








Could the reproductive system explain the stability and long-term persistence in a natural hybrid zone of *Petunia* (Solanaceae)?

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ABSTRACT

The long-term success of populations is dependent on individual reproductive success, and in general, increased population size and genetic diversity contribute to population maintenance, reducing the risk of local extinction. Interspecific hybridization has consequences that can vary according to the hybrids' fate, which can be strongly influenced by the reproductive capacity of hybrids and canonical individuals from the contact zone. We examined the reproductive biology and morphology of two closely related *Petunia* (Solanaceae) species and their interspecific hybrids from Serra do Sudeste, Southern Brazil, and we measured their reproductive success under controlled conditions based on seed production and germination from five pollination treatments. We found differences in self-compatibility degree among individuals, lineages, and pollination treatments based on high total seed production (> 204,000 seeds) and germination (630 seedlings evaluated). No correlation was observed between corolla colour and reproductive success or between floral morphological traits and compatibility. High self-compatibility and inter-lineage compatibility can explain the hybrid populations' maintenance and origin, favouring the two analysed species' introgression.

Keywords: reproductive success, interspecific hybridization, mating system, germinability, *Petunia*

Introduction

The long-term success of populations is dependent on individual reproductive success, and in general, increased population size and genetic diversity contribute to population maintenance, reducing the risk of local extinction (Wubs *et al.* 2010). Mainly for pioneer plants that are colonising new environments, self-fertilization and inbreeding may result in a reduction in fitness but, on the other hand, can ensure successive generations (Keller & Waller 2002).

Interspecific hybridization is the basis of angiosperm diversification (Soltis & Soltis 2009), and its consequences can vary in the function of the hybrids' fate (Ellstrand *et al.* 2013; Todesco *et al.* 2016). Closely related species that occur in sympatry may experience reciprocal pollen flow that may or may not lead to hybrid descendants' formation. In turn, hybrids may show morphological differences and shifts in the mating system or pollinator interaction compared with their parents (Hopkins 2013), and their viability and reproductive potential would determine the success of these novel lineages (Vilà *et al.* 2000). Potential outcomes

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for hybrids can be heterosis, which is established when hybrids show enhanced growth or function, or outbreeding depression when hybrids have lower fitness than parents (Oakley *et al.* 2015). Hybridization can lead to severe floral morphology changes, which may cause shifts in pollinator attractive traits (Teixeira *et al.* 2020) with a priori unpredictable impact on population or species' fate. Changes in pigmentation, scent emission, ultraviolet light response, nectar production or composition, and floral morphology, in general, are associated with evolutionary shifts leading to speciation (Esfeld *et al.* 2018; Rodrigues *et al.* 2018b), and all these traits can be affected by interspecific hybridization.

Variation in herkogamy or operative distance (*i.e.*, the distance among rewards and anthers and stigma) and other floral traits (*e.g.*, corolla shape and size, corolla colour and aroma) can have different effects on the pollinator's visitation, mating success, and fitness gain dependently on the mating systems (Karron *et al.* 2012). Differences in fruit or seed quantities and seedling viability have been observed as mating systems dependent, and evolutionary issues on the advantage of selfing *versus* outcrossing mating strategies to establish or maintain a population have been debated for a long time (Cheptou 2019). Several studies in plants have used fruit formation, seed quantity, and seedling survival as parameters to estimate the population or species fitness (Bigio *et al.* 2017), thus assessing the stability and potential persistence of a population or species.

The genus *Petunia* (Solanaceae) encompasses species with high floral morphology diversity (Knapp 2010) that are mainly associated with different pollination syndromes (Fregonezi *et al.* 2013). Despite the lack of intrinsic barriers against hybridization, at least under controlled conditions (Dell' Olivo & Kuhlemeier 2013), natural hybrids are scarcely found (Lorenz-Lemke *et al.* 2006). *Petunia exserta* and *P. axillaris* exhibit perceived contrasting red and white corolla colours by the human eyes, respectively, and individuals with intermediate corolla colour are found in a sympatric zone between them. The intermediate-coloured individuals were considered hybrids based on genetic data (Lorenz-Lemke *et al.* 2006; Segatto *et al.* 2014; Turchetto *et al.* 2015b; 2019a; b; Teixeira *et al.* 2019) and they also display different corolla shape compared to the species, not necessarily intermediate between the canonical morphologies of the two species (Teixeira *et al.* 2020). As the canonical type, we considered plants displaying all traits according to the species description, respectively (Stehmann *et al.* 2009). To date, *P. axillaris* has an erect habit, salverform white corolla, yellow pollen, and inflexed pedicel in fruit stage, with scented flowers at dusk; *P. exserta* shows red (red-orange) corolla and distinct exerted stamens and stigma, and share with *P. axillaris* the erect habit, salverform corolla, yellow pollen, and inflexed stalk. Moreover, *P. axillaris* is only found in open and sunny environments, whereas *P. exserta* individuals grow in shady places (Lorenz-Lemke *et al.* 2006).

