*Acta Botanica Brasilica* 33(4): 724-733. October-December 2019.
doi: 10.1590/0102-33062019abb0124

# Species boundary and extensive hybridization and introgression in *Petunia*

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Received: April 5, 2019
Accepted: July 8, 2019

## ABSTRACT

Studying the role of hybridization in the speciation of plants is one of the most thrilling areas of evolutionary biology. Hybridization in natural populations can act in opposition to divergence, contribute to adaptation through introgression or foster the emergence of new lineages via hybrid speciation. Species of the plant genus *Petunia* grow in open areas in southern South America. Some natural interspecific hybrid events have been described for the genus, such as between the endemic *P. exserta* and the widespread *P. axillaris*. Both species occur in sympatry in Serra do Sudeste (Brazil), where they occur in diverse habitats and exhibit floral divergence, which has been related to the attraction of different primary pollinators. The present study evaluates the maintenance of the species boundaries front of hybridization and introgression. Direct and indirect methods of estimating gene exchange employed genotyping 720 reproductive plants and 611 progenies of both species with eight microsatellite loci. Gene exchange was found to be frequent and bidirectional between the species, indicating that introgression changes their genetic constitution in areas of sympatry. Limits of the studied species are being maintained because of the high level of inbreeding and backcrosses that are habitat-dependent.

**Keywords**: gene exchange, hybridization, inbreeding, introgression, microsatellite, *Petunia*, species limits

## Introduction

Gene exchange between closely related species is considered an important driver of diversity in angiosperms (Abbott *et al.* 2016). The consequences of interspecific hybridization range from blurring species' limits (Soltis & Soltis 2009) to neutral effects (Arnold 2006) in extreme situations, passing for introgression (Kenney & Sweigart 2016) and genetic divergence with new phenotypes and adaptations (Meier *et al.* 2016). Hybrids' fates are dependent on their genetic combinations, demographic distribution and population structure, as well as their reproductive ability (Yan *et al.* 2017).

In Serra do Sudeste, Rio Grande do Sul, Brazil, two closely related species of the *Petunia* genus are found (Fig. 1). These species share many morphological traits (Stehmann *et al.* 2009) and display close evolutionary relationships (Reck-Kortmann *et al.* 2014). Deeper differentiation between *P. axillaris* subsp. *axillaris* (Fig. 1C) and *P. exserta* (Fig. 1E) is translated into ecological aspects, ranging from pollination syndrome to habitat and geographical distribution. *Petunia axillaris* subsp. *axillaris* (hereafter *P. axillaris*) has a very broad distribution, occupying open areas in Pampas grasslands in Brazil, Argentina, and Uruguay (Turchetto *et al.* 2014a; b). *P. exserta* is endemic to the Serra do Sudeste, more specifically to the region known as Guaritas (Fig. 1B; Segatto *et al.* 2014), where both species occupy different

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microhabitats: *P. axillaris* occurs on the top and faces of arenitic towers, in sunny and open patches (Fig. 1D), while *P. exserta* is found inside small cavities (shelters) in those towers (Fig. 1F), with individuals growing totally protected from direct sunlight and rain. Both species present long and salverform (hypocrateriform) corolla tubes, erect growth habit, and yellow pollen (Stehmann *et al.* 2009), but they differ in morphological characteristics associated with their pollination syndromes, such as corolla color, scent production, and UV response. *Petunia axillaris* corollas are white, scented, and UV-absorbent, with reproductive organs included on the corolla, traits associated with hawkmoth-pollination (Venail *et al.* 2010; Klahre *et al.* 2011); flowers of *P. exserta* are red, nonfragrant, UV-reflectant and have exserted stamens, characteristics usually attributed to hummingbird-pollination syndrome (Lorenz-Lemke *et al.* 2006).

Despite different floral morphology and pollinators, individuals with intermediary corolla color and other floral traits are found inhabiting the shelter side-by-side with typical *P. exserta* plants. Several studies have been conducted in Guaritas seeking to understand the origin of those individuals and their impact on typical lineages of *P. axillaris* and *P. exserta*. Plastid markers determined via a phylogeographical approach revealed high polymorphism-sharing between both species but pointed to an elevated risk of extinction to *P. exserta* because of introgression (Lorenz-Lemke *et al.* 2006). Combining plastid with nuclear markers in an enlarged sample compared to this previous work's results showed that despite hybridization, the species and especially *P. exserta* probably preserve their boundaries through a certain limiting of gene exchange or reproductive strategy (Segatto *et al.* 2014). Interspecific hybridization was confirmed for a small number of intermediary colored individuals (Turchetto *et al.* 2015b), whereas at least for *P. axillaris* from the contact zone, high levels of inbreeding were suggested as an effective barrier against gene exchange with *P. exserta* in Guaritas (Turchetto *et al.* 2015a).

Moreover, molecular characterization of genes and processes involved in floral syndromes shifts among *Petunia* species in general and *P. axillaris* and *P. exserta* species in particular (Amrad *et al.* 2016; Sheehan *et al.* 2016; Esfeld *et al.* 2018; Rodrigues *et al.* 2018), demonstrating the importance of a prezygotic barrier to reproductive isolation to maintain the species limits of this group. On the other hand, strong evidence of interspecific hybridization between *P. axillaris* and *P. exserta* was also obtained through molecular and morphological characterization, mostly at two sites in Guaritas (Turchetto *et al.* 2019; LM Caballero-Villalobos unpubl. res.; MC Teixeira unpubl. res.; CK Schnitzler unpubl. res.), confirming introgression in both species.

