

Contact zones and their consequences: hybridization between two ecologically isolated wild *Petunia* species

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Plant hybridization is frequently observed in nature and considered an important driver of angiosperm diversity. Species are thought to arise through the accumulation of morphological and genetic differences that promote their evolutionary independence, even in the presence of hybridization. Natural hybrid zones yield an excellent system to study the outcomes of hybridization in terms of species diversity. Two recently diverged species, *Petunia axillaris* and *P. exserta*, show floral differentiation attributed to attraction of varying pollinators. Previous studies suggested natural hybridization between these species to explain morphological floral polymorphisms found among individuals of *P. exserta*. Here, we analyse genetic and morphological diversity in plants from the contact zone between these species and from isolated populations of each species to evaluate natural hybridization and its consequences. We found that the species' integrity is maintained despite interspecific hybridization and introgression that drove the origin of a new lineage in *P. exserta* in the contact zones.

KEYWORDS: floral morphology – genetic diversity – introgression – Pampas.

INTRODUCTION

Natural secondary contact zones between closely related species constitute an ideal opportunity to study the pace and mode of interspecific hybridization and introgression. Studying the extent and direction of introgression and gene flow in these areas can help unravel the historical dynamics of hybrid zones (Cinget *et al.*, 2015) and contribute to understanding the evolutionary patterns driving angiosperm diversity (Soltis & Soltis, 2009; Yakimowski & Rieseberg, 2014). The emergence of stable and hybrid-derivate lineages opens questions about the maintenance of the involved taxa and whether efforts to restore their genetic structure or their conservation should be made in the face of the process in due course (Marques *et al.*, 2014).

The consequences of interspecific hybridization on species persistence can vary as a function of the genetic, demographic or geographical structure of each involved taxon (Yan *et al.*, 2017) and may promote blurring the boundaries between species and evolutionary noise (Soltis & Soltis, 2009) and genetic erosion through introgression (Rieseberg & Wendel, 1993; Kenney & Sweigart, 2016), but can also cause adaptation and genetic divergence (Ellstrand, 2014; Meier *et al.*, 2016) or have neutral effects (Arnold, 2006), with each species maintaining its limits. In Neotropical orchids, evolutionary success is attributed to the emergence of hybrid lineages (Pinheiro *et al.*, 2010; Veja *et al.*, 2013; Marques *et al.*, 2014; Leal *et al.*, 2016; Szlachetko *et al.*, 2017), suggesting hybridization plays an important role in increasing diversity in this region, despite the fact that only a few plant groups have been studied.

In flowering plants, the relationships among features relevant to attracting pollinators (pollination

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syndromes; Fenster *et al.*, 2004) can be used to illustrate why recently diverged species may have strong differentiation in floral traits (Cronk & Yang, 2016). In this sense, such differences may contribute to genetic isolation between species (Johnson, 2006; Imbert *et al.*, 2014). However, interspecific crosses between divergent floral morphs resulting in viable and highly fertile hybrids have been observed in nature (Milano, Kenney & Juenger, 2016). Hybrid success can be related to the attraction of a more diverse set of pollinators (Veja *et al.*, 2013). The analysis of morphological data may provide information about the hybridization phenomena, including the understanding of pre- and post-zygotic barriers. However, not all morphological variation can be attributed to hybridization between taxa and, in some systems, morphological variation can alternatively be related to genetic drift, disruptive selection or phenotypic plasticity (Leal *et al.*, 2016). In addition, hybrids do not always have intermediate morphology relative to the parental species (Szlachetko *et al.*, 2017).

Petunia Juss. (Solanaceae) is a South American endemic genus (Stehmann *et al.*, 2009) with a recent diversification history (Lorenz-Lemke *et al.*, 2010; Särkinen *et al.*, 2013) whereby the diversification of species is related to pollination syndromes and geographical isolation (Fregonezi *et al.*, 2013). Divided into two main clades (Reck-Kortmann *et al.*, 2014), the genus has species with long and short corolla tubes. Among the species with long corolla tubes, three pollination syndromes are observed and these species feature all the floral traits traditionally associated with them.

Petunia exserta Stehmann and *P. axillaris* (Lam.) Britton, Sterns & Poggenb. are herbaceous and annual plants with contrasting floral traits (Fig. 1A–D); the former has bright red and non-fragrant flowers with exserted styles and anthers (typical traits of the hummingbird pollination syndrome), whereas the latter has white flowers that are strongly fragrant at the dusk (characteristics of the hawkmoth pollination syndrome) (Stehmann *et al.*, 2009; Venail, Dell’Olivo & Kuhlemeier, 2010; Klahre *et al.*, 2011; Amrad *et al.*, 2016; Sheehan *et al.*, 2016). *Petunia exserta* is a self-compatible (SC) species and *P. axillaris* was initially considered to be a self-incompatible species (SI) (Stehmann *et al.*, 2009). Studies using molecular markers to map the pollen flow in one generation showed that *P. exserta* shows high rates of selfing along its entire geographical distribution and *P. axillaris* has a mixed mating system in the geographical area where it co-occurs with *P. exserta* (Turchetto *et al.*, 2015a; Turchetto *et al.*, unpublished data).