In this study, we examined the reproductive biology and morphology of floral traits of the *P. exserta*, *P. axillaris*, and interspecific hybrids from the contact zone between these species in southern Brazil under controlled conditions. Our goal was to evaluate differences in reproductive success among species and their hybrids to determine whether the corolla colour classes, which correspond to the morphological description previously observed for canonical and hybrid individuals (Teixeira *et al.* 2020), are interfertile and self-compatible. To accomplish this goal, we assessed differences in fruit production, seed quantity (number of seeds per fruit), and seed germination (number of seedlings that reached true-leaves stage) derived from controlled pollination experiments in a greenhouse; we also evaluated the association of these results with the corolla tube length and the distance between the stigma and dorsal anthers. We are motivated by the fact that these hybrid populations are observed for a long time with no changes in size or genetic polymorphism (Schnitzler *et al.* 2020), even with most individuals displaying characteristics that are not related to the canonical species (Teixeira *et al.* 2020) and, therefore, not included in parental floral syndromes.

Materials and methods

Study system

Petunia exserta Stehmann and *P. axillaris* (Lam.) Britton Sterns & Poggenb are herbaceous and annual species that grow in South America's grasslands (Stehmann *et al.* 2009; Fregonezi *et al.* 2013). The red-flowered *P. exserta* is endemic to a locality formed by sandstone towers with 200 m to 500 m in elevation, known as Guaritas in Serra do Sudeste, Southern Brazil (Stehmann *et al.* 2009). *Petunia axillaris* has a white corolla colour and is widespread throughout the Pampas and Chaco ecoregions and in adjacent mountains in the Andean areas (Turchetto *et al.* 2014b); three subspecies are proposed to *P. axillaris* based mainly on floral morphology and distribution areas (Ando 1996; Kokubun *et al.* 2006). These subspecies constitute different evolutionary lineages based on molecular and ecological data (Turchetto *et al.* 2014a) and *P. axillaris* subsp. *axillaris* (hereafter referred to as *P. axillaris*) only grows in the Pampas' open fields in southern Brazil, Uruguay, and Argentina. The geographic distribution of this subspecies overlaps with the range of *P. exserta* in Serra do Sudeste. Although *P. axillaris* and *P. exserta* grow in this same location, these two taxa occupy different microhabitats. *Petunia exserta* is found inside small cavities in the towers (caves or shelters), entirely protected against direct sunlight and rain, on shallow soil (Lorenz-Lemke *et al.* 2006; Segatto *et al.* 2014). *Petunia axillaris* grows in the open areas of grasslands on towers' top or faces (Turchetto *et al.* 2014a; 2015a). These species share some morphological traits, such as a hypocrateriform corolla,



yellow pollen, and capsule fruits with hundreds of seeds. On the other hand, they show specific floral traits related to the main effective pollinators for each species, as they are responsible for attracting the pollinator and allowing pollen removal and deposition, indicating the respective floral syndromes (Etcheverry & Alemán 2005). Whereas *P. exserta* is a hummingbird-pollinated species (Lorenz-Lemke *et al.* 2006) displaying bright red petals, exserted anthers, and non-fragrant and UV-reflective corollas, *P. axillaris* is a hawk moth-pollinated plant (Galetto & Bernardello 1993; Ando *et al.* 2001; Venail *et al.* 2010) with white and smelling corollas that absorb UV light. Pollination experiments indicated that *P. exserta* is a self-compatible (SC) species (Stehmann *et al.* 2009), and *P. axillaris* was considered a self-incompatible (SI) species (Ando *et al.* 1998). A study based on a pollen flow conducted in the Guaritas area (Turchetto *et al.* 2015a) revealed that *P. axillaris* has a mixed mating system, at least in populations from this region.

Two sites in the Guaritas region are particularly intriguing because, during several years, individuals of *P. axillaris* and *P. exserta* were found close to individuals displaying flowers with different shades of intermediate colours (Fig. 1). The individuals' floral morphology in those

sites ranges from canonical *P. exserta* to canonical *P. axillaris*, at extremes, with five intermediate corolla colours between them (Teixeira *et al.* 2020). As commonly observed with other *Petunia* species, these sites comprise small patches with few individuals closely distributed. The few works conducted to evaluate reproductive compatibility in *Petunia* species revealed that even the self-compatible species are dependent on a pollen vector (Ando *et al.* 1998; Turchetto *et al.* 2015a; Rodrigues *et al.* 2018a) because there is no autonomous self-pollination neither fruit production by apomixis.