The role that hybridization plays in the evolutionary history of different taxa is variable and may appear as periods of introgression, hybrid speciation, and/or adaptive introgression (Taylor & Larson 2019). What happens after hybridization occurs is dependent on reproductive barriers between hybrid individuals and their parental taxa. There is widespread evidence for hybridization between species pairs with contrasting mating systems, but the consequences of introgression for each of these members is related to the potential of recurrent backcrossing of hybrids to the parents to allow genetic exchange and, at least for animal-pollinated species, if hybrid self-fertilization increases (Jordan *et al.* 2017) and stable populations are established.

To evaluate the extension of gene flow and introgression as well as species boundaries between *P. axillaris* and *P. exserta* in their cooccurrence area, we sampled populations covering the entire *P. exserta* distribution and all populations of *P. axillaris* found in Guaritas, and investigated genetic polymorphism based on microsatellites. First, we ascertained the presence of hybrid plants through indirect methods, then we tested for different hybrid classes and purebred individuals based on Bayesian assignments. We also applied direct methods to estimate gene flow through pollen movement between species in the same reproductive season.

## Materials and methods

### Plant material

We collected fresh leaves from 720 adult individuals of *Petunia axillaris* (Lam.) Britton, Sterns and Poggenb (252 individuals from 23 populations) and *P. exserta* Stehmann (468 individuals from 30 populations) in Guaritas, Serra do Sudeste (Tab. 1; Fig. 1B). We used a Global Positioning System (GPS) to obtain geographic coordinates per population, and because of the proximity among individuals, we recorded the geographic coordinates only per population per species (Tab. 1). All individuals were collected in the same phenophase at the same time during the spring in 2011 (September to December).

We also collected mature fruits of 15 plants of *P. axillaris* and 19 plants of *P. exserta* to sample open-pollinated progeny arrays in that reproductive season for these two species. One to three fruits per mother-plant were collected, and their seeds were pooled. Thirty seeds per mother-plant pool were randomly selected for cultivation. The seeds were pretreated for 24 h in the dark at 4 °C with a solution of 100 μM gibberellic acid (GA4; Sigma-Aldrich Co., St. Louis, USA) dissolved in 1 mL DMSO (dimethyl sulfoxide; Sigma-Aldrich) and diluted in water before planting to break any physiological dormancy associated with seasonal weather changes. The seeds were cultivated in a growth chamber under controlled temperature (25 °C) and luminosity (12-h light:12-h dark). Even after two rounds of germination, some mother-plants produced just a few seedlings that resulted in 285 seedlings of *P. axillaris* (16-21 per mother plant) and 326 progenies of *P. exserta* (4-21 per mother-plant).

