hoc Student Newman Keuls test for each seed parent group; different letters indicate significant difference among pollination type.

Seed×pollen parent	*N* crosses	*N* capsules	Capsule rate (%)	Mean±SE	Capsule weight (mg)
P. axillaris×*P. axillaris*	33	23	69.7	53.4±5	a
P.axillaris×*P. integrifolia*	49	6**	12.2	15.7±4	b
P.axillaris×(*P. axillaris*+*P. integrifolia*)	21	14	66.7	51.4±4	a
P.axillaris×(*P. integrifolia*+*P. axillaris*)	29	20	69.0	29.8±5	b
P. integrifolia×*P. integrifolia*	23	22	95.6	18.6±2	a
P. integrifolia×*P. axillaris*	43	2**	4.6	1.4±0	b
P. integrifolia×(*P. integrifolia*+*P. axillaris*)	14	14	100	16.5±2	a
P. integrifolia×(*P. axillaris*+*P. integrifolia*)	20	18	90	18.0±2	a

time to flower of those generated plants. We used seeds obtained from pollinations of wild species as seed plants and hybrid pollen (BC1 seeds). We calculated the *RI* based on germination rate of seeds as follows: $RI_{seed-ger\,BC1} = 1 -$ (seed germination BC1 crosses/seed germination homospecific crosses). We calculated the RI based on time to flower as: $RI_{time\,to\,flower\,BC1} = 1 -$ (time to flower BC1 plants/time to flower parent plants).

CALCULATING ABSOLUTE CONTRIBUTION OF ISOLATION BARRIERS

Isolation barriers come into play in a linear temporal order. This allows representing isolation barriers as a sequence of filters reducing gene flow between two species. We followed the method of Coyne and Orr (Coyne and Orr 1989) with modifications (Ramsey et al. 2003; Husband and Sabara 2003) to estimate the effect of the single barriers and their relative contribution to total reproductive isolation. Reproductive isolation (*RI*) values represent the strength of isolation between the species and vary from one (complete isolation) to zero (no isolation). The absolute contribution (*AC*) of a component of reproductive isolation (*RI*) at a certain stage in the life history and total isolation was calculated as in Ramsey et al. (2003).

Results

SPATIAL ISOLATION

We identified 33 allopatric populations of *P. integrifolia*, 203 allopatric populations of *P. axillaris*, and four sympatric populations. Thus $RI_{spatial}$ was estimated as 0.892 for *P. integrifolia* and 0.981 for *P. axillaris*. These results indicate a high degree of habitat isolation between the two *Petunia* species. Due to the ephemeral nature of *Petunia* populations, the true spatial overlap of both species may be fluctuating and thus difficult to estimate.

To test whether geographic isolation is associated with parameters characteristic of habitat differentiation such as elevation, we tested for differences in elevation between *P. axillaris* and *P. integrifolia* populations. The four *P. integrifolia* and 27 *P. axillaris* sites did not differ significantly in elevation above sea level (mean = 58 m for *P. axillaris*, with minimum = 0 m, maximum = 269 m; and mean = 63.5 m for *P. integrifolia*, minimum = 0 m, maximum = 224 m). This was not surprising as Uruguay is a rather flat country (highest elevation 514 m).

HYBRIDIZATION AND POLLINATOR CONSTANCY

Seeds were collected from two sympatric populations and germinated under optimal growth conditions. In a total of 1914 *P. axillaris* and 290 *P. integrifolia* derived seeds, not a single hybrid plant was found.

Previous work has established that *P. integrifolia* flowers are visited by bees and diurnal butterflies during the day and no pollination occurred at night (Hoballah et al. 2007). In contrast, *P. axillaris* flowers were effectively visited by several hawkmoths species at night, but also by bees and beetles during the day. Exclusion experiments showed that capsule formation is dependent on animal pollinators and therefore both species are fully self-incompatible. Observations of pollinator constancy indicated high constancy of diurnal pollinators.

At the Uruguayan study site native pollinators behaved similarly. A total of 110 diurnal pollinators were observed of which the majority (68.2%) visited only a single flower (34 on *P. integrifolia* and 44 on *P. axillaris*). A total of 35 pollinators visited more than one flower (22 from *P. integrifolia* to *P. integrifolia*, three from *P. integrifolia* to *P. axillaris*, 10 from *P. axillaris* to *P. axillaris*, 0 from *P. axillaris* to *P. integrifolia*). On the basis of these very few switches between flowers, we estimate of $RI_{pollinator-ax} = 0.880$ for the direction from *P. integrifolia* to *P. axillaris*. No switches