Plant sources

We collected all mature fruits found in the two sites in Guaritas (30° 53'47" S - 53° 25'15" W and 30° 50'12" S - 53° 30'15" W) where canonical and intermediate coloured individuals are found (Fig. 1). We pooled the seeds together based on the mother plant corolla colour (identified as classes A, B, C, D, and E with intermediate corolla colour, based on a Red-Green-Blue (RGB) colour chart according to Teixeira *et al.* (2020), *P. axillaris*, and *P. exserta*). We used an RGB-based classification to identify the hybrid morphologies

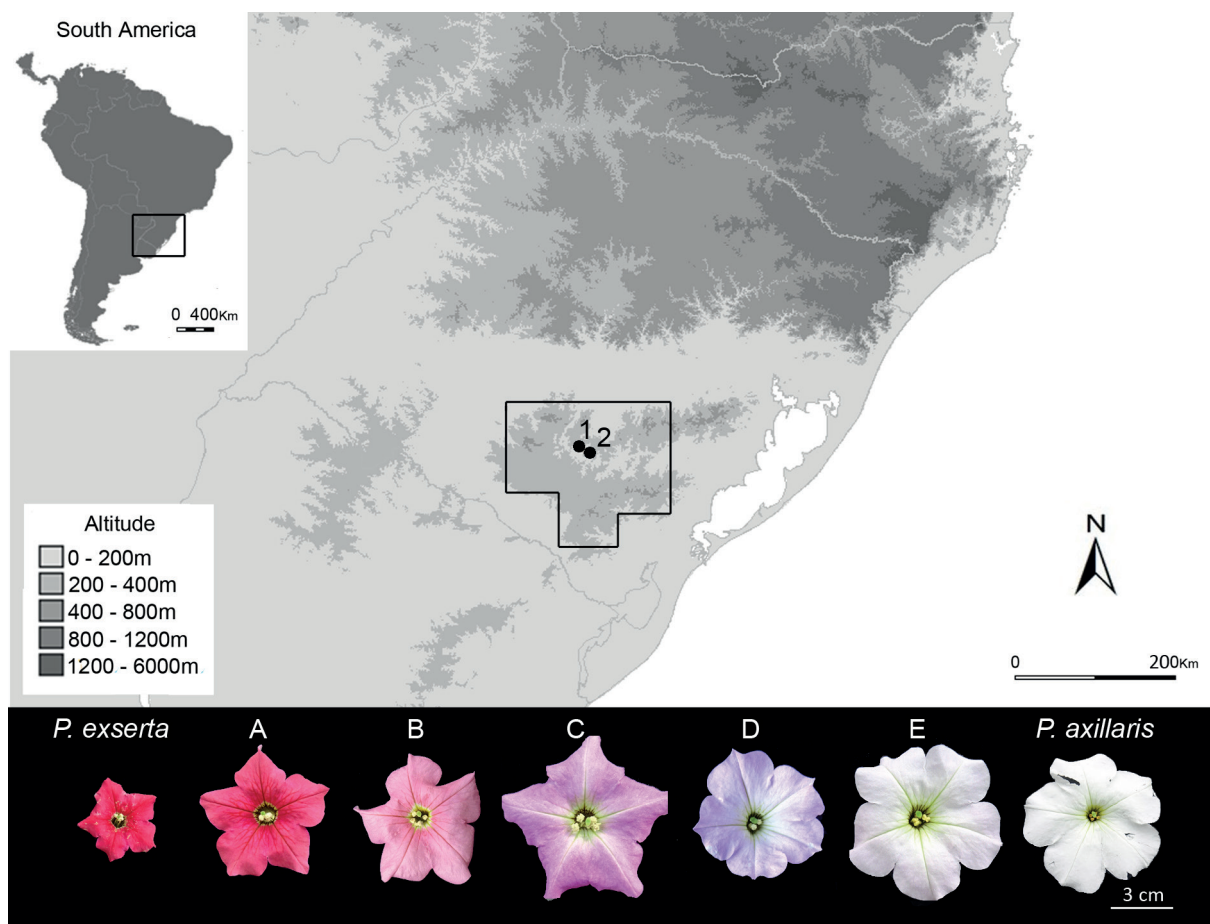


Figure 1. The original collection sites' geographical location in nature and visible colour of flowers that are found in the contact zone between *P. exserta* (left) and *P. axillaris* (right). Intermediate coloured individuals were classified into five classes according to an RGB (red-green-blue) chart as A to E (center) for human eye perception.