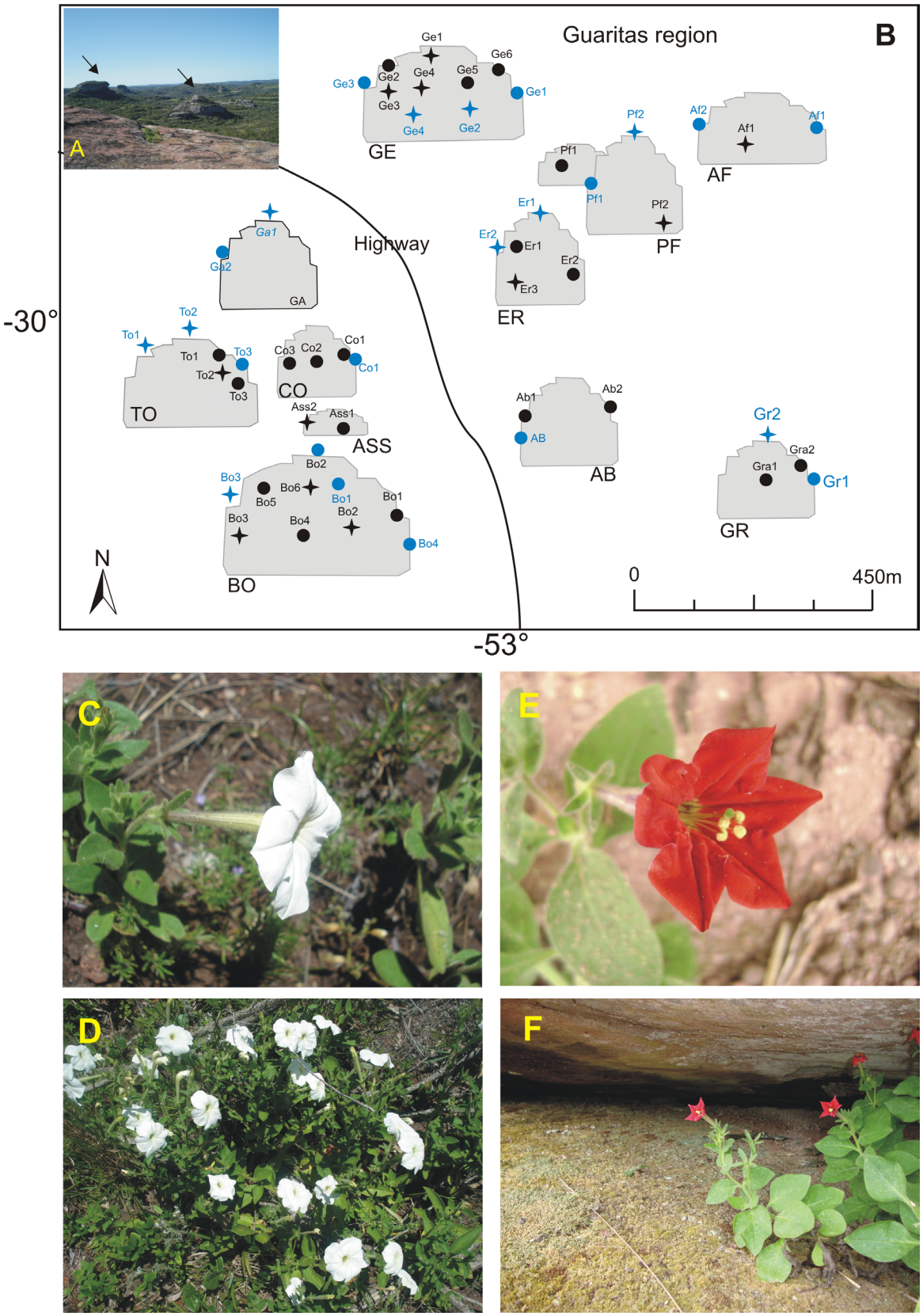


**Figure 1. A.** Landscape of Serra do Sudeste / Guaritas (Brazil). Arrows indicate arenitic towers that characterise the region; **B.** Schematic representation of 12 towers indicating 30 *Petunia exserta* (black) and 23 *P. axillaris* (blue) populations studied; crosses indicate populations from which open-pollination seeds were sampled; **C, D.** *Petunia axillaris* morphology and environment; **E, F.** *Petunia exserta* corolla flower, individual, and habitat inside shelter.

**Table 1.** Sampling information: geographical coordinates of collection sites, sample identity, and number of analysed individuals of *P. exserta* and *P. axillaris*. In bold – populations from which mother-plants were collected; N – number of adult individuals; n – number of mother-plants. Population codes follow Figure 1.

| Sp | Pop Code | N | n | Geographical Coordinates |
|---|---|---|---|---|
| *Petunia exserta* | GE01 | 22 | 2 | 30°49'52.19"S 53°30'11.65"W |
| | GE02 | 4 | | 30°49'54.07"S 53°30'12.39"W |
| | GE03 | 5 | 1 | 30°49'54.11"S 53°30'10.89"W |
| | GE04 | 9 | 1 | 30°49'53.21"S 53°30'10.87"W |
| | GE05 | 9 | | 30°49'51.27"S 53°30'09.02"W |
| | GE06 | 21 | | 30°49'50.49"S 53°30'08.53"W |
| | PF01 | 17 | | 30°49'55.13"S 53°29'59.78"W |
| | PF02 | 14 | 4 | 30°50'00.12"S 53°29'52.25"W |
| | AF01 | 40 | 2 | 30°49'56.23"S 53°29'46.39"W |
| | ER01 | 3 | | 30°50'04.22"S 53°30'03.16"W |
| | ER02 | 2 | | 30°50'04.89"S 53°30'00.61"W |
| | ER03 | 4 | 1 | 30°50'06.12"S 53°30'01.85"W |
| | GRa01 | 2 | | 30°50'18.43"S 53°29'43.09"W |
| | GRa02 | 4 | | 30°50'18.90"S 53°29'42.35"W |
| | AB01 | 14 | | 30°50'14.09"S 53°29'54.72"W |
| | AB02 | 1 | | 30°50'17.97"S 53°29'57.96"W |
| | BO01 | 4 | | 30°50'22.59"S 53°30'07.18"W |
| | BO02 | 17 | 2 | 30°50'22.92"S 53°30'12.59"W |
| | BO03 | 17 | 1 | 30°50'25.82"S 53°30'19.19"W |
| | BO04 | 4 | | 30°50'23.91"S 53°30'18.14"W |
| | BO05 | 17 | 2 | 30°50'23.63"S 53°30'23.48"W |
| | BO06 | 22 | 1 | 30°50'19.00"S 53°30'16.18"W |
| | ASS01 | 49 | | 30°50'13.76"S 53°30'15.04"W |
| | ASS02 | 22 | 1 | 30°50'13.84"S 53°30'16.67"W |
| | CO1 | 34 | | 30°50'10.25"S 53°30'16.48"W |
| | CO2 | 18 | | 30°50'13.76"S 53°30'15.04"W |
| | CO3 | 10 | | 30°50'13.11"S 53°30'19.23"W |
| | TO01 | 12 | | 30°50'12.17"S 53°30'22.49"W |
| | TO02 | 62 | 1 | 30°50'12.42"S 53°30'22.48"W |
| | TO03 | 9 | | 30°50'14.61"S 53°30'23.96"W |
| Total | | 468 | 19 | |
| *Petunia axillaris* | CO1 | 1 | | 30°83'66.41"S 53°50'50.14"W |
| | TO1 | 12 | 2 | 30°83'74.97"S 53°50'68.97"W |
| | TO2 | 8 | | 30°83'76.58"S 53°50'69.69"W |
| | TO3 | 14 | | 30°83'72.22"S 53°50'66.67"W |
| | GA1 | 8 | 1 | 30°83'42.54" 53°50'48.23"W |
| | GA2 | 29 | | 30°83'43.92"S 53°50'52.62"W |
| | BO1 | 8 | | 30°83'86.12"S 53°50'44.96"W |
| | BO2 | 5 | | 30°83'89.70"S 53°50'32.59"W |
| | BO3 | 18 | 1 | 30°83'97.27"S 53°50'52.92"W |
| | BO4 | 4 | | 30°83'82.36"S 53°50'26.69"W |
| | AF1 | 3 | | 30°83'10.29"S 53°49'56.35"W |
| | AF2 | 1 | | 30°83'22.85"S 53°49'62.20"W |
| | PF1 | 1 | | 30°83'28.85"S 53°49'84.07"W |
| | PF2 | 25 | 3 | 30°83'28.61"S 53°49'83.28"W |
| | GR1 | 8 | | 30°83'82.26"S 53°49'50.84"W |
| | GR2 | 27 | 1 | 30°83'83.71"S 53°49'51.15"W |
| | ER1 | 4 | 1 | 30°83'42.77"S 53°50'04.51"W |
| | ER2 | 45 | 3 | 30°83'42.94"S 53°50'02.03"W |
| | GE1 | 7 | | 30°83'03.74"S 53°50'21.16"W |
| | GE2 | 7 | 2 | 30°83'07.88"S 53°50'22.83"W |
| | GE3 | 7 | | 30°83'21.89"S 53°50'36.86"W |
| | GE4 | 5 | 1 | 30°83'12.53"S 53°50'32.77"W |
| | AB | 5 | | 30°83'72.64"S 53°49'93.77"W |
| Total | | 252 | 15 | |