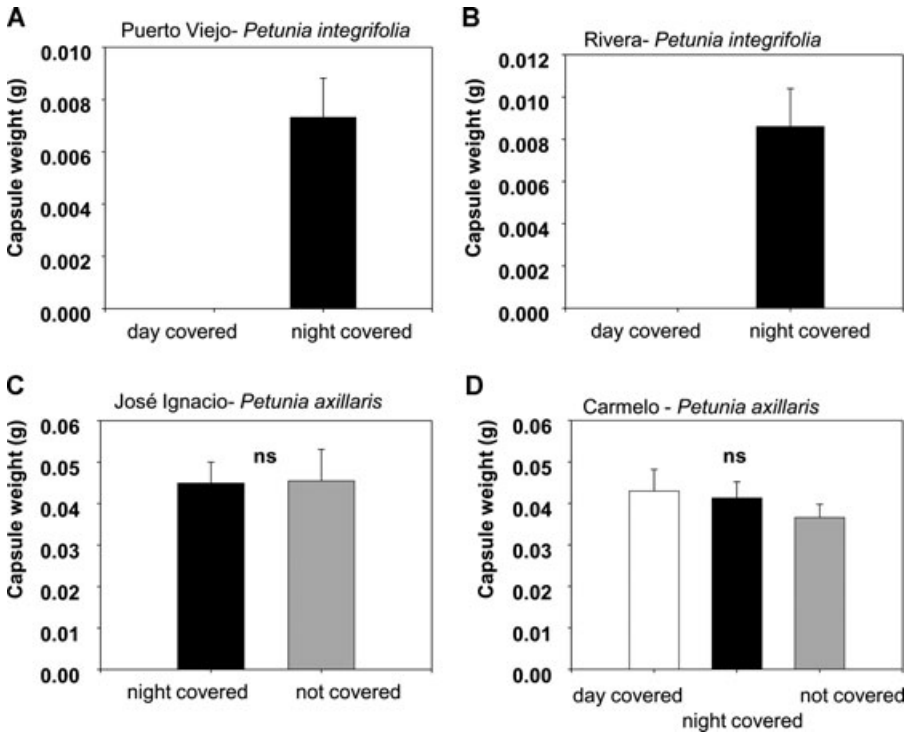

Figure 1. **Effective pollination of *P. integrifolia* and *P. axillaris* by nocturnal and diurnal visitors. Mean ± SE weight of capsule in allopatric populations of *Petunia integrifolia* (A: Puerto Viejo, $n = 27$ for day covered and $n = 21$ for night covered; and B: Rivera, $n = 26$ for day covered and $n = 28$ for night covered) and of *P. axillaris* when covered during the day or during the night (see also Hoballah et al. 2007). *Petunia axillaris* treatments were tested using ANOVA; There was no significant difference. C = Carmelo (ns, $P = 0.523$, $F = 0.658$ $P = 0.943$, $F = 0.005$, $n = 24$ for night covered and $n = 13$ not covered) and D = José Ignacio ($P = 0.943$, $F = 0.005$, $n = 10$ for day covered and $n = 21$ night covered and $n = 20$ not covered).**

in the opposite direction were observed implying $RI_{pollinator-int} = 1$, and overall $RI_{pollinator}$ was 0.914. It is noteworthy that the pollinator constancy studies in native and nonnative habitats yielded very similar results. For *P. integrifolia,* diurnal insects are the only effective pollinators because day-covered plants did not produce capsules (Fig. 1). The weight of *P. axillaris* capsules was similar for day-covered, night-covered, and not-covered plants indicating that diurnal and nocturnal pollinators can achieve the same total seed-set (Fig. 1). In José Ignacio, we lack results for day-covered plants because of mowing by farmers.

NONCOMPETITIVE POSTPOLLINATION ISOLATION

Noncompetitive heterospecific pollinations produced significantly lower seed capsule set and CW than homospecific crosses (Table 1). Of 43 pollinations of *P. integrifolia* as seed parent with *P. axillaris* as pollen donor only two capsules with only a few seeds were obtained. Of 49 pollinations of *P. axillaris* with *P. integrifolia* pollen only six capsules of reduced weight were obtained. Furthermore, the capsules obtained from heterospecific pollinations were significantly lower in weight, indicating a lower production of seeds (Table 1). On the basis of CW, $RI_{non-comp\,postpoll\,CW}$ was estimated as 0.706 for *P. axillaris* and 0.925 for *P. integrifolia*. On the basis of capsule rate, $RI_{non-comp\,postpoll\,CR}$ was estimated as 0.825 for *P. axillaris* and 0.952 for *P. integrifolia*. These results indicate strong gametic and/or early postzygotic barriers. Because in the interspecific *Petunia* cross, seed set is reduced in both directions, reduced pollen growth cannot be (the only) cause.

POLLEN GERMINATION AND POLLEN TUBE GROWTH

To discriminate between pre- or postzygotic barriers, germination and growth of pollen grains was tested in vitro and semi in vivo. In the in vitro experiment, pollen was placed on synthetic growth media and the percentage of germination was determined. We found no significant difference in pollen germination after 2-h incubation whereas after 24 h *P. axillaris* pollen had germinated slightly better than *P. integrifolia* (Fig. 2A,B).