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because it corresponds to a set of shape, measures, and genetic traits easily discriminated by humans' observation, despite that it is not necessarily the same for pollinators. Vouchers for these sites were deposited at ICN herbarium (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil): *P. exserta*—ICN185146; *P. axillaris*—ICN185145; Hybrid A—ICN 200858; Hybrid B—ICN 200859; Hybrid C—ICN 200860; Hybrid D—ICN 200861; and Hybrid E—ICN 200862. One seed per pot was sown in 5-mL plastic pots, filled with commercial soil, and maintained in a growth chamber at 22 °C in a 12-h light: 12-h dark cycle. Before the germination process, the seeds were pre-treated with gibberellic acid: dimethyl sulfoxide solution, according to Rodrigues *et al.* (2018a). We transferred the 60-day-old seedlings to a greenhouse in 0.6-L plastic pots filled with commercial soil (70 %): vermiculite (15 %): sterile sand (15 %), maintained siblings under uniform humidity, natural luminosity, and an air temperature of 26± 5 °C, and cultivated these plants until blooming. A commercial nutritive solution (4% nitrogen:14% phosphorous:8% potassium) was added to the soil every 15 days. The germination and cultivation conditions are successfully employed to obtain wild *Petunia* plants in greenhouse experiments (Turchetto *et al.* 2015a; Rodrigues *et al.* 2018b; Teixeira *et al.* 2020). We selected a set of 21 plants by the visual assessment of the corolla colour encompassing the five intermediate colour classes, *P. exserta*, and *P. axillaris* (Fig. 1), three plants per colour class.

Floral traits

We measured two floral characteristics, corolla tube length and distance between the stigma and dorsal anthers, to evaluate whether they could be associated with different pollination treatment or plant visible colour phenotypes in the greenhouse. Based on previous experimental tests in a greenhouse and personal observations in the field that indicate low variability among individuals in each colour class, we measured three flowers on the first day-open stage per plant (63 flowers in total) and used the mean values as the colour classes measure to evaluate: (1) the variation in measures of hybrid plants compared with those observed in canonical individuals of each species; (2) the success in fruit production from spontaneous self-pollination related to the difference between the stigma and the dorsal anthers; and (3) the correlation between reproductive success (fruit production) and tube length. We employed such evaluations to verify whether differences in flower tube length and distance between stigma and anthers favor spontaneous selfing, ensuring hybrid in the absence of pollen vectors.

Pollination experiments

We performed manual crosses under controlled conditions (pollination treatments) for canonical *P. exserta* and *P. axillaris* individuals and each intermediate corolla colour class in a greenhouse. Hand pollination was

conducted by removing one anther of a donor flower with a metallic pin and placing this anther on the recipient flower's stigma. All hand pollinations were performed in the same floral phase (newly opened flowers), and, to prevent accidental selfing, we emasculated the recipient buds just before anthesis. We conducted five pollination treatments: (1) spontaneous self-pollination—intact buds were bagged (SS); (2) hand self-pollination—manually fertilised with self-pollen (HS); (3) autonomous apomixis—the anthers were removed, and the buds were bagged (APo); (4) hand geitonogamy—the flowers were manually fertilised with pollen from another flower of the same individual (GEi); and (5) hand cross-pollination—the flowers were manually pollinated with pollen from other plants from the same or other colour classes. We analysed three plants per colour class in each experiment and performed all bidirectional crosses as each colour class provided to and received pollen from each other phenotype (seven colour classes). We used only three plants because the wild *Petunia* populations usually encompassed just a few individuals, especially in the contact zone between *P. axillaris* and *P. exserta* (Schnitzler *et al.* 2020). Moreover, each fruit produces hundreds of small seeds, which display high viability (Rodrigues *et al.* 2018a; also see Results section). All these facts ensure population survival across generations (Rodrigues *et al.* 2019). In total, we analysed 231 flowers of 21 plants. Fruit formation was recorded, and seed quantity was estimated through the seed mass for 100 seeds per fruit evaluated in precision balance in grams. Green fruits were bagged, and we collected all seeds per mature fruit per pollination treatment in standard plastic tubes. The total seed content was then weighed, and the total number of seeds was estimated proportionally to the mass of 100 counted seeds per fruit.

Germinability

To evaluate seeds' viability from the pollination treatment, we performed a germinability test using 30 randomly selected seeds per individual in each colour class per pollination treatment. We used the same previously described pre-treatment and germination protocols. We followed the seeds for 21 days and counted how many germinated. In general, *Petunia* mature seeds from nature spend five days to germinate, and seedlings have the first true leaves expanded on the 21st day.

Statistical analyses

We compared phenotypes along pollination treatments for the production and germinability of seeds based on the total of seeds or seedlings obtained in each colour class/pollination treatment combination using a chi-square test for homogeneity and residuals. We considered hand self-pollination, hand geitonogamy, and crosses between individuals of the same colour class as intra-class crossings; crosses between hybrids of different colour classes as interclass crossings; crosses between hybrids and canonical



individuals as backcrosses with *P. exserta* and *P. axillaris*, respectively; and crosses between *P. exserta* and *P. axillaris* as hybridization. We conducted comparisons among crosses based on the number of produced seeds and seedlings. To cope with multiple testing issues, we used a significance level of 0.0001 (Bonferroni's correction) in the analyses of Chi-square residuals. For all other tests, the result was considered statistically significant if $p < 0.05$. We used a Kruskal-Wallis test to compare the two morphological traits among colour classes, whereas Pearson's coefficient measured the correlation between these two traits and seed quantity or germinability. The analyses were performed using PASW Statistics (SPSS) for Windows v. 18.0 (IBM Corp., Armonk, USA).