## DNA extraction and microsatellite genotyping

The leaves of each adult individual collected in nature and of progenies obtained in the growth chamber were dried in silica gel, ground in liquid nitrogen, and stored at -20 °C until processing. The genomic DNA was isolated using a CTAB (cetyl-trimethylammonium bromide)-based protocol (Roy *et al.* 1992). All samples (Tab. S1 in supplementary material) were genotyped with eight polymorphic microsatellite loci (PM8, PM21, PM167, PM173, PM177, PM188, PM192, and PM195) covering five of seven *Petunia* chromosomes (Bossolini *et al.* 2011). The selected loci are highly discriminant among *Petunia* species (Turchetto *et al.* 2015b) and were successfully used to study the breeding structure in *P. axillaris* (Turchetto *et al.* 2015a).

The PCRs were performed according Turchetto *et al.* (2015b). The DNA fragments were denatured and size-fractionated utilizing capillary electrophoresis on a MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, USA). The manufacturer's software was applied to determine the alleles per locus with manual inspections and reference samples for *P. axillaris* and *P. exserta* (Turchetto *et al.* 2015b) in each run. Genotyping errors from stutter bands, allele dropout, and null alleles were verified with Micro-Checker software (Oosterhout *et al.* 2004).

## Genetic diversity in each species

Considering adult individuals of both species, we estimated the number of alleles, number of private alleles, allele richness, and inbreeding coefficient ($F_{IS}$; Weir & Cockerham 1984) per loci via FSTAT (Goudet 1995). We also estimated observed and expected heterozygosity under Hardy-Weinberg equilibrium after Bonferroni correction and analysis of molecular variance (AMOVA; Excoffier *et al.* 1992) with 10 000 permutations using ARLEQUIN 3.5 (Excoffier & Lischer 2010).

## Admixture analyses though indirect methods

To estimate the levels of hybridization, proportion and direction of introgression, we carried out three complementary analyses. First, we performed model-based Bayesian clustering employing Structure 2.3.2 software (Pritchard *et al.* 2000) to estimate admixture proportions among *P. exserta* and *P. axillaris* adult individuals from the entire sampled area (overall, 720 individuals considering both species). Then, we ran two separated analyses pooling all seedlings of each species (totaling 285 samples in *P. axillaris* and 326 progenies in *P. exserta*) and including all adults of both species per comparison to test the admixture proportion in a second generation of each species. Structure analyses were carried out according to an admixture model assuming correlated allelic frequencies, and no *a priori*

population assignment was used in the analyses. Each run was conducted with $2.5 \times 10^5$ burn-in periods and 106 Markov chain Monte Carlo (MCMC) repetitions after burn-in. Three independent runs were performed per comparison, and the results were examined for convergence across runs. We identified the number of gene pools through the most likely number of genetic clusters obtained using the maximum value of $\Delta K$ (Evanno *et al*. 2005) as implemented in Structure Harvester 0.6.93 (Earl & Holdt 2012) and the estimate of Pr(X | K) from Structure. Pophelper, an online *R* package (Francis 2017), was utilized to summarize the output of the optimal *K*-value from Structure and generate the bar plots. Further, Structure was used to classify individuals as parental species or hybrid. A threshold of *Q* value > 0.10 (individuals of *P. exserta* that displayed at least *Q* = 0.10 of a genetic component found in *P. axillaris* and individuals of *P. axillaris* that presented at least *Q* = 0.10 of a genetic component found in *P. exserta*) was leveraged to identify hybrid plants. Based on the fact most molecular markers were in HWE disequilibrium (Tab. 2), we also ran multivariate discriminate analyses of principal components (DAPC; Jombart *et al*. 2010) as implemented in Adegenet of R 3.3.0 (R Development Core Team 2016) based on microsatellite data including all adults of both species and their respective progenies. These analyses were performed to identify genetic clusters within the data set and compare to the Structure results from *K* = 2 (two species). This method makes no assumption about data structure or underlying population genetic model.

To infer the genetic composition of hybrids, we performed a Bayesian clustering analysis implemented in Newhybrids 1.1 software (Anderson & Thompson 2002), which assigns individuals into different genotypic classes (parental species, F1, F2 and back-crosses with each parental species). The analyses were run considering adult individuals and families independently. For the families' analyses, we included mother-plants as representative of each species' genetic constitution. For each comparison, we ran two independent analyses using Jeffrey's priors with uniform priors that included 105 steps as burn-in followed by 106 MCMC interactions performed to assure the convergence of chains and homogeneity across runs. Analyses were performed without previous information on populations or taxonomic identity (Vähä & Primmer 2006).

## *Direct measures of gene flow between* ***P. exserta*** *and* ***P. axillaris***

We performed parentage analyses to estimate the pollen flow between *P. exserta* and *P. axillaris*. To determine the most likely pollen donors for seedlings of *P. exserta,* we included all adult individuals of both species as pollen donor candidates (720 individuals of both species growing at the same site and sampled in the same flowering season) and the families derived from *P. exserta* mother plants. Additionally, we re-analyzed the paternity assignments of *P. axillaris'* 285 seedlings from Turchetto *et al.* (2015a), including all *P. exserta* adults, as potential pollen donors.