These two species grow in an overlapping area (Lorenz-Lemke *et al.*, 2006) in rock formations inside the Pampas in South America (Guaritas region, Brazil; Fig. 1F, G). *Petunia exserta* is endemic to the Guaritas region, whereas *P. axillaris* is widely distributed through the South American grasslands and the Guaritas region constitutes the eastern edge of its distribution (Fig. 2A, B). In Guaritas, the two species occur as isolated populations among the sandstone rock towers ranging at 200–500 m elevation, and each species occupies a specific habitat. *Petunia exserta* plants are located strictly inside the shady cracks of sandstone towers that constitute shallow shelters of a few centimetres in depth and height (Fig. 1D); inside these shelters, *P. exserta* individuals grow on shallow soil and are protected from direct rain and sunlight (Segatto *et al.*, 2014). *Petunia axillaris* individuals only grow in patches of grassland in open and sunny habitats (Fig. 1G) on tower tops or faces (Turchetto *et al.*, 2014a). Across an area of c. 3000 km² in Guaritas, two sites (Fig. 2C) deserve more attention because plants of both species grow close together and plants presenting corolla colour ranging from dark pink to slightly pinkish have also been found inside shelters (Fig. 1E) every year since 2002 (Turchetto *et al.*, 2015b). These two sites are considered contact zones for the species. Despite this, certain individuals with an atypical flower colour, relative to the description for species, have also been found among other *P. exserta* populations, they have not been seen every year or in all populations. The occurrence of atypical flower colour has been hypothesized to be due to interspecific hybridization and introgression (Lorenz-Lemke *et al.*, 2006) based on plastid markers or as a natural polymorphism in *P. exserta* (Segatto *et al.*, 2014) based on genetic polymorphisms from plastid sequences and some nuclear CAPS (cleaved amplified polymorphic sequences). Whereas the first study identified strong hybridization between these species and identified a resultant extinction risk for *P. exserta*, the second raised the possibility of morphological polymorphism with selection against hybrid condition across the distribution of *P. exserta*. These previous works did not focus on stable contact zones either performed a systematic analysis on morphological data, which could have hampered the achievement of clear patterns about extension and dynamics of hybridization between these species.

In this study, we used an integrative approach combining genetic and morphological evaluation to address the origin of intermediate coloured individuals between *P. axillaris* and *P. exserta* from Guaritas region. We used microsatellite markers to characterize genetic differentiation and population structure and measurements of floral traits to evaluate floral differentiation between species and among

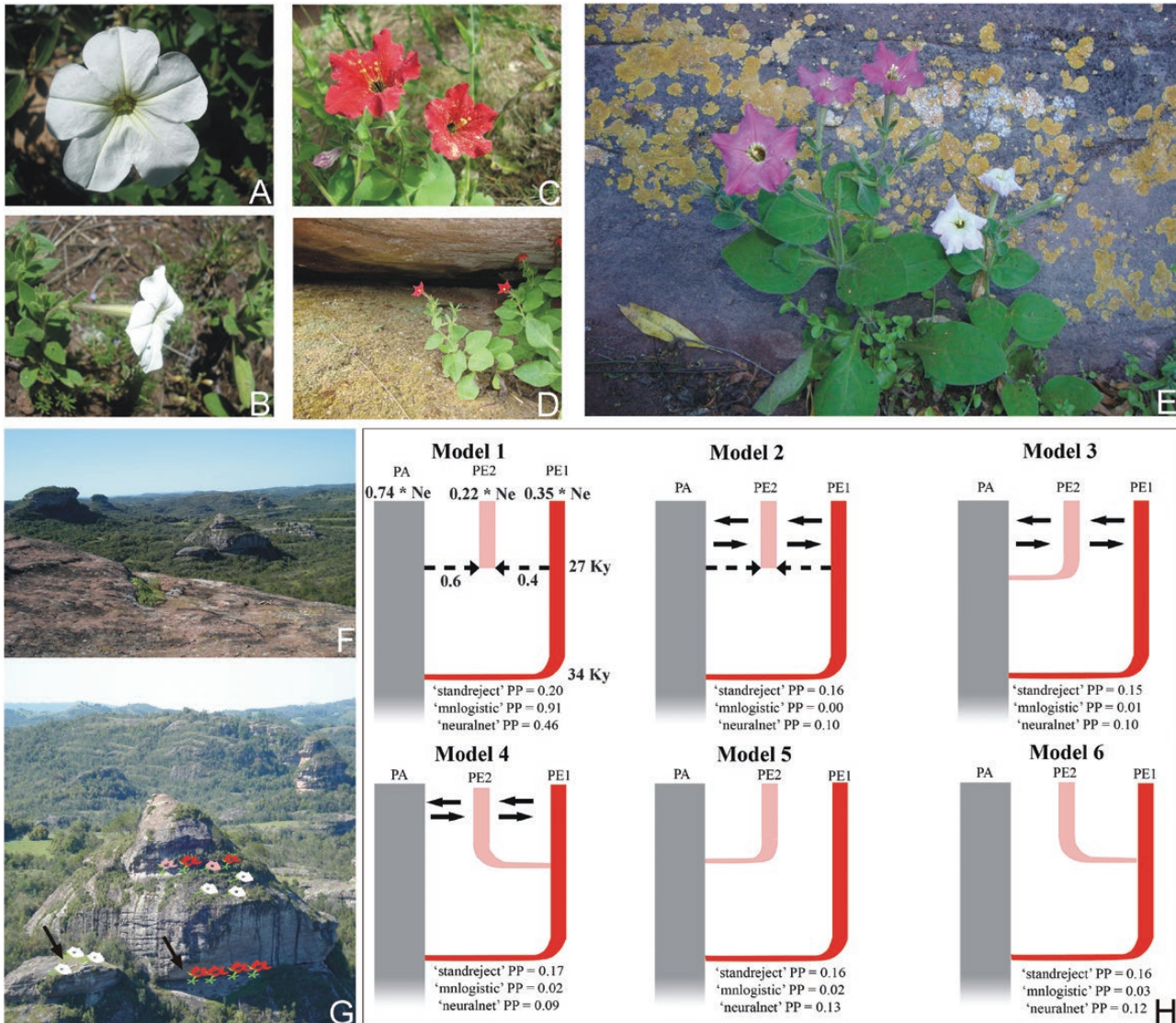


Figure 1. Morphologies, habitats and ABC approach models: A, B, typical phenotype of *P. axillaris*; C, D, typical phenotype and habitat of *P. exserta*; E, atypical corolla coloration representative found in a co-occurring population; F, landscape in Serra do Sudeste region; G, sandstone tower with a schematic representation of co-occurring populations (atypical phenotypes grow inside or on the edge of shelters together; *P. exserta* and *P. axillaris* plants grow outside shelters); arrows indicate isolated populations per species; H, graphical representation of six coalescent models assessed with ABC analysis to test the atypical morphologies (PE2) origin from *P. axillaris* (PA) and *P. exserta* (PE1) co-occurring populations; below each model, posterior probabilities for three rejection algorithms implemented. Model 1 was the best-supported coalescent model (posterior estimates of parameters of effective population sizes, admixture and divergence times are indicated).

flower colour classes (white, intermediate and red) from the two contact sites and from isolated populations of each species. If the morphological intermediate coloured individuals are hybrids in any generation, we expect an admixture of genetic components of both species in these individuals, whereas if the morphological polymorphism is a prevalent phenomenon, no admixture is expected and individuals would tend to show the genetic component of one species only.