Results

Floral trait variation

The tube length was not significantly different among colour classes and species (Kruskal-Wallis test, $H = 14.29$; $p = 0.270$; Tab. 1). Although it was not possible to conduct pairwise statistical comparisons between colour classes due to the small number of flowers *per* colour class or species, in the sampled flowers of classes C and E, we observed the longest corolla tubes on average compared with those of the other five classes. The shortest tubes were observed in *P. exserta* flowers, whereas flowers of classes A, B, and D showed intermediate tube lengths. Concerning distance between the stigma and dorsal anthers, the highest and lowest individual values were observed in *P. exserta* individuals, and only two individuals displayed stigmata positioned below the anthers (Tab. 1). We did not find significant differences in herkogamy values among the colour classes (Kruskal-Wallis test, $H = 6.09$; $p = 0.413$).

Crossing experiments: fruit and seed production

In general, all the pollination treatments were successful considering all colour classes, with high fruit production. No fruits were produced by apomixis, regardless of colour class or plant individual fruits, independently of colour class or individual. Most plants (14/21) in spontaneous self-pollination treatment did not produce fruits, and only one individual that display the stigma positioned below the anthers produced fruit by SS. Most plants (19 from 21) in hand geitonogamy pollination treatment produced fruits. All fruits produced seeds, but the number of seeds varied among pollination treatments and colour classes (Tab. S1 in supplementary material). We observed a statistically significant association between colour classes and pollination treatments (Chi-squared test $p < 0.001$) for the total number of produced seeds from three random flowers of each pollination treatment, but no clear pattern could be discerned in this association.

Table 1. Floral traits measurements (mm) per plant and corolla colour class. Each value corresponds to the mean of three flowers per individual.

Colour class	Plant ID	Corolla tube length		Distance between stigma and dorsal anthers	
		Individual values	Class mean	Individual values	Class mean
Red (RGB 240:33:76)	Ex1	37.69	35.92	1.88	1.62
	Ex2	35.32		4.48	
	Ex3	34.75		-1.51	
A (RGB 251:94:76)	A1	44.77	46.39	1.76	0.62
	A2	47.23		0.66	
	A3	47.18		-0.55	
B (RGB 225:147:188)	B1	49.46	46.17	2.04	1.43
	B2	47.23		0.74	
	B3	41.83		1.50	
C (RGB 243:183:248)	C1	47.58	49.67	0.07	0.32
	C2	49.48		0.88	
	C3	51.94		0.02	
D (RGB 243:214:252)	D1	45.34	46.38	3.78	2.17
	D2	48.45		1.90	
	D3	45.36		0.83	
E (RGB 238:233:243)	E1	50.69	51.86	1.06	0.63
	E2	53.48		1.38	
	E3	51.41		2.27	
White (RGB 243:243:246)	Ax1	50.75	47.97	2.71	1.91
	Ax2	46.27		1.62	
	Ax3	46.89		1.40	

Red:Green:Blue chart is according to Teixeira *et al.* (2020) and follows Figure 1; A to E – intermediate colour classes; Ex – *P. exserta* (red) colour class; Ax – *P. axillaris* (white) colour class; plant ID 1 to 3 – number of individuals per class.

Concerning the intermediate coloured individuals under different pollination treatment (Fig. 2A; Tab. S2 in supplementary material), hybrid mothers produced a higher number of seeds when mother plant and pollen donor were both hybrids (average contribution of pollination treatments intra-class = 32 % and interclass = 48 % of all produced seeds) and backcrosses contributing with smaller amounts. For Class A mother plants, the seed production decreased in intra-class crossings (21 % of its production) compared to other intra-class crossings colour classes (34 % - 37 %). On the other hand, while for Class B mother plants the intra-class: interclass crossings odds in seed production ranged from 34 % to 53 %, to Classes C, D, and E mothers, the contrast between intra-class and interclass crossings was differently balanced (37 %:45 %; 36 %:46 %; and 34 %:44 %, respectively).

Backcrosses were less productive than intra-class and interclass crossings when the mother plant was a hybrid (Fig. 2A; Tab. S2 in supplementary material). On average, the percentage of seeds produced was 12 % considering backcrosses with *P. exserta* and about half of this (7 %) for backcrosses with *P. axillaris*. Again, this pattern varied according to the hybrid mother plant colour class. In Classes A and D, backcrosses with *P. exserta* produced two percent points more seeds than the average (14 % versus 12 %, respectively).