We employed the Bayesian method to conduct paternity analysis with Cervus 3.0.6 (Marshall *et al*. 1998; Kalinowski *et al*. 2007). To determine the likely pollen donors for seedlings of each species, all adult individuals found in the same season were used as a pollen donor candidate for each offspring in equal probability. As selfing is possible, mother plants were also considered father candidates. To define a critical LOD (logarithm of likelihood ratios) score, we ran the simulation of paternity analysis using the allele frequencies calculated for adults as a reference and thus we estimated the Δ statistic (critical Δ; Marshall *et al*. 1998). Afterwards, we conducted a parentage analysis to assign the father candidate to each offspring. The most likely pollen donor was evaluated relative to the critical Δ scores as determined in previous simulations. The individual with the highest Δ value was accepted as father of the seedling when the difference between its LOD score and the LOD score of the second most likely candidate was above the critical Δ (threshold value). For parentage analysis, the following parameters were used: 10 000 repetitions; 0.9840 proportion of typed loci; and

**Table 2.** Diversity indices based on eight microsatellite per species. N – number of alleles; E – number of private alleles; R – allele richness; *HE* – expected heterozygosity; *HO* – observed heterozygosity; *FIS* – inbreeding coefficient; * - significant HWE values after Bonferroni correction.

| Sp | | PM8 | PM21 | PM173 | PM167 | PM192 | PM188 | PM195 | PM177 | Average |
|---|---|---|---|---|---|---|---|---|---|---|
| *P. axillaris* | N | 6 | 7 | 17 | 11 | 12 | 9 | 7 | 24 | 11.63 |
| | E | 1 | 2 | 10 | 1 | 2 | 0 | 3 | 6 | 3.13 |
| | R | 6.00 | 6.98 | 16.97 | 11.00 | 12.00 | 9.00 | 7.00 | 23.99 | 11.62 |
| | $H_E$ | 0.57 | 0.61 | 0.56 | 0.82 | 0.77 | 0.79 | 0.54 | 0.91 | 0.70 |
| | $H_O$ | 0.31* | 0.31* | 0.32* | 0.52* | 0.42* | 0.48* | 0.24* | 0.40* | 0.38 |
| | $F_{IS}$ | 0.46 | 0.49 | 0.42 | 0.36 | 0.45 | 0.39 | 0.57 | 0.56 | 0.46 |
| *P. exserta* | N | 6 | 5 | 8 | 11 | 13 | 9 | 8 | 21 | 10.13 |
| | E | 1 | 0 | 1 | 1 | 1 | 0 | 2 | 3 | 1.13 |
| | R | 5.52 | 4.68 | 7.35 | 9.74 | 12.42 | 8.56 | 7.12 | 18.99 | 9.30 |
| | $H_E$ | 0.23 | 0.50 | 0.75 | 0.67 | 0.84 | 0.35 | 0.35 | 0.82 | 0.57 |
| | $H_O$ | 0.08* | 0.16* | 0.27* | 0.24* | 0.24* | 0.12* | 0.10* | 0.25* | 0.18 |
| | $F_{IS}$ | 0.72 | 0.68 | 0.64 | 0.65 | 0.71 | 0.67 | 0.73 | 0.70 | 0.68 |

99 % (strict) and 95 % (relaxed) levels of confidence. We considered 90 % of parents sampled in the area and a 1 % genotyping error. The minimum number of loci required to determine the paternity of a seedling was fixed at six.

# Results

## Genetic diversity

The eight microsatellite loci produced 93 alleles in *P. axillaris* and 81 alleles in *P. exserta*. Most markers presented private alleles in each species. The means of allele richness were higher in *P. axillaris* than *P. exserta* (Student's t-test, $P < 0.05$). The two species presented high and positive $F_{IS}$ values. Almost all loci were in Hardy-Weinberg disequilibrium displaying homozygote excess (Tab. 2). AMOVA analyses revealed a genetic differentiation between species (20.34 %, $P < 0.001$). Both species presented positive and elevated *FIS* values, suggesting high levels of inbreeding that were greater in *P. exserta* (Student's t-test, $P < 0.05$).

## Genetic composition of species and hybrids

The best $K = 2$ was obtained in Structure Bayesian clustering analysis according to the criterion of Evanno *et al.* (2005), which grouped individuals of each species (Fig. 2). Most adult individuals of *P. axillaris* and *P. exserta* presented *Q* values > 0.90, but ca. 2 % of individuals previously assigned as *P. axillaris* (six among 252) or *P. exserta* (12 among 468) were now identified as hybrids and had *Q* values > 0.10. Two populations of *P. exserta* had the highest number of hybrid individuals (GE01 and CO2, with five and three putative hybrids, respectively), and four populations had only one hybrid individual (GE06, AF01, BO02, and CO3). Population CO2 was the only shelter in which we detected a few individuals with color variation, thereby suggesting hybridization through phenotype (data not shown). One mother plant from GE01 was established as a hybrid based on its genetic profile (E1630). Four populations of *P. axillaris* had one hybrid individual (GR1, BO3, CO1, and TO1), and the GA2 population had two hybrid individuals. No *P. axillaris* mother plants were identified as hybrids based on their genotypes, and no indication of hybrid morphological phenotype was observed among adults of this species.

We detected the *P. axillaris* genetic component in three families of *P. exserta* (Fig. S1 in supplementary material): two from the GE01 population, from which mother plants also showed the genetic component of *P. axillaris,* and one from the PF02 population. Among the progenies of *P. axillaris* (Fig. S2 in supplementary material) we found several individuals presenting the genetic component of *P. exserta*, especially from two populations (GR2 and GE2).

In the DAPC analysis (Fig. 3), we observed two clusters of individuals as observed in the Structure analyses. Several individuals among adults presented genetic component of both clusters. We observed *P. axillaris*' genetic component in progenies of *P. exserta* from one family (FM_05). However, five progenies of *P. axillaris* from different families presented mixed genetic components. Several individuals among adults and progenies featured genetic components of both clusters, whereas others had genetic profiles not concordant with their morphological group or spatial distribution (floral morphology corresponding to a given species as well as position relating to shelters, but genetic profile as another species).