In the semi in vivo experiment, pollen was placed on the stigma and the emergence of pollen tubes from the cut style was observed. Pollen tubes of either species grew faster through a homospecific style (Fig. 2C,D). These results indicate a prezygotic gametic barrier due to reduced pollen tube growth in heterospecific styles in *P. axillaris* and *P. integrifolia*. On the basis of pollen tube growth, $RI_{non-comp\,postpoll\,PTL}$ was estimated as 0.504 for *P. axillaris* and 0.961 for *P. integrifolia*.

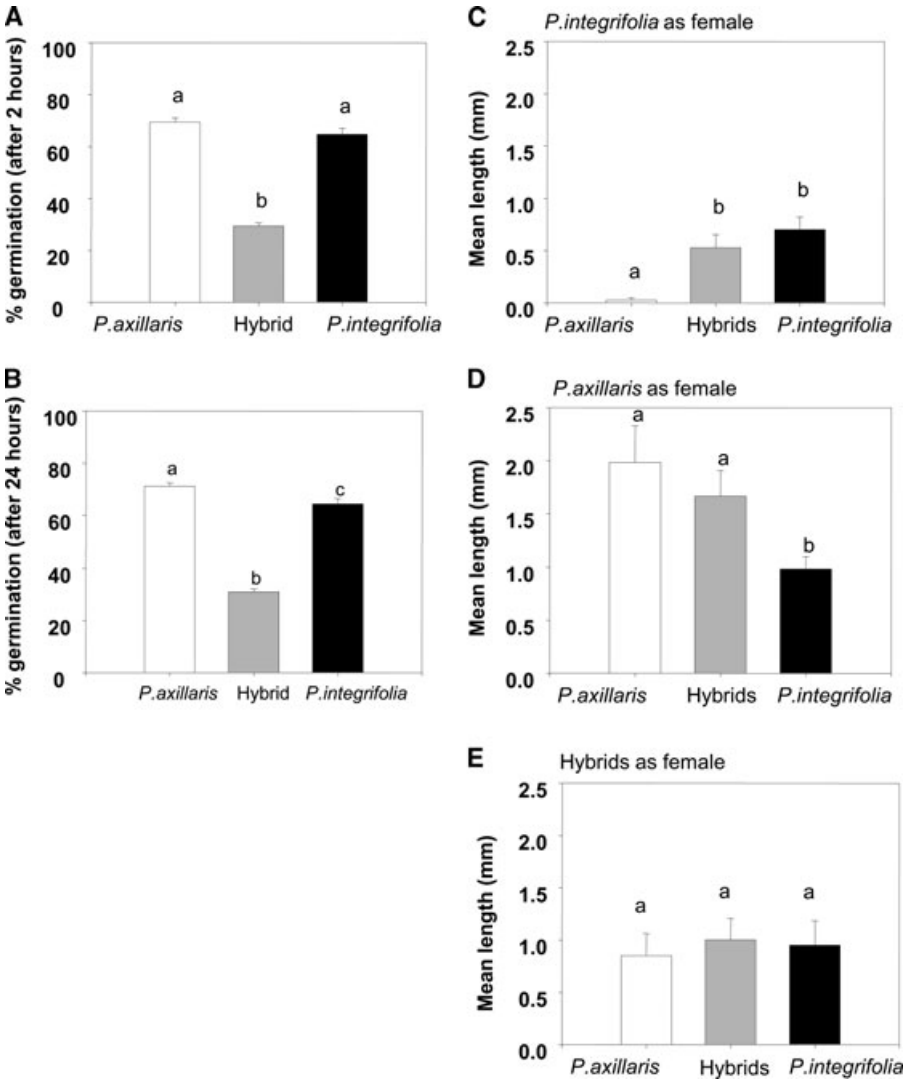

Figure 2. **In vitro and in vivo germination and growth of pollen tubes from *Petunia integrifolia*, *P. axillaris*, and F1 hybrids. (A,B) In vitro pollen germination. Pollen was germinated on a synthetic medium and the mean percentage of pollen tube germination was determined after (A) 2 h and (B) 24 h. Different letters above bars indicate a significant difference between treatments. (A) Kruskal–Wallis; $P = 0.0001$; $\chi^2 = 101.931$. (B) Kruskal–Wallis; $P = 0.0001$; $\chi^2 = 111.499$. $n = 60$ for each plant. (C–E) In vivo pollen tube growth. Pollen of the two parental types and their F1 hybrid were placed on the stigma and the length of the pollen tubes emerging from the cut style after 15-h incubation was measured (mean ± SE). (C) *P. integrifolia* as female, Kruskal–Wallis; $P = 0.003$; $\chi^2 = 13.538$. $n = 2$ for *P. axillaris* as male, $n = 7$ for F1 hybrid as male and $n = 9$ for *P. integrifolia* as male. (D) *P. axillaris* as female, Kruskal–Wallis; $P = 0.051$; $\chi^2 = 8.097$. $n = 10$ for each plant. (E) ANOVA, $P = 0.876$, $F = 0.129$. F1 hybrid as female, $n = 8$ for *P. axillaris* as male, $n = 8$ for F1 hybrid as male and $n = 7$ for *P. integrifolia* as male.**