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Figure 2. Schematic representation for each pollination treatment contribution on the seed production as estimated through Pearson's chi-square and considering intermediate coloured (A) and canonical (B) individuals. For individual and significance values, see Tables S2 and S3 in supplementary material.

respectively), whereas other colour classes produced around the average. The proportion of seed production of Classes A and E mothers in backcrosses with *P. axillaris* was much higher (12 %) than the average (7 %), whereas mothers from Classes C, D, and E produced seeds below the average.

The general summary of the results (Fig. 2A; Tab. S2 in supplementary material) revealed that Class A mother plants seem to produce more seeds than the Class average for all pollination treatments if pollen comes from interclass crossings or any kind of backcrosses (Adj. Res. = 10.9 to 30.4). The Class B mother plants were more effective in intra-class and interclass crossings (Adj. Res. = 7.1 and 17.7), whereas Class C individuals generated more seeds from intra-class crossings (Adj. Res. = 20.9). Concerning the Class D mothers, intra-class and backcrosses with *P. exserta* were the crosses that yielded more seeds. Finally, Class E flowers were especially productive in backcrosses with *P. axillaris*.

Examining seed production of crosses where the ovule donors were plants of the two *Petunia* species (Fig. 2B;

Tab. S3 in supplementary material), the most productive pollination treatment was backcross (*P. exserta* with 54 % of all produced seeds and *P. axillaris* with 63 %). Hybridization between these *Petunia* species was the least effective pollination treatment, contributing with only 9.5 %, on the average, of the total seed production of these plants.

Crossing experiments: seed germination

Seed germination (Tab. S4 in supplementary material) was low (< 50 %) on average, except for *P. axillaris* that had more than 75 % of sown seeds reaching the developmental stage of true leaves. Concerning hybrid plants (Fig. 3A; Tab. S5 in supplementary material), the pollination treatments impacted seed germination only for mother plants from Classes D and E. A lower than the average percentage of viable seedlings was observed in intra-class for Class D mother plants (19 % versus 32 %) and in interclass for Class E plants (22 % versus 33 %). In contrast, intra-class crossings were responsible for higher germination for Class E individuals (42 % versus 32 %).

Comparing the species (Fig. 3B; Tab. S6 in supplementary material), *P. exserta* mother plants produced more viable seedlings (46 %) when involved in intra-class crossings, followed by backcrosses (32 %). For *P. axillaris* as a pollen receiver, a higher success was observed in backcrosses (66 %), with much lower percentages of germinated seeds with the other pollination treatments. Hybridization between these *Petunia* species was the least effective pollination treatment for both species: on average, only 18 % of all seedlings came from this kind of crosses (22 % for *P. exserta* mothers; 16 % for *P. axillaris* plants).

Correlation between floral traits and pollination treatments

We did not observe correlation either between corolla tube length/seed quantities or between tube length/stigma-dorsal anthers distance ($r = -0.14$ and -0.09 , respectively, $p = 0.518$). There was no correlation also between these floral traits and germinability ($r = 0.01$ and 0.30 , respectively, $p = 0.235$). Floral traits were also not associated ($r = -0.07$; p

$= 0.768$). To check whether these results were not due to variable distribution issues, we calculated Spearman's rank correlations, and no statistical significance was achieved (all six p values > 0.14).

Discussion

Herein, we investigated individuals' reproductive compatibility from a contact zone between *P. exserta* and *P. axillaris* through a range of hand pollination tests conducted under controlled and uniform conditions. Our experiments revealed that *P. exserta*, *P. axillaris*, and their interspecific hybrids are self-compatible. Individuals of *P. exserta* and hybrids are also occasionally autogamous. We observed no significant barriers for mates between individuals and between lineages because almost all pollination treatments produced fruits with viable seeds. Seedling's viability is equally low for all phenotypes, but it seems enough to maintain the populations as no differences in the number