MATERIAL AND METHODS

STUDIED POPULATIONS

During the spring season of 2011, we collected all adult individuals from the two secondary contact zones, hereafter referred to as CO1 and CO2 (grey squares; Fig. 2C), respectively, and totalling 43 individuals. In this area, we observed individuals with corollas ranging from pure red to pure white through different

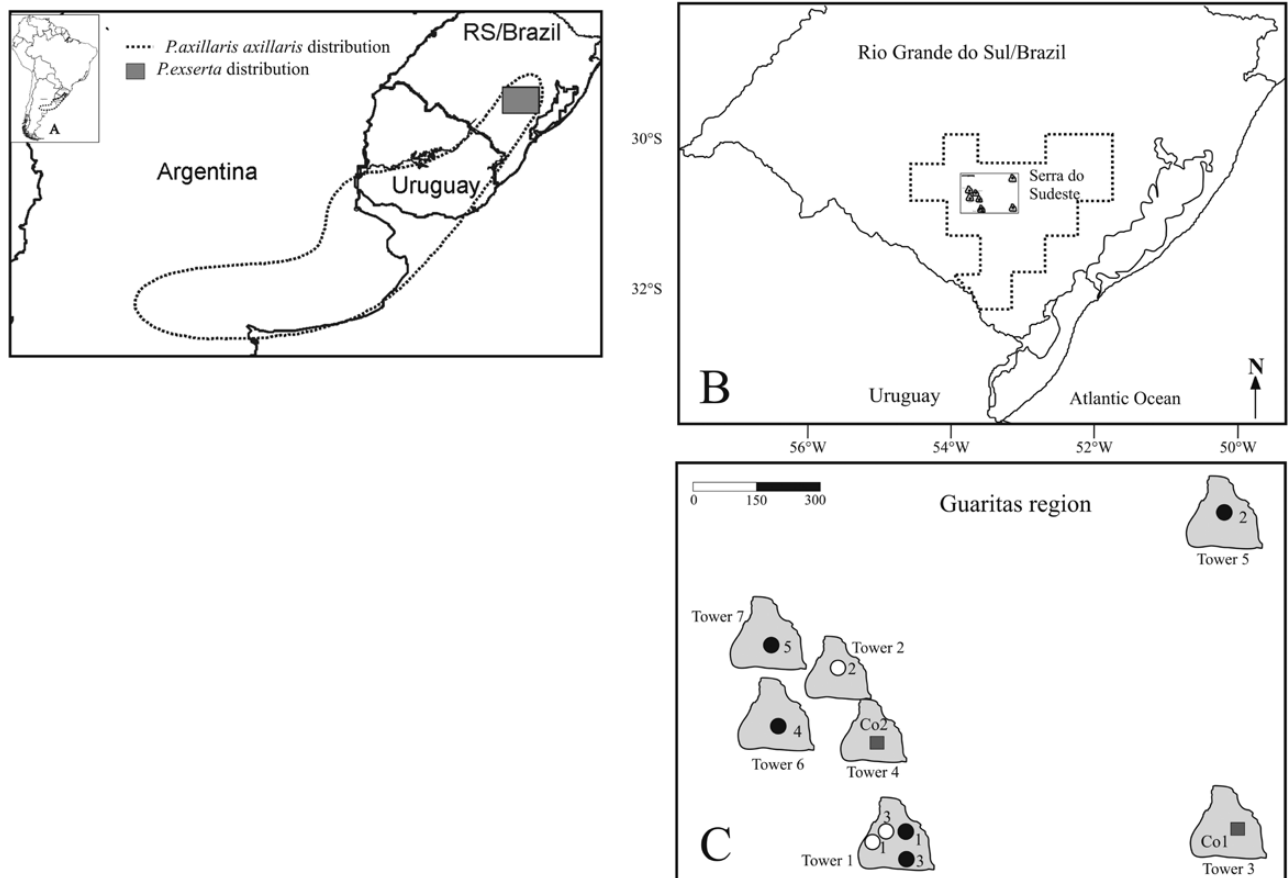


Figure 2. Sampling geographical distribution: A, entire occurrence area of *P. axillaris* (dotted line) and *P. exserta* (grey square) species; B, Serra do Sudeste in Rio Grande do Sul / Brazil and C, tower and population spatial distribution in the Serra do Sudeste region: co-occurring populations (CO1 and CO2, grey squares), *P. axillaris* isolated populations (Pa_IS; white circles) and *P. exserta* isolated populations (Pe_IS; black circles). Population and tower numbers in accordance with Table 1.

hues of pink (Table S1). In both CO1 and CO2, all red- and pink-coloured individuals were found inside shelters and all white-flowered individuals were found outside. We also collected all adult individuals from isolated sites (IS; sites where only one species occurs) for *P. axillaris* (three sites, 47 individuals in total) and *P. exserta* (five sites, 51 individuals in total) (Fig. 2). These populations were considered as purebred individuals in all analyses because there is no evidence of hybridization and only one species occurs. One voucher per site was collected (Table 1).

MORPHOLOGICAL MEASUREMENTS AND ANALYSES

We measured five floral traits in nature from one flower in the post-anthesis phase of each sampled individual using digital callipers: corolla tube length (X1); corolla diameter (X2); total corolla length (X3); length of the exerted segment of anthers and pistils (from the edge of the corolla to the stigma; X4) and distance

between anthers of the longest and middle-length stamens (didynamous stamens; X5). Measurements were recorded in millimetres with a precision of two decimal places. Corolla colour was recorded with a digital camera (Table S1). These morphological traits have been employed previously to discriminate among subspecies of *P. axillaris* (Ando, 1996; Turchetto et al., 2014b). We focused on these characteristics, because floral traits, such as corolla shape, length and colour and the position of reproductive organs are traits associated with specific pollination syndromes (Hermann et al., 2013, 2015), and morphological variability can be useful to detect putative hybrids (McIntosh et al., 2014). The variation in floral traits among sites and species/phenotype was analysed using a Kruskal–Wallis multiple-comparisons test performed in SPSS 11.0 software ($K = 4$ corresponding to *P. axillaris* and *P. exserta* isolated sites, CO1 and CO2).