POLLEN COMPETITION

We next examined whether homospecific pollen might have a competitive advantage in mixed pollinations. Applying pollen from both species in an alternating order to the same stigma resulted in similar rates of capsule formation as in homospecific crosses (Table 1). CW was significantly reduced in *P. axillaris* when heterospecific pollen was applied before homospecific pollen (Table 1). In 11 capsules obtained from mixed pollinations with *P. integrifolia* as female no hybrid plants were found. In contrast, of 15 capsules from mixed pollinations with *P. axillaris* as female four capsules contained hybrids. In one case only hybrids were produced and the other three contained a mixture of F1 hybrids and *P. axillaris* seeds. The results suggest that *P. axillaris* pollen cannot successfully compete in a *P. integrifolia* style, whereas in *P. axillaris* as seed parent hybrids can be formed in presence of interspecific pollen competition.

Table 2. Vigor of hybrids. Mean±SE percentage of seed germination (20 seeds per replication) and mean±SE number of days to flower (five plants per replication). *N*=number of replications. ANOVA seed germination: for *P. axillaris* group was *P*=0.329 and *F*=1.140, ANOVA for *P. integrifolia* group was *P*=0.001 and *F*=8.463. ANOVA time to flower: for *P. axillaris* was *P*=0.168 and *F*=1.869, ANOVA for *P. integrifolia* group was *P*=0.184 and *F*=1.795. Letters after the *N* represent the results of the post hoc Student Newman Keuls test for each seed parent group, different letters indicate significant difference among pollination type.

Seed×pollen parent	% seed germination	*N*		*N* days to flower	*N*	
P. axillaris×*P. axillaris*	45.75±7.3	20	a	61.62±1.1	19	a
P. axillaris×*P. integrifolia*	24.00±14.3	5	a	64.18±2.2	4	a
P. axillaris×Hybrid	44.52±5.7	21	a	64.62±1.2	18	a
P. integrifolia×*P. integrifolia*	50.71±6.3	14	a	66.59±0.7	14	a
P. integrifolia×*P. axillaris*	89.16±5.8	2	b	65.00±3.2	2	a
P. integrifolia×Hybrid	83.98±5.7	17	b	64.26±0.9	17	a

F1 HYBRID SEED GERMINATION AND TIME TO FLOWERING

Comparing seed germination rate of F1 hybrid seeds (derived from heterospecific crosses) to seeds from either parental species revealed an increased germination rate for F1 hybrid seeds (*P. integrifolia* × *P. axillaris*) compared to *P. integrifolia* (Table 2). F1 seeds derived from crosses with *P.axillaris* as seed parent tended to have lower germination rate, although this trend was not significant due to large variance in the F1 hybrid seed germination rate. We estimate isolation at the F1 hybrid seed germination stage to be $RI_{seed-ger\,F1} = 0.475$ for *P. axillaris* and -0.7582 for *P. integrifolia.*

Under greenhouse conditions, time to flower was similar for F1 hybrids and both wild species (Table 2). As both species mostly behave like annual weeds in the field, a short time to flowering can be used as a proxy for vigor. Hence, vigor in F1 hybrids was not reduced.

POLLINATOR ISOLATION OF F1 HYBRIDS

Observations on pollinator visits to F1 hybrids and species wild types in allopatric populations of both species in Uruguay showed that *P. integrifolia* received significantly more visits than F1 hybrids (Fig. 3). No nocturnal pollinators were observed visiting either the F1 hybrids or *P. integrifolia* at night. F1 hybrids were not preferred over *P. axillaris* by diurnal pollinators. During the night, hawkmoths visited fewer hybrids than wild *P. axillaris* (Fig. 3), although this difference was statistically nonsignificant. It is noteworthy that the frequency of pollinator visits during the day was much lower in the *P. axillaris* than in the *P. integrifolia* habitat.