The NewHybrids analyses (Fig. 4) revealed the different classes of crosses among adults as well as progenies, with inbreeding being the most frequent type in both species. Among adults, the second most frequent class was $F_2$, whereas backcrosses with *P. axillaris* were high among *P. axillaris* offspring. Backcrosses with *P. axillaris* were also observed among *P. exserta* progenies, and some individuals of this offspring were classified as *P. axillaris* according their microsatellite profiles. Despite the low frequency, F1 individuals were observed among adults and progenies. In the family of one *P. axillaris* mother plant (A1809) from the GRA2 population, we, observed four individuals as a backcross with *P. exserta* (A2072, A2082, A2084, and A2091).

## Pollen flow between ***P. exserta*** and ***P. axillaris***

In the paternity analysis of the 326 *P. exserta* offspring, considering all *P. exserta* (468) and *P. axillaris* (252) adult plants as pollen donor candidates, no *P. axillaris* individual was assigned as the most likely father (critical value $\Delta = 2.95$; 95 % confidence interval). When we considered a relaxed

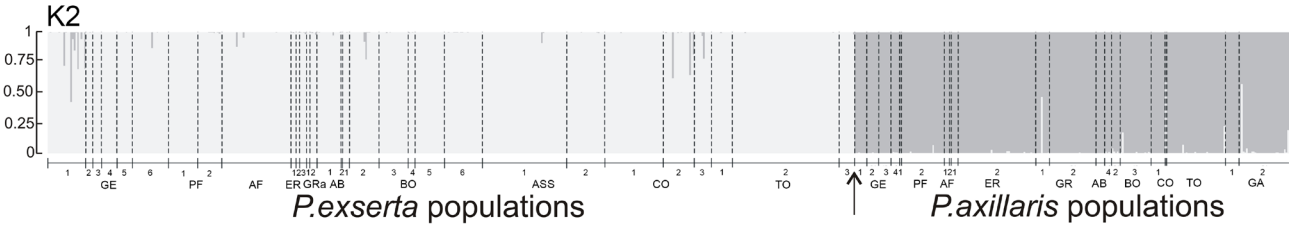


**Figure 2.** Structure bar plot under admixture coefficients model based on eight microsatellite loci and 720 adult individuals of *Petunia exserta* and *P. axillaris*. Each bar represents individuals and black-dotted vertical lines indicate each population; different colours correspond to genetic components ($K = 2$) and individuals' membership.

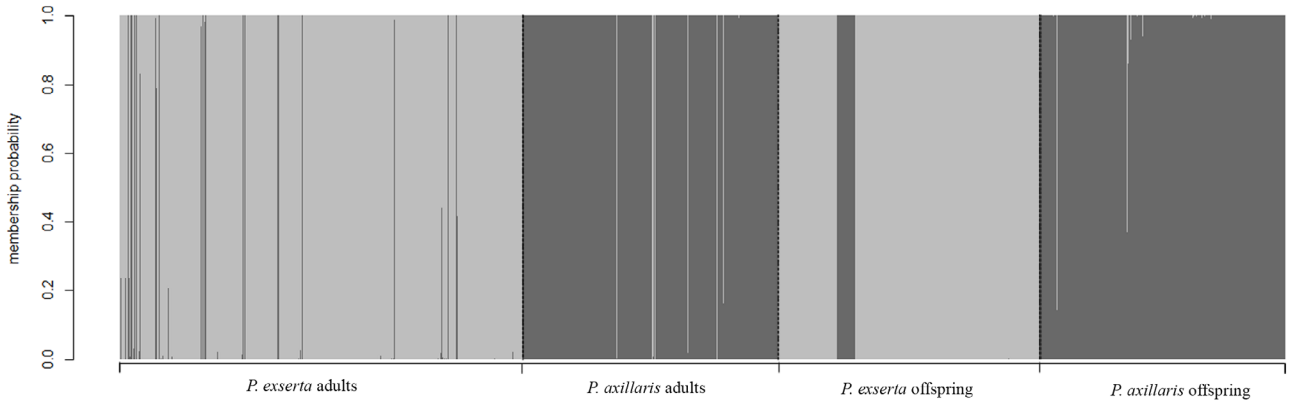


**Figure 3.** Clustering of individuals as revealed in DAPC analysis ($K$ = 2) performed based on microsatellite genotypes of adults and progenies of *P. exserta* and *P. axillaris*. Black-dotted lines separate individuals' groups (each species adults or progenies); each vertical bar corresponds to an individual. Different colours indicate membership probability of each species per individual.