Discriminant analysis of principal components (DAPC; Jombart, Devillard & Balloux, 2010) in

Table 1. Collection sites and sample information

Site	Geographical coordinates	<i>N</i>	Voucher
Pa_IS1	30° 50' 20.938"S / 53° 30' 14.914"W	17	BHCB140443
Pa_IS2	30° 50' 10.886"S / 53° 30' 18.512"W	15	BHCB140434
Pa_IS3	30° 50' 20.430"S / 53° 30' 13.390"W	15	BHCB140431
CO1	30° 53' 48.153"S / 53° 25' 16.080"W	25	ICN185145
CO2	30° 50' 13.761"S / 53° 30' 15.036"W	18	ICN185146
Pe_IS1	30° 50' 20.190"S / 53° 30' 12.318"W	6	ICN158647
Pe_IS2	30° 49' 56.000"S / 53° 29' 47.000"W	7	ICN158542
Pe_IS3	30° 50' 22.000"S / 53° 30' 12.000"W	8	ICN158647
Pe_IS4	30° 50' 12.422"S / 53° 30' 22.484"W	18	BHCB79896
Pe_IS5	30° 50' 09.000"S / 53° 30' 24.000"W	12	ICN158643
Total		141	

Pa_IS – *P. axillaris* isolated sites 1 - 3; Pe_IS – *P. exserta* isolated sites 1 - 5; CO – contact zones 1 and 2; N – number of individuals per site. BHCB – Universidade Federal de Minas Gerais herbarium (Belo Horizonte, Brazil); ICN – Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil)

the package ADEGENET of R v.3.3.0 (R Core Team, 2016) was applied to determine whether the species and intermediate coloured individuals could be distinguished based on these floral traits. The DAPC relied on data transformation using principal component analysis (PCA) as a prior step to discriminant analysis (DA), maximizing the separation among groups.

GENETIC DATA ACQUISITION

Young leaves of each individual were sampled, dried in silica gel, ground in liquid nitrogen and stored at –20 °C until processing. Genomic DNA was extracted following a standard CTAB (cetyl trimethyl ammonium bromide) protocol (Roy *et al.*, 1992), and all individuals were genotyped with eight polymorphic microsatellite loci (PM8, PM21, PM167, PM177, PM173, PM183, PM188 and PM195) scattered across five of the seven *Petunia* chromosomes (Bossolini *et al.*, 2011). Polymerase chain reactions (PCRs) were conducted following the protocol described for these species (Turchetto *et al.*, 2015b). The DNA fragments were denatured and size-fractionated using capillary electrophoresis on a MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, PA, USA). The manufacturer's software was used to determine the alleles for each locus with manual inspections. Genotyping errors from stutter bands, allele dropouts and null alleles were verified in MICRO-CHECKER (van Oosterhout *et al.*, 2004).

GENETIC DIVERSITY AND DIFFERENTIATION

Descriptive statistics, including the number of alleles (N), number of private alleles (E), allelic richness (R) and inbreeding coefficient (F_{IS}), were calculated for all

populations and loci using FSTAT v.2.9.3.2 (Goudet, 1995). Observed (H_o) and expected (H_e) heterozygosity under the Hardy–Weinberg equilibrium (HWE, after Bonferroni's correction) were calculated in ARLEQUIN v.3.5.1.2 (Excoffier & Lischer, 2010). We also performed allelic richness analysis as implemented in HP-RARE v.1.0 (Kalinowski, 2005), where the statistical technique of rarefaction compensates for sampling disparity. Deviation from linkage equilibrium (Goudet *et al.*, 1996) was tested with Bonferroni's correction, also in ARLEQUIN. Analysis of molecular variance (AMOVA) (Excoffier, Smouse & Quattro, 1992) and pairwise F_{ST} among the collection sites were estimated with ARLEQUIN with 10 000 permutations to assess significance. The variation of diversity indices was statistically tested with Student's test.

ANALYSIS OF GENETIC STRUCTURE

As with morphological traits, we ran a DAPC analysis in ADEGENET of R based on microsatellite data for isolated sites of each species and both contact zones. We also used NEWHYBRIDS v.1.1 (Anderson & Thompson, 2002) software to estimate the probability of individuals belonging to distinct hybrid or purebred classes (F1, F2, backcrosses with *P. exserta* and backcrosses with *P. axillaris*). These two alternative Bayesian-based methods have been employed to assess hybrid status.

For the NEWHYBRIDS analysis, we ran two independent analyses using Jeffrey's priors with uniform priors that included 10^5 steps as burn-in followed by 10^6 Markov chain Monte Carlo (MCMC) interactions performed to assure the convergence of chains and homogeneity across runs.

To estimate the direction and rate of recent immigration among the 12 combinations of collection

sites and species/morphology, we employed a Bayesian multilocus genotyping procedure implemented with MCMC methods in BAYESASS v.3.0 (Wilson & Rannala, 2003). In this analysis, the level of gene flow was calculated based on the migration rate within the last one to three generations, providing estimates most accurately when migration rates were low and population genetic differentiation was high (Kane *et al.*, 2009). The results of the BAYESASS analysis can be interpreted with confidence with an $F_{ST} > 0.05$ (Faubet, Waples & Gaggiotti, 2007), an assumption that holds true in this study (see Results). BAYESASS analyses were run with a 100 sampling frequency, a chain length of 3×10^6 iterations and 3×10^5 iterations as burn-in period settings. Runs were carried out in duplicate to assess convergence of the MCMC. The program TRACER was used to validate data convergence.