Pollinator exclusion experiments in the natural habitat of *P. integrifolia*, showed that no capsules were formed when diurnal pollinators were excluded (Fig. 4), suggesting either the absence of hawkmoths in this habitat or the inefficiency of the F1 hybrid plants to attract hawkmoths. In the natural habitat of *P. axillaris*,

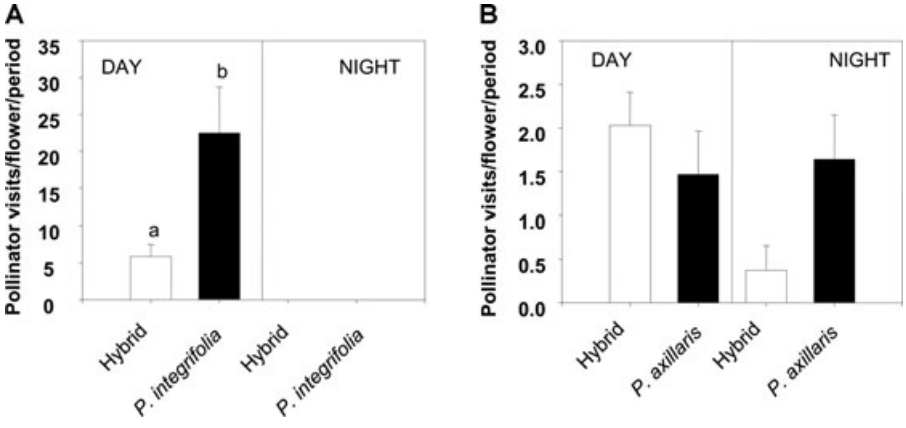

Figure 3. Pollinator visitation of *Petunia integrifolia*, *P. axillaris,* and F1 hybrids. Mean ± SE of pollinator visits per flower per period. (A) F1 hybrids vs. wild *Petunia integrifolia* in Puerto Viejo. Different letters above bars indicate a significant difference between treatments. Mann–Whitney, $Z = -2.619$, $P = 0.009$. $n = 7$ replications. (B) F1 hybrids vs. *P. axillaris* in Minas (Uruguay). Left graph showing day observations (*t*-test, $F = 0.522$, $P = 0.497$) and right graph night observations (*t*-test, $F = 0.811$, $P = 0402$). $n = 4$ day and 5 night replications.

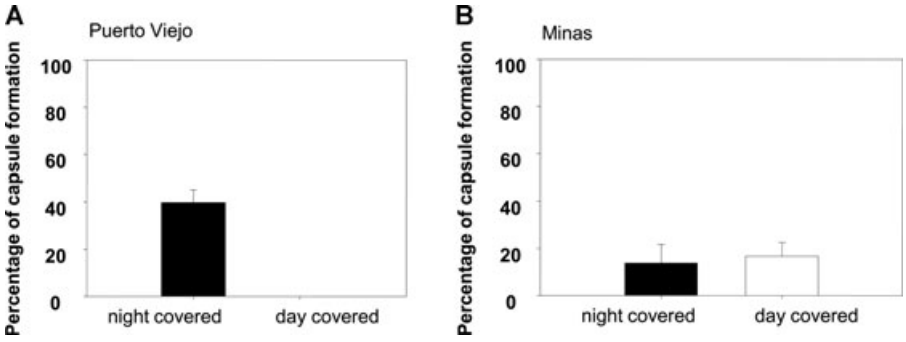

Figure 4. Pollinator isolation in F1 hybrids. (A) Mean ± SE percentage of capsule formation of hybrids covered during the night (black bar) or during the day (white bars) in a *Petunia integrifolia* habitat in Puerto Viejo and (B) in a *P. axillaris* natural habitat in Minas (*t*-test, $F = 0.086$, $P = 0.779$). The Puerto Viejo data were not statistically analyzed due to insufficient replication of day-cover experiments ($n = 2$).

capsules were formed in F1 hybrid plants when covered either during the day or at night (Fig. 4). Thus hybrids are capable of attracting both diurnal and nocturnal pollinators (Fig. 3) and are efficiently pollinated by them (Fig. 4).

F1 HYBRID POLLEN GERMINATION AND POLLEN TUBE GROWTH

In vitro pollen germination after 2 h and after 24 h of incubation was significantly lower in hybrids than in both parental species (Fig. 2A,B). The pollen germination isolation barrier index was $RI_{pollen-ger\,F1} = 0.566$ for *P. axillaris* and $RI_{pollen-ger\,F1} = 0.520$ for *P. integrifolia*. When pollen tube growth was assessed under semi in vivo conditions, F1 hybrid pollen tube growth was not different from that of the parent pollen tubes (Fig. 2C,D). There was also no difference in pollen tube growth of the two parental species and hybrids on styles of F1 hybrids (Fig. 2E). The F1 hybrid gametic noncompetitive isolation barrier index was $RI_{non-comp\,postpoll\,PTL\,F1} = 0.162$ for *P. axillaris* and 0.247 for *P. integrifolia*.

HYBRID GAMETIC FITNESS

To analyze the effect of the postpollination barriers against F1 hybrids we measured capsule formation obtained from pollinating the two wild species with pollen from F1 hybrids (male postpollination index) and from pollinating F1 hybrids with pollen from each species (female postpollination index). Capsule set of all crosses was high (>85%, Table 3). Interestingly, pollen from F1 hybrids applied to stigmas of either parent produced capsules of reduced weight. Thus, the isolation index for F1 hybrid male postpollination isolation based on CW $RI_{male-postpoll\,CW\,F1}$ is 0.301 for *P. axillaris* and 0.275 for *P. integrifolia*.