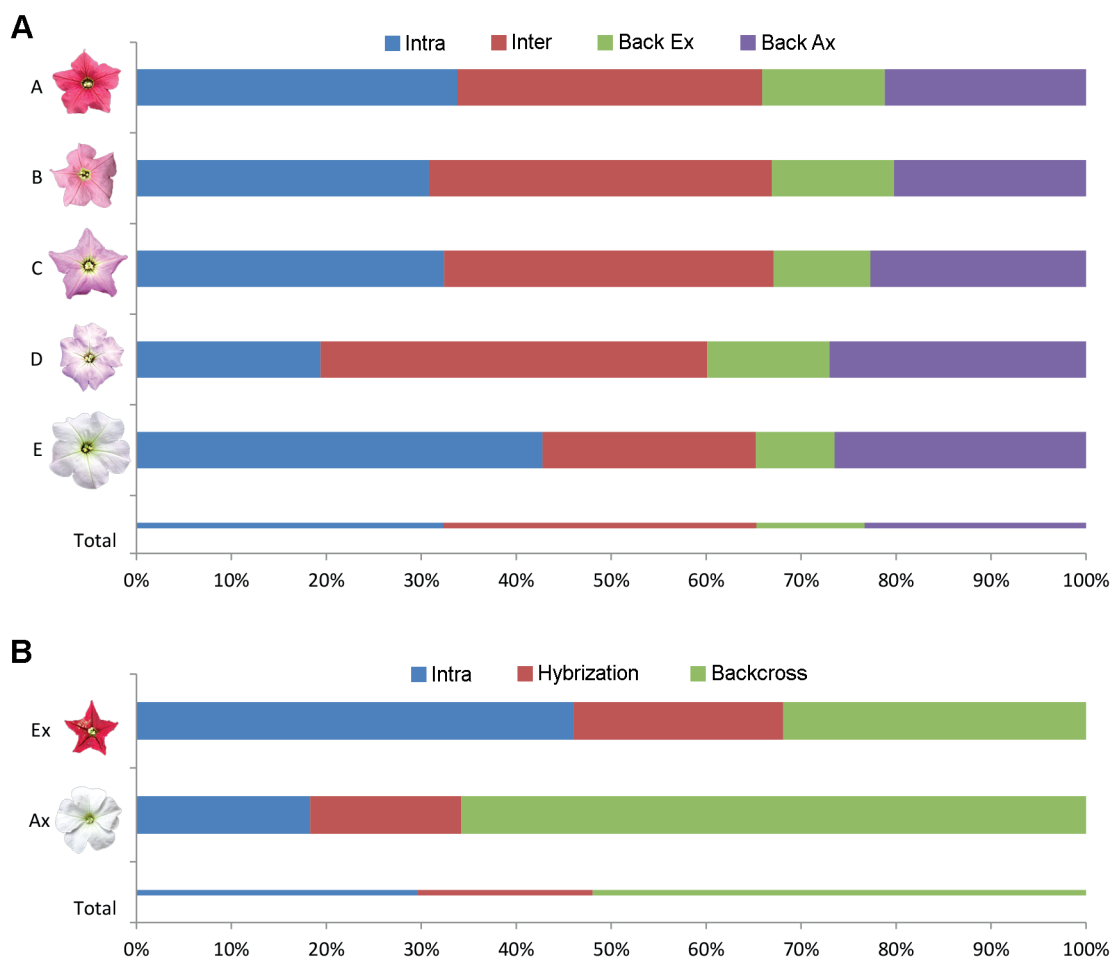


Figure 3. Schematic representation for each pollination treatment contribution on the seed germination as estimated through Pearson's chi-square and considering intermediate coloured (A) and canonical (B) individuals. For individual and significance values, see Tables S5 and S6 in supplementary material.

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of individuals have been observed along time (Schnitzler *et al.* 2020).

As previously described (Watanabe *et al.* 2001), we observed higher reproductive success (translated as seed production) in interspecific crosses when *P. exserta* acts as the mother plant. Under controlled crosses and cultivation, *P. exserta* mother plants produced proportionally more viable seedlings (22 %) than *P. axillaris* (16 %), which evidenced a unilateral interspecific incompatibility that is commonly observed in Solanaceae species (Watanabe *et al.* 2001) and consists of several mechanisms to restrict pollination between different species (initially proposed by Lewis & Crowe 1958). Such mechanisms would protect *P. axillaris* species integrity more than *P. exserta*, which can be expected considering the evolutionary history of these two taxa (Turchetto *et al.* 2019b) as *P. exserta* has been originated from *P. axillaris*. In nature, just a few hybrid morphotypes were found outside shelters together with canonical *P. axillaris* individuals (Turchetto *et al.* 2019a; b; Schnitzler *et al.* 2020; Teixeira *et al.* 2020), which reinforces the idea of a well-established system protecting *P. axillaris* from gene exchanging with a sympatric congener. Moreover, the same gene set that allowed *P. exserta* divergence and adaptation to a particular environment could restrict hybrids' development outside the shelters (Caballero-Villalobos *et al.* 2021).

The structure and fate of a population are strongly influenced by the reproductive potential of its individuals (Ghazoul 2005) and shifts in the floral traits of pollinator-dependent plants have been implicated in the establishment of reproductive barriers between individuals and lineages (Peakall & Whitehead 2014; van der Niet *et al.* 2014) that may put the population's survival at risk. In the contact zone between *P. exserta* and *P. axillaris*, we have followed two sites encompassing canonical individuals as well as interspecific hybrids that have several changes in morphology (Teixeira *et al.* 2020) and genetic profiles (Turchetto *et al.* 2019b) comprising stable populations, despite being reduced in size (Lorenz-Lemke *et al.* 2006; Segatto *et al.* 2014; Turchetto *et al.* 2015b). Recent analyses based on these sites' genetic composition showed that individuals are mainly F_2 hybrids or products from backcrosses with canonical individuals of both species across different flowering seasons (Schnitzler *et al.* 2020). Hybrids were always found inside shelters (Turchetto *et al.* 2019b; Schnitzler *et al.* 2020), close to *P. exserta* plants, and F_1 hybrids were rarely observed. Our current results concerning the compatibility among hybrids and backcrosses support the hypothesis that this contact zone represents a stable population, promoting introgression (Turchetto *et al.* 2019a; b) and increasing morphological variability (Teixeira *et al.* 2020) in both *P. exserta* and *P. axillaris*.