TEST OF ALTERNATIVE EVOLUTIONARY SCENARIOS

Taking into account our assignment results based on morphological and microsatellite data, three main clusters were identified and named PA, PE1 and PE2. We therefore implemented a model-based coalescence method to investigate the origin of the PE2 cluster, which encompassed almost all intermediate coloured individuals. Thus, we used the approximate Bayesian computation (ABC) method (Beaumont, Zhang & Balding, 2002) to assess the posterior probabilities (PPs) of six plausible coalescent scenarios. The first model (M1) represented the emergence of the PE2 genetic cluster by hybridization between PA and PE1 clusters through a single admixture process after the divergence between PA and PE1. The second model (M2) was similar to M1, although it included maintenance of bidirectional gene flow between PE2 and the other two clusters. The third and fourth models

(M3 and M4) represented isolation-migration scenarios in which PE2 originated from PA or PE, respectively. The fifth and sixth models (M5 and M6) represented simple divergence scenarios in which PE2 originated from PA or PE, respectively (Fig. 1H). The scenarios corresponded to the most reliable evolutionary histories that could have led to the current status of these species considering what is already known about the system (Reck-Kortmann *et al.*, 2014; Segatto *et al.*, 2014; Turchetto *et al.*, 2014a, b).

We simulated 10^6 data sets for each model using MS software (Hudson, 2002) implemented with customized Python scripts (modified from Perez *et al.*, 2016). Prior parameter values were considered random variables drawn from uniform distributions (Table 2). Prior distribution divergence times were based on previous studies with *P. exserta* (Segatto *et al.*, 2014) and *P. axillaris* (Turchetto *et al.*, 2014a). Each simulated genealogy was translated into a microsatellite dataset using MICROSAT software (<http://massey.genomicus.com/software.html#microsat>) assuming a single-step mutation model (SMM). We used ARLSUMSTAT v.3.5.1.3 (Kalinowski, 2005) to calculate the summary statistics (SuSt) for each simulation. We computed a set of 16 SuSt, including the number of alleles, genetic diversity and genetic differentiation (Table S2). For initial inspection of the differences among scenarios and in comparison with the observed SuSt, we implemented a PCA for 10^4 randomly collected simulated SuSt of each model using the *prcomp* function in R.

We calculated the PPs for each coalescent model employing the ABC package (Csilléry, François & Blum, 2012) in R. We assessed three model selection methods: (1) the standard rejection method ('standreject'), which approximates the PP of each model and the proportion of the simulations retained within the distance threshold relative to the observed SuSt; (2) the

Table 2. Prior distributions of model parameters in the ABC analysis

Parameter	Distribution	Minimum	Maximum
Theta (Θ)	Uniform	0.1	20
SSR Mutation rate (μ)	Uniform	0.000001	0.001
Effective population size (N_e)	$\Theta / 4 * \mu$		
NPa (proportion of N_e)	Uniform	0.1	1
NPe1 (proportion of N_e)	Uniform	0.1	1
NPe2 (proportion of N_e)	Uniform	0.1	1
Divergence time NPa–NPe1	Uniform	10 000	78 000
Divergence time NPe2	Uniform	1	Time divergence NPa–NPe1
Generation time	1 generation / year		
Migration ($4N_e m$ per generation)	Uniform	1	20
Proportion of Pa contributed to Pe2	Uniform	0.1	0.9

PA: *P. axillaris* lineage; PE1: *P. exserta* lineage 1 (red coloured flowers); PE2: *P. exserta* lineage 2 (intermediate coloured flowers)

multinomial logistic regression method ('mnlogistic') and (3) the neural network approach ('neuralnet'). The last two methods employ adjustment-based nonlinear approaches that minimize departures from linearity and homoscedasticity and allow for a reduction in the dimensionality of the SuSt via internal projections on lower-dimensional subspaces (Blum & François, 2010). We used a threshold level of 0.1, resulting in 6×10^5 simulations retained to estimate PPs for each model.

We used the model with the highest PP to calculate posterior parameter distributions. We performed post-rejection adjustments within the prior parameter value bounds (Blum & François, 2010) according to the 'abc' function of the ABC package in R. We integrated a posterior predictive verification, taking the 1000 best simulations to draw a new parameter distribution. We then executed 10^4 simulations for the model with the highest PP using MS software implemented with customized Python scripts. We plotted the histograms of the calculated SuSt of the posterior simulations to assess fit to the observed SuSt. Finally, we implemented a posterior PCA for 10^4 SuSt randomly collected from simulations of the six tested models and the model with the highest PP based on the posterior parameter distribution and observed SuSt.

RESULTS

MORPHOLOGICAL VARIATION

Overall, we analysed 141 individuals. From isolated sites, the 51 individuals collected inside shelters had the a priori *P. exserta* morphology (dark pink or red flowers and exserted anthers and stamens), and the 47 individuals found outside shelters had the typical white flowers of *P. axillaris* species with no exserted anthers. From the contact zones, based on flower colour, 17 individuals showed intermediate corolla colour: 12 from CO1 (C08, C21, C23, C27, C30, C64, C66, C70, C72, C73, C80 and C98) and five from CO2 (E1072, E1078, E1088, E1090 and E1222), all sampled inside or on the edge of a shelter and the corolla colour ranged from dark pink to slightly pinkish. Reproductive organs position was intermediate compared to that observed in the species from IS and from CO sites (Table S1). In CO1, only two individuals had red flowers (typical colour of *P. exserta*), whereas this colour was prevalent (12 plants) among individuals inside the shelter in CO2. From CO1 and CO2, 11 individuals and only one individual, respectively, had white flowers; all were collected outside shelters (Table S1).

Flowers of *P. exserta* and *P. axillaris* from the isolated sites were significantly different for all five morphological traits (Kruskal–Wallis test, $H = 2.52$, $P = 2.8$). The contact zones differed significantly

from each other only with respect to the length of the exserted reproductive organs (X4), the same trait that differed CO1 from *P. exserta*, whereas all traits were significantly different comparing contact zones and *P. axillaris* from isolated sites (Fig. 3). High levels of variability were observed among individuals from each collection site for all traits (Table S1).