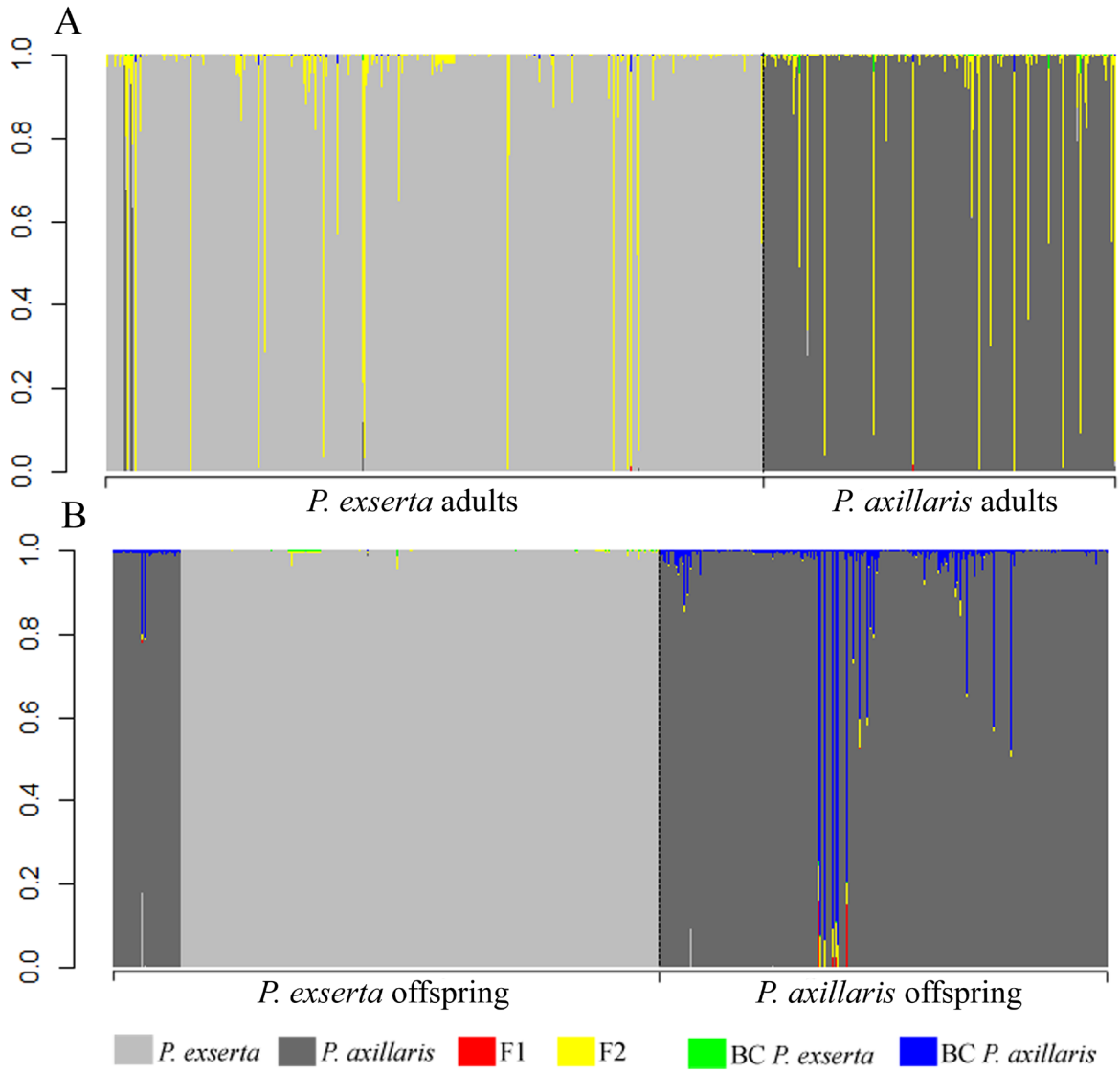


**Figure 4.** Posterior probabilities (q) for all analysed plants using NewHybrids assigned to six classes following the legend for pure parental species (*P. axillaris* or *P. exserta*), $F_1$, $F_2$, backcrosses (BC) with *P. exserta*, and backcrosses with *P. axillaris*. **A.** Adults and **B**. progenies of each species. Vertical black dotted lines separate species or progenies.

confidence interval (critical value Δ = 0.69; 85 % confidence interval), two offspring of EM1624 had one *P. axillaris* plant indicated as pollen donor (from the BO2 population) and one offspring of EM1630 had equal LOD score values for one *P. exserta* and one *P. axillaris* individual as the most likely pollen donor (both EM1624 and EM1630 are from the GE01 population of *P. exserta*). These two mother plants (EM1624 and EM1630) and all their offspring were placed in the *P. axillaris* cluster through Structure analysis (Fig. 2 and Fig. S1 in supplementary material). For *P. axillaris*, two of 285 seedlings (mother plant from the GRA2 population) had *P. exserta* as the most likely pollen donor, considering the strict confidence interval (critical value Δ = 2.95; 95 % confidence interval). Furthermore, considering the relaxed confidence level (critical value Δ = 0.69; 85 % confidence interval), eight seedlings from the same population (GRA2) had one *P. exserta* as a pollen donor coming from five different populations of *P. exserta* (CO2, GE06, ASS02, GE01 and PF01). Four of these progenies were also indicated in the NewHybrids analyses as resulting from backcrosses with *P. exserta*. Unlike *P. exserta*, the GRA2 mother plant was assigned to the cluster of *P. axillaris* within the Structure analyses that included all *P. axillaris* offspring and some of the offspring presenting mixed ancestry (Fig. S2 in supplementary material). These results showed that although pollen flow between *P. axillaris* and *P. exserta* is rare, it happens in both directions. In this reproductive season, the interspecific pollen flow occurred more frequently from *P. exserta* to *P. axillaris*. However, in the Bayesian analysis of adult individuals, hybrid ancestry was observed more frequently among *P. exserta* individuals (Fig. 2). These results suggested that fertilization between these species failed when pollen flows from *P. exserta* to *P. axillaris* or that the hybrid progeny has a lower success of developing until the adult stage when it is in the *P. axillaris* microhabitat. Only a few adult individuals were identified as hybrids based on the genetics and no morphological intermediary phenotypes were found among *P. axillaris* populations. By contrast, some hybrid individuals, as indicated by genetic markers and intermediary corolla color, were collected growing in the *P. exserta* microhabitat and might effectively reproduce primarily by self-fertilization, as observed with the mother plant from the GE1 population that had a hybrid origin (Fig. 2).

## Discussion

Herein, we estimated gene exchange in a contact zone between two closely related *Petunia* species that, despite interspecific hybridization, are preserving their species boundaries. Gene flow is low but introgression occurs in both directions and, contrary to previously thought (Lorenz-Lemke *et al.* 2006), introgression is also high among individuals of *P. axillaris*. Hybrids are predominantly found in the same microenvironment as *P. exserta* (inside shelters), although individuals with both genetic components exist among *P. axillaris* plants, in sunny and opened fields. In this latter case, hybrids present the same morphology as canonical *P. axillaris*, whereas when they are observed inside shelters, they usually exhibit intermediary corolla color. The direct and indirect estimates of gene exchange between these two species revealed high levels of inbreeding in both, with backcrosses with the spatially closer parental being more frequent than other kinds of crossing.