Comparing wild species and F1 hybrids pollinated by the same wild species pollen showed that F1 hybrids produced lower seed CW than *P. axillaris* (Table 3). The isolation index for F1 hybrid female postpollination isolation based on CW $RI_{female-postpoll\,CW\,F1}$ is therefore 0.783 for *P. axillaris* (Table 1, compare lines 1 and 5). For *P. integrifolia* the $RI_{female-postpoll\,CW\,F1}$ is 0.044 (Table 1, compare lines 3 and 6). Thus, F1 hybrid female gametic isolation is virtually nonexistent for *P. integrifolia*, whereas for *P. axillaris* the barrier is relatively high. This suggests that the pollen of *P. axillaris* does not efficiently fertilize the hybrid. In addition, we noted that almost no capsules were formed when hybrids were selfed (of 124 self-pollinations only four capsules contained only few seeds) indicating that self-incompatibility does not break down in the F1 hybrids. Hence, in nature F1 hybrids would depend on cross-pollination by insects.

Table 3. Cross-compatibility between the wild species and their F1 hybrids. No significant differences between treatments when analyzed in a 2×2 contingency test (Fischer's exact test) comparing the number of crosses and number of capsule set of the homospecific crosses with the backcross of F1 hybrids. However, seed capsule weight was significantly lower in the backcrosses than in homospecific crosses.

	Seed×pollen parent	*N* crosses	*N* capsules	Capsule formation rate (%)	Mean±SE capsule weight (mg)	*t*-test
1	*P. axillaris*×*P. axillaris*	20	19	95	72.0±4.23	P=0.002
2	*P. axillaris*×Hybrid	20	20	100	50.3±5.41	F=10.631
3	*P. integrifolia*×*P. integrifolia*	20	17	85	22.5±1.85	P=0.013
4	*P. integrifolia*×Hybrid	20	18	90	16.3±1.64	F=6.925
5	Hybrid×*P. axillaris*	92	83	90.2	13.6±0.73	P=0.0001
6	Hybrid×*P. integrifolia*	79	78	98.7	21.5±2.15	F=12.795

Table 4. Quantitation of reproductive barriers. Reproductive isolation (RI) and absolute contribution (AC) of individual reproductive barriers including (+habit.) and excluding habitat isolation (−habit.) in *Petunia axillaris* (ax) and *P. integrifolia* (int).

Isolation barrier	RI ax	RI int	AC ax (+habit.)	AC int (+habit.)	AC ax (−habit.)	AC int (−habit.)
Habitat/Spatial	0.98	0.892	0.98	0.892		
pollinator	0.88	1	0.0176	0.108	0.88	1
Noncomp postpoll	0.706	0.928	0.0016944	0	0.08472	0
F1 germination	0.475	−0.758	0.00033516	0	0.016758	0
F1 noncomp postpoll	0.301	0.275	0.000175959	0	0.00879795	0
BC1 germination	0.0269	−0.656	0.0000923785	0	0.004618924	0
Total			0.999897897	1	0.994894874	1

There was a clear effect on seed CW of F1 hybrids pollinated with *P. integrifolia* pollen giving rise to heavier seed capsules than when pollinated with *P. axillaris* pollen (Table 3).

HYBRID BREAKDOWN

When *P. integrifolia* was used as seed parent, first backcross (BC1) seeds germinated better than *P. integrifolia* seeds (Table 2). There was no difference in the comparisons with *P. axillaris* as seed parent. The resulting $RI_{seed-ger\,BC1}$ indexes are 0.027 for *P. axillaris* and −0.656 for *P. integrifolia*. Number of days needed to plants for flower was very similar for BC1 and parent plants (Table 2). Isolation index due to time to flower was $RI_{timetoflower\,BC1} = -0.049$ for *P. axillaris* and 0.035 for *P. integrifolia*. There was no hybrid breakdown in the BC1 generation.

TOTAL REPRODUCTIVE ISOLATION

The analysis of total isolation (Table 4) showed that there is complete isolation between these two *Petunia* species. Excluding the effect of habitat isolation still yielded total isolation values close to 1 suggesting complete isolation in sympatric populations, with pollinator isolation being the strongest barrier and gametic isolation also being high (Table 4). Postzygotic isolation barriers are relatively low and play hardly any role in reducing gene flow between both species.

Discussion

The assessment of the relative contribution of different reproductive isolation barriers is important to understand how populations become genetically isolated and how species maintain their integrity (Ramsey et al. 2003; Husband and Sabara 2003). Recent studies suggest that prezygotic isolation barriers are stronger than postzygotic barriers in flowering plants and that postmating barriers are highly asymmetric (reviewed by Rieseberg and Willis 2007; Lowry et al. 2008; Widmer et al. 2009). This view is based mainly on studies of individual reproductive isolation barriers in various species. However, there are only a limited number of studies analyzing multiple isolation barriers in a single system (Lowry et al. 2008). The present study provides a comprehensive study of multiple isolation barriers and supports these hypotheses.