The DAPC analysis based on five floral traits revealed two main groups made up preferentially of the individuals from IS populations of each species (Fig. 4A), which we called Pe_IS and Pa_IS clusters, respectively. Considering only the IS populations of both species, we observed a perfect match between taxonomic classification and corolla colour, morphological trait measures and spatial distribution (inside/outside) in relation to shelter. Concerning individuals from the co-occurrence areas, this observation was not necessarily true and individuals were not always intermediate between canonical colours or morphological measures of each species. Intermediate coloured individuals from CO1 mainly formed an intermediate group in the morphospace, with only two individuals included in the Pe_IS group and nine in the Pa_IS cluster, which had red or white corollas, respectively; from CO2, intermediate coloured individuals were preferentially grouped into the Pe_IS cluster, with only three occupying an intermediate position and two superimposed onto the Pa_IS group. From the contact zones, there was no correlation between corolla colour and measures or taxonomic

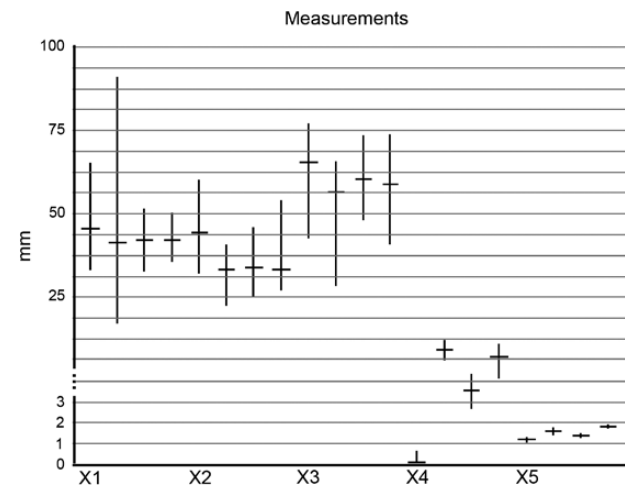


Figure 3. Morphological measurements for five floral traits considering mean and intervals. From left to right, Pa_IS, Pe_IS, CO1 and CO2, respectively. X1 – corolla tube length, X2 – corolla diameter, X3 – total corolla length, X4 – length of the exserted segment of anthers and pistils (from the edge of the corolla to the tip) and X5 – distance between anthers of the longest and middle-length stamens – didynamous stamens.

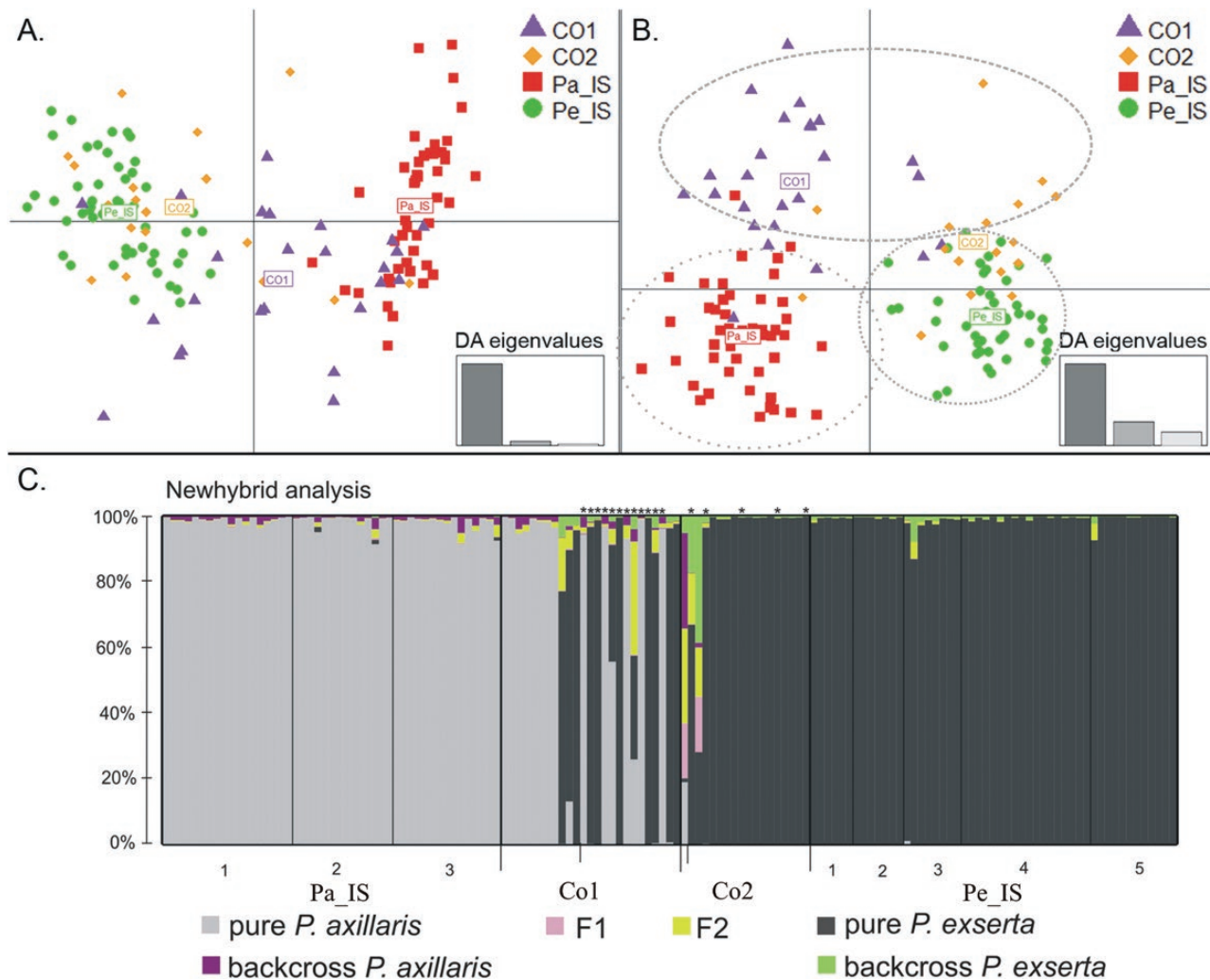


Figure 4. Clustering of individuals: A, DAPC scatterplot performed based on five floral traits considering four groups; B, DAPC scatterplot performed based on SSR genotypes for four populations and C, PPs of NEWHYBRIDS of individuals of *P. axillaris* and *P. exserta* belonging to six classes (pure parental species F_1 or F_2 hybrids and backcrosses). Vertical black lines separate collection sites. *Atypical floral colour a priori classified based on visual assessment.

classification. We considered all morphological variables as not correlated.