These two *Petunia* species present suites of floral traits strongly related to their respective floral syndromes, hawkmoth in *P. axillaris* and hummingbirds in *P. exserta* (Stehmann *et al.* 2009). No systematic pollination studies have been conducted in the field to identify which pollinators the intermediary colored plants attract and, except their pink shades that usually attract bees in *Petunia* species (Rodrigues *et al.* 2018), other clues on pollinators remain unidentified.

Hummingbirds were observed visiting natural populations of *P. exserta* (Lorenz-Lemke *et al.* 2006). Moreover, these birds were also described as visitors in *P. axillaris* populations in Uruguay (Gübitz *et al.* 2009). Floral morphology and spatial distribution of individuals of both species in Guaritas suggest that these animals could be responsible for pollen exchange among these individuals. Pollinators tend to forage on nearby flowers when a flower offers rewards (Tremblay *et al.* 2005) and especially in the absence of other nectar sources. Hummingbirds, in general, can differ in their foraging strategies and degree of specialization regarding floral resources.

Many species of these birds feed along trapping lines following repeated foraging circuits among successive flowers or clumps (Snow & Snow 1972; Colwell 1973; Linhart 1973). Furthermore, the circuit may be long distance and high reward by following a regular route and particular sequence of plants (Feinsinger 1983). Other groups of hummingbirds exhibit greater variety regarding degree of specialization, and most species are territorial only during the bloom peak when flowers are densely packed (Maglianesi *et al.* 2015b). Although these *Petunia* species usually bloom in the same period without seasonal isolation (Stehmann *et al.* 2009), field observations in Serra do Sudeste revealed a certain level of difference in the flowering time between these cooccurring species, with *P. axillaris* usually blooming earlier than *P. exserta*, along with some overlaps (MC Teixeira, unpubl. res.). In early spring, and afterwards the reverse, many individuals of *P. axillaris* can be found with flowers, whereas few individuals of *P. exserta* are flowering.

The foraging preferences of hummingbird species involve the morphological matching between the bill and floral traits and are also a consequence of resource abundance (Maglianesi *et al.* 2015a; b). In fact, specialized interactions have been observed in many plant-hummingbird pairs and are implicated as important drivers of plant diversification in the Neotropics (Nunes *et al.* 2016; Serrano-Serrano *et al.* 2017). *Petunia exserta* and *P. axillaris* present several floral traits in common (Stehmann *et al.* 2009), such as

a long and salverform corolla tube with nectar chambers at the base, which also exist in hybrid individuals. Nectar volume and sugar concentration vary among *Petunia* species; characteristics of nectar from *P. exserta* flowers are not known, but this species displays other traits related to hummingbirds (Gübitz *et al.* 2009; Stehmann *et al.* 2009). The nectar volume and sugar concentration in *P. axillaris* (Gleiser *et al.* 2014) are similar to those described for bird-pollinated species (Baker 1975; Proctor *et al.* 1996).

Selfing and backcrosses are strategies that can reinforce barriers against the gene flow between species and therefore contribute to species cohesion (Kamran-Disfani & Agrawal 2014; Segatto *et al.* 2014). Here, we found that pollination was one primary barrier to gene exchange between these two cooccurring species, as inbreeding was the most frequent mating system. This prezygotic barrier can be associated with the specific interaction between pollinators and morphological traits of each species (Huber *et al.* 2005) and may be implicated in adaptation to microenvironmental conditions (Hu 2015) with selection against hybrids in one or another parental environment (Toews & Brelsford 2012), such as the high amount of backcrosses observed, especially among *P. axillaris* progenies that grow in sunny and open patches, ecological conditions totally different from the environment inside shelters. Although such prezygotic barriers can prevent interspecific pollen flow, it was permeable, as indicated by the events of interspecific hybridization among progenies of both species, which were also observed among adults of both species and previously hypothesized (Lorenz-Lemke *et al.* 2006; Segatto *et al.* 2014; Turchetto *et al.* 2015a).

Seed dispersal can also influence the genetic structure and pollen movement within populations of these two species because in *Petunia* species, seeds usually fall near the mother plant (Pijl 1982), and thus each population inside shelters as well as in open areas mostly encompasses related individuals. Self-fertilization and biparental inbreeding rates observed might be a consequence of pollinator behavior, with behavior influenced by the local abundance of flowers of each species in each tower.

Speciation can be thought of as a continuous process (Nosil & Feder 2012). Plant species are typically isolated not by a single factor but by a large number of different barriers in complex interactions (Widmer *et al.* 2009) that act in a hierarchical order (Dell'Olivo *et al.* 2011). The *Petunia* genus is a young group of endemic species that has low genetic differentiation (Reck-Kortmann *et al.* 2014) and underwent allopatric speciation during the Pleistocene (Lorenz-Lemke *et al.* 2010); microenvironmental and pollinator diversity have also been suggested as important factors driving their diversification (Fregonezi *et al.* 2013). In the current work, we put forth several results that support these statements for two of these species, which are found in sympatry, share many morphological and genetic polymorphisms and have interspecific hybridization as a strong driver of diversification.

## Acknowledgements

The authors thank J.N. Fregonezi, A.M.C. Ramos-Fregonezi, G. Mäder, and A.L.A. Segatto for assistance during field collection; J.R. Stehmann for help with taxonomic identification; and J. Beduschi for help in DNA extraction**.** This project was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). C.T. was supported by PNPD-CAPES/PPGBM, UFRGS.

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