SPATIAL ISOLATION

Spatial isolation is rarely taken into account when analyzing what causes genetic isolation between species (Lowry et al. 2008). Our analysis showed that geographic isolation is the most important factor isolating *P. integrifolia* and *P. axillaris*. Spatial isolation can be produced by simple vicariance, but in cases of parapatry such as described here, the explanation for spatial isolation is almost certainly differential adaptation (Mayr 1963). There is little information on the historical biogeography of the Southeastern parts of South America. In the case of Uruguay, a country with a relatively flat relief, elevation does not explain the distribution of the two species. Our preliminary data on soils where *Petunia* species occur in Uruguay did not reveal any obvious factor that could explain the species distributions.

Morphological plant characters or seed size sometimes can explain why a species can compete in a particular environment. *P. axillaris* produces taller plants with broader leaves than *P. integrifolia* suggesting that both species may compete differently at varying vegetation densities. Further, *P. integrifolia* produces fewer but larger seeds than *P. axillaris.* This may imply that *P. axillaris* has better seed dispersal but may be less competitive at the seedling stage. Although more detailed ecological analysis may provide an explanation for the distribution of both species, in the present absence of an autecological explanation, synecological factors such as the availability of suitable pollinators of each species should also be considered. Reciprocal transplant experiments, testing for plant survival and fitness in different environments, could directly assess whether ecological conditions determine the distribution of both species.

POLLINATOR ISOLATION

The two *Petunia* species feature radically different pollination syndromes—bee and hawkmoth pollination. However, field

studies have shown that *P. axillaris*, which is pollinated by hawkmoths, is also effectively pollinated by pollen-collecting bees (Hoballah et al. 2007). The main pollinators of *P. integrifolia* are small bees that appear to be closely associated with this species and hardly visit *P. axillaris* (reviewed in Gübitz et al. 2009). Hence, although both *Petunia* species are visited by bees, there appears to be little overlap in the groups of bees visiting both species. This could explain the low frequency of cross-pollinations observed in the natural and artificial populations. Although assaying pollinator constancy suggests that minor pollen transfer may take place, pollinator isolation is the strongest isolating factor after geographic isolation.

Although the phylogeny of *Petunia* is not well resolved, we may assume that the ancestral species was bee-pollinated as *P. integrifolia* (Ando et al. 2005; Kulcheski et al. 2006) because the sister genus *Calibrachoa* features mainly bee-pollination. The molecular evolution of the transcription factor *AN2* controlling flower anthocyanin production supports this view by indicating that white flowers in *P. axillaris* were acquired by multiple loss-of-function events in *AN2* (Quattrocchio et al. 1999; Hoballah et al. 2007). Hence, *P. axillaris* is thought to have recruited a new pollinator type (i.e., nocturnal hawkmoths) which does not visit the ancestral bee-pollinated species. It is of interest that, although morphological adaptation to hawkmoth pollination such as a long floral tube excludes nectar foraging bees in *P. axillaris* from reaching the nectar reward, pollen-collecting bees still visit both *Petunia* species. If pollinator isolation were the only isolation mechanism, a low degree of pollen flow would still occur between species. This suggests that the emergence of divergent pollination syndromes alone is unlikely to lead to complete genetic isolation.

GAMETIC ISOLATION

In the absence of temporal-spatial or pollinator isolation, pollen from one species will land on the stigma of another species. In this case gametic isolation mechanisms may reduce gene flow between two taxa considerably. In plants, gametic isolation barriers may play a major role in diploid speciation (Rieseberg and Willis 2007; Lowry et al. 2008; Widmer et al. 2009). In the present study we find evidence that gametic isolation is high in the focal *Petunia* species (Table 4). Gametic isolation factors can be distinguished according to stages: pollen germination, pollen tube growth, and ovule fertilization. These are difficult to analyze separately.

Our competitive and noncompetitive pollination experiments (comparing seed set in different pollination scenarios) combined with studies on pollen germination in vitro and pollen tube growth semi-in-vivo, suggest that there is a strong gametic isolation barrier in *Petunia* species. This suggests that gametic isolation is stronger in *Petunia* than, for example in *Mimulus*, where heterospecific crosses set about half of the seeds of homospecific crosses (Ramsey et al. 2003).