GENETIC DIVERSITY AND DIFFERENTIATION

Collection sites and some loci exhibited a significant departure from HWE and positive F_{IS} values. Analysis considering only isolated sites of each species showed that *P. axillaris* had greater allele richness and more private alleles than *P. exserta* (29 alleles in *P. axillaris* and eight alleles in *P. exserta*). Private alleles were also observed among plants collected in CO1 (Table 3), and for almost all loci the most frequent allele was different for each collection site (data not shown). *Petunia axillaris* featured patterns of allele richness similar to co-occurrence sites, whereas *P. exserta*

showed the lowest values (Table 3; $P < 0.05$ Student's t -test).

The AMOVA analysis demonstrated significant variation among the four populations ($\sim 20\%$; $P < 0.005$). The variance estimated between species, considering only isolated sites for each species, was $c. 21\%$ ($P < 0.001$). The pairwise genetic distance (as measured by F_{ST}) ranged from 0.109 (Pe_IS vs. CO2) to 0.243 (Pe_IS vs. Pa_IS), respectively (Table 4).

DAPC analysis demonstrated there are three main groups: the *P. axillaris* cluster that was composed of all individuals from isolated sites and those from contact zones that presented white flowers and were collected outside shelters (termed the PA genetic lineage in Table S1) and correspond to the left quadrant (Fig. 4B). The majority of individuals of *P. exserta* from

Table 3. Genetic polymorphisms based on nuclear microsatellites

Site/loci		PM177	PM188	PM195	PM21	PM183	PM8	PM167	PM173	Average
Pa_IS	<i>N</i>	18	8	4	3	13	4	6	7	7.9
	<i>E</i>	9	3	2	1	7	1	2	4	3.6
	<i>R</i>	13.7	7.5	3.6	3.0	10.1	4	5.3	5.2	7.8 [#]
	<i>H_E</i>	0.90	0.83	0.57	0.60	0.87	0.38	0.70	0.52	-
	<i>H_O</i>	0.38*	0.47*	0.23*	0.43*	0.68*	0.38*	0.45*	0.30*	-
	<i>F_{IS}</i>	0.58	0.44	0.60	0.30	0.22	0.41	0.37	0.42	0.42
Pe_IS	<i>N</i>	11	5	4	3	6	2	4	6	5
	<i>E</i>	2	0	2	1	0	0	0	3	1
	<i>R</i>	9.3	3.8	3.0	2.3	5.2	2.0	3.7	5.5	3.8
	<i>H_E</i>	0.87	0.32	0.18	0.42	0.76	0.27	0.59	0.75	-
	<i>H_O</i>	0.18*	0.08*	0.04*	0.10*	0.59*	0.04*	0.16*	0.28*	-
	<i>F_{IS}</i>	0.80	0.75	0.79	0.77	0.23	0.85	0.74	0.64	0.65
CO1	<i>N</i>	10	6	6	4	7	4	7	7	6
	<i>E</i>	1	0	1	0	1	0	1	0	0.5
	<i>R</i>	8.7	5.8	5.8	3.9	6.5	3.9	6.5	6.6	6
	<i>H_E</i>	0.84	0.62	0.78	0.46	0.77	0.70	0.67	0.82	-
	<i>H_O</i>	0.38*	0.12*	0.34*	0.24*	0.60*	0.46*	0.21*	0.36*	-
	<i>F_{IS}</i>	0.56	0.81	0.58	0.48	0.22	0.35	0.70	0.57	0.53
CO2	<i>N</i>	9	5	5	3	5	3	6	5	5.13
	<i>E</i>	1	0	0	0	0	0	0	0	0.13
	<i>R</i>	9	5	5	3	5	3	6	5	5.12
	<i>H_E</i>	0.76	0.63	0.37	0.44	0.72	0.474	0.72	0.64	-
	<i>H_O</i>	0.29*	0.06*	0.29	0.11*	0.35*	0.333	0.50*	0.41*	-
	<i>F_{IS}</i>	0.62	0.92	0.20	0.75	0.52	0.304	0.31	0.37	0.51

N = number of alleles; *E* = number of private alleles; *R* = allele richness; *H_E* = expected heterozygosity; *H_O* = observed heterozygosity; *F_{IS}* = inbreeding coefficient. * = Hardy–Weinberg equilibrium deviation significance per locus after Bonferroni correction at *P* = 0.05; Pa_IS = *P. axillaris* isolated sites; Pe_IS = *P. exserta* isolated sites; CO = contact zones 1 and 2. # *P* < 0.05 Student's *t*-test.

Table 4. Pairwise *F_{ST}* values estimated for the four analysed populations

POP	Pa_IS	CO1	CO2	Pe_IS
Pa_IS	-			
CO1	0.139	-		
CO2	0.206	0.199	-	
Pe_IS	0.243	0.230	0.109	-

All values were significant (*P* < 0.01); Pa_IS = *P. axillaris* isolated sites; Pe_IS = *P. exserta* isolated sites; CO = contact zones 1 and 2.

the isolated sites and almost all individuals from CO2 comprised the second genetic cluster (called the PE1 genetic cluster; lower right quadrant in Fig. 4B). The third cluster grouped almost all individuals with intermediate coloured flowers collected inside or at the edge of shelters from CO1 and CO2 plus a few individuals of *P. exserta* from IS sites (named PE2 genetic cluster in Table S1; the upper right quadrant in Fig. 4B). Low superimposition of individuals from different collection sites was observed (Fig. 4B). DAPC results indicated that there were different patterns

of genetic compounds for the two contact zones: CO2 was closer to *P. exserta*, whereas CO1 showed a more differentiated genetic constitution than CO2 or either parental species.

The NEWHYBRIDS analysis was not highly successful for determining the classes to which putative hybrids belong (Fig. 4C). We did not observe evidence of any individual being an *F₁* hybrid, even those from the co-occurring populations and displaying intermediate coloured flowers. The majority of individuals from the contact zones, independent from morphology, failed to be classified as purebred and were not clearly assigned to any other class. Estimates of recent immigration with BAYESASS indicated that <10% of individuals in each site corresponded to recent immigrants from another locality, and high recent immigration rates were observed from Pe_IS to CO2 (Table 5).