In general, small populations suffer from pollen limitation, which occurs when individuals receive less pollen than necessary to reach their full reproductive success that, if chronic, can lead to the development of selfing

strategies (Runquist & Moeller 2014). Self-fertilization is also usually seen in contact zones between closely related species (Brys *et al.* 2014), as in *P. axillaris* (Turchetto *et al.* 2015a). Furthermore, species with limited dispersal and spatially structured populations may have reduced genetic neighborhood, implying an increase in mating between relatives (Carrillo-Angeles *et al.* 2011), as observed in *P. secreta* (Rodrigues *et al.* 2019), another *Petunia* species from the same geographical distribution and closely related to the *P. exserta* and *P. axillaris* (Reck-Kortmann *et al.* 2014).

Self-fertility increases when *P. hybrida* (Flaschenreim & Ascher 1980) plants are exposed to reduced light intensity similar to that inside shelters where *P. exserta* and intermediate coloured individuals are found. An alternative hypothesis is that small populations could experience an absence of pollinators as was already observed in other studies (Vesprini & Galetto 2000; Amorim *et al.* 2014). Moreover, in populations with a small number of individuals and few flowers per plant, such as those of the *Petunia* species, self-pollination may be increased because pollinators tend to visit conspecific flowers less frequently than they do in large populations (Levin 2012).

If, on the one hand, self-pollination increases the reproductive chances of an invasive species or those living under adverse ecological conditions (Levin 2012), inbreeding can increase the expression of deleterious allele combinations that may also contribute to inbreeding depression (Charlesworth & Willis 2009). However, the magnitude of the endogamic depression effect depends on the mating system and the number of generations under inbreeding (Luijten *et al.* 2002). Here, we observed that selfing pollination treatments did not significantly reduce the seed viability, which usually seems low in these two *Petunia* species and their hybrids, suggesting that these mating systems may have stabilized the populations in the contact zone between *P. exserta* and *P. axillaris*. Moreover, it was previously suggested that inbreeding keeps the species borders (Segatto *et al.* 2014; Turchetto *et al.* 2015a; 2019a). However, species' integrity can also be maintained by postzygotic isolating that limits the fitness of hybrids (Coyne & Orr 2004). The most limiting barrier for these *Petunia* species and their hybrids establishment seems to be the environmental differential selection (Caballero-Villalobos *et al.* 2021) that could prevent the development of *P. axillaris* inside caves and almost totally prevent hybrids growth outside shelters.

In monoecious plants, such as *Petunia* species, it is useful to distinguish the mating system to understand population permanence and diversity (Charlesworth 2006). Here, we described high self-compatibility levels in three co-occurring lineages that also produce viable seedlings in hand-crosses under controlled conditions. Such potential could, at least in part, explain the population stability as a secondary contact zone between *P. exserta* and *P. axillaris* and the hybrids' occurrence among generations, genetic



diversity in those populations (Turchetto *et al.* 2019b), and introgression-generating morphological diversity (Teixeira *et al.* 2020).

In both *P. exserta* and *P. axillaris*, there is a set of genes with tight genetic linkage that provides a mechanism for rapid switches between their distinct pollination syndromes in response to changes in pollinator availabilities (Hermann *et al.* 2013) and have molecular mechanisms of colour differences that are related to the pollinator preferences (Hoballah *et al.* 2007; Sheehan *et al.* 2016). Moreover, occasional visits of hummingbirds have been reported for *P. axillaris* populations (Lorenz-Lemke *et al.* 2006; Gübitz *et al.* 2009), and flowers of these two species share some scent compounds that can be attractive to bees (Rodrigues *et al.* 2018b). However, we did not observe *Pseudagapostemon* sp. collecting pollen from *P. axillaris*, *P. exserta*, or hybrids between them. The hybrids display several pink shades, which are attractive to bees. Such corolla colour shifts are attributed to the loss and regain of just one gene in *Petunia* species (Esfeld *et al.* 2018). Thus, the formation and maintenance of hybrid zones between *P. exserta* and *P. axillaris* can be associated with incomplete discrimination by pollinators and high compatibility levels among hybrids and canonical individuals.

In summary, high self-compatibility and seed viability in these three lineages (canonical individuals of each species and interspecific hybrids) that occur in a contact zone promote the establishment of stable populations and facilitate gene exchange and introgression in these populations. Combined with previous studies on the genetic constitution and morphological characterization, the present results contribute to understanding the *Petunia* diversification process.

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