ASYMMETRY IN GAMETIC ISOLATION

Petunia axillaris is more easily pollinated by *P. integrifolia* than the reverse. Such directionality in gene flow has been reported in many species (Tiffin et al. 2001). Although theory would suggest that isolation barriers should evolve asymmetrically by initially disrupting gene flow from the ancestral species to the derived species, some empirical data contradict this hypothesis. The mechanism causing directionality in F1 hybrid formation in *Iris* (Hodges et al. 1996) was hypothesized to be similar to that found in animals, where asymmetrical mate choice predicts that males from an ancestral taxon will mate with females from a derived taxon but not vice versa (Tiffin et al. 2001). As *P. axillaris* is most likely derived from a *P. integrifolia*-like ancestor (Gübitz et al. 2009), our results are in line with this hypothesis.

In *Rhododendron* and *Nicotiana* section *Alatae* pollen growth rate was greater for species with longer pistils (Williams and Rouse 1990; Lee et al. 2008). Although this relationship holds in our *Petunia* system when pollen growths in homospecific styles (Fig. 2C,D), this is not the case in heterospecific styles. The same has been found in other plant systems (e.g., the genus *Iris*) (Emms et al. 1996). In *Iris*, heterospecific pollen tubes grew slower than conspecific pollen tubes and this affected the frequency of hybridization (Carney et al. 1996). Crosses of *Helianthus* species produce few hybrids and showed an asymmetry in gene flow, although not due to differential pollen growth rate (Rieseberg et al. 1995). A possible explanation for this may be found in *Petunia*'s collaborative nonself recognition system, which involves multiple pollen F-box genes, one or several of which specifically inhibit the style's S-RNase (Kubo et al. 2010). This models implies that a complete set of F-box proteins is required for realizing full compatibility of a self-incompatibility (SI) type with other SI types (Kubo et al. 2010). As a consequence, the model would imply that SI types coevolve. If SI loci evolve and diverge further in isolated populations (or species) this may result in gametophytic incompatibility. Mutations leading to divergence (or even loss of function) are more likely to occur in the founding population of the derived species—in our case *P. axillaris*—(as it is likely to harbor a reduced set of SI alleles and hence stabilizing selection on some the F-box genes may be reduced). We propose that this mechanism could generate the observed asymmetry in pollen tube growth and ultimately result in asymmetric pollen flow.

Overall, these and our data suggest that barriers at the gametic level are more relevant at the noncompetitive than at the competitive level and could be the most relevant gametic isolation factor.

POSTZYGOTIC ISOLATION

There are no isolation barriers associated with F1 hybrid seed germination, growth or time to flowering. Also the postzygotic gametic isolation barriers between *P. axillaris* and *P. integrifolia*

appear to be generally low (Table 4). The contribution of postzygotic isolation barriers to total isolation is minor. In *P. axillaris* the postzygotic isolation indices are in general higher than the indices for *P. integrifolia*. These data suggest an asymmetry in reproductive isolation between the two *Petunia* species. In nature, pollen flow between hybrids and wild plants is possible. For pollinator-mediated divergent selection to occur, hybrids must have low pollination success (Campbell 2003). We observed fewer pollinator visits on F1 hybrids than on *P. integrifolia* but the same number of pollinator visits on F1 hybrids and *P. axillaris* (Fig. 3). This suggests a higher postzygotic barrier in *P. integrifolia* than in *P. axillaris*. Extreme directionality in pollen flow has been reported in two *Nicotiana* species, where one species was the seed parent of 97% of the F1 offspring, resulting from either an asymmetry in pollen delivery or postpollination processes (Ippolito et al. 2004). Hybrid pollen also germinated less well than pollen of the parental species (Fig. 2A,B). However, pollen tube growth was not significantly different from that of the parental plants (Fig. 2C,D) and capsule rate formation and weight was in some cases higher for hybrids than for wild species (Table 3). This was also found in *Iris*, were hybrids performed as well as, or significantly better than, both of their parents (Burke et al. 1998). Furthermore, in our study, hybrid seeds germinated well and there was no indication of hybrid breakdown (Table 2). In summary, these data suggest that some postzygotic isolation barriers between *P. axillaris* and *P. integrifolia* are existent but low.

CONCLUSION

Our study has shown that ecogeographic isolation is an important isolating barrier between *P. integrifolia* and *P. axillaris*. However, even in sympatric populations no hybrids were found in nature. This lack of hybridization can be explained by differences in pollination syndromes (i.e., pollinator isolation) and gametic isolation between the two *Petunia* species. In plants (in particular spermatophyta) a general picture in which reproductive isolation barriers prevent gene flow between plant species is just emerging (Rieseberg and Willis 2007; Lowry et al. 2008; Widmer et al. 2009). This area of research is still in need of more data if general rules for reproductive isolation in angiosperms shall be achieved. Detailed analysis of pollinator isolation and gametic isolation are also prerequisites for further work unraveling the underlying molecular mechanisms. Molecular characterization of prospective "speciation genes," such as AN2, may help to elucidate the order in which isolation mechanisms have evolved. The genetic and experimental analysis of pollination syndromes in *Petunia* is currently underway.

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