EVOLUTIONARY MODEL SELECTION

Six coalescent models were simulated with the ABC approach (Fig. 1H) and selected through three different methods. Despite certain differences among method results ('standreject' PP = 0.20; 'mnlogistic'

Table 5. Estimates of recent immigration according to BAYESASS analysis

MIGRATION TO	MIGRATION FROM			
	Pa_IS	CO1	CO2	Pe_IS
Pa_IS	0.97 (0.01)	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)
CO1	0.03 (0.02)	0.95 (0.03)	0.01 (0.01)	0.01 (0.01)
CO2	0.02 (0.01)	0.03 (0.02)	0.68 (0.01)	0.27 (0.03)
Pe_IS	0.01 (0.01)	0.01 (0.01)	0.01 (0.01)	0.97 (0.01)

Values in parenthesis – means of posterior distribution of m = migration rate per collection site; values along the diagonal indicate residents.

PP = 0.91; ‘neuralnet’ PP = 0.46), the estimates of PP were higher for M1 than for any other model (Fig. 1H), suggesting that the PE2 cluster is the result of an ancient admixture process between PA and PE1 clusters (Fig. 1H). The scatterplot of the simulated SuSt showed greater differentiation among the models with migration (M2, M3 and M4) and without migration (M1, M5 and M6), as reflected in the low values of PP obtained with the ‘standreject’ algorithm for all tested models (Figs 1H and S1).

Posterior parameter distribution was obtained from 0.001 of the posterior samples (10^3 simulations) of the M1 model with post-rejection adjustments using the ‘neuralnet’ method. Posterior predictive verification showed that simulations using parameter distributions obtained with posterior adjustments under the best-supported model (M1) were able to recover SuSt values better fitted to the observed SuSt as seen in the posterior PCA and in the SuSt distribution plots obtained with the posterior simulations (Figs S2 and S3).

The estimated time of the genetic cluster associated with hybridization between *P. axillaris* and *P. exserta* (Table S3) was *c.* 27 kya (6.7–58.4 kya; 95% CI), which corresponded to the early stages of the divergence between them *c.* 33 kya (13.9–72.3 kya; 95% CI). The intermediate coloured individuals emerged adapted to the same ecological conditions as *P. exserta* inside shelters.

DISCUSSION

Hybridization plays an important role in plant evolution (Abbott, Barton & Good, 2016), and its consequences may differ among taxa, probably reflecting the biological differences between pairs of species, such as speciation time and mode (Payseur & Rieseberg, 2016). Thus, recently diverged species that occur at the same site provide a unique opportunity to study the evolutionary process involved in speciation.

The results of this study demonstrate that strong differentiation in floral traits clearly groups individuals and corresponds to species designation, with intermediate coloured individuals representing a different lineage intermediate between the species. This intermediate lineage identification was possible only combining morphological and genetic data. Previous studies, based only in genetic information (Lorenz-Lemke *et al.*, 2006; Segatto *et al.*, 2014), failed to explain the morphological variation in the absence of strong hybridization. Overall, molecular evidence indicated that the two species are clearly separated based on nuclear microsatellites and the better-supported evolutionary model uncovered that an ancient hybridization process established a new lineage as a polymorphism into the *P. exserta* profile.

Interspecific gene exchange between *Petunia* spp. is rarely documented in nature (Ando *et al.*, 1995; Kokubun *et al.*, 1997), although all species are still capable of producing at least partially fertile interspecific hybrids in controlled crosses (Gerats & Vandebussche, 2005). Moreover, quite a few species have overlapping ranges and, when they are found in sympatry or close to each other, the species show niche species specificities or have different pollinators that prevent frequent hybridization (Stehmann *et al.*, 2009; Fregonezi *et al.*, 2013; Segatto *et al.*, 2017). The differences in the floral characters of *Petunia* spp. have been attributed to adaptations to different pollinators (Dell’Olivo *et al.*, 2011): *P. exserta*, a bird-pollinated species (Lorenz-Lemke *et al.*, 2006) has red flowers that are UV-reflectant and unscented (Sheehan *et al.*, 2016) and an elongated pistil and stamens (Stehmann *et al.*, 2009); *P. axillaris* has UV-absorbing and scented white flowers with short pistils and stamens that are pollinated by hawkmoths (Stuuman *et al.*, 2004; Venail *et al.*, 2010). In fact, extreme differentiations in floral morphology in recently diverged plants are associated with adaptations to specific pollinators, even when substantial gene exchange between these floral morphs results in fertile hybrids (Cronk & Yang, 2016). At the same time, hybrid individuals can attract new

pollinators, differing from those attracted by parental species and/or changing the dynamic of interaction with visitors, which can help to promote homoploid hybrid speciation (Ma, Zhou & Milne, 2016; Marques *et al.*, 2016). The detection of homoploid hybrid speciation is a challenge because no changes in the chromosome number occur and parental species are frequently closely related (Abbott *et al.*, 2013). In this context, it is necessary to analyse reproductive isolation in field coupled with molecular data and studies on pollen DNA barcoding, pollen tube growth, reproductive fitness between parental species and hybrids using artificial pollination can be useful to understand the process (Ma *et al.*, 2016). For *P. exserta* and *P. axillaris*, detailed studies on pollination systems and pollinator biology will be necessary to comprehend how the new *P. exserta* lineage is maintained and whether it could be a speciation event in progress.

Analyses of simulated and empirical data using the ABC framework have proved successful in distinguishing between models of admixture from those of simple divergence with migration even when based on just a few microsatellite loci (Sousa *et al.*, 2012). By comparing different evolutionary scenarios, our genetic data supported that hybridization was involved in the origin of the PE2 lineage and this has happened in early divergence stages of *P. axillaris* and *P. exserta*. This new lineage encompasses the majority of individuals with atypical phenotypes that grow in the CO populations and share the microenvironment with *P. exserta* canonical individuals. These results suggest that hybridization has been involved in the demographic history of *P. exserta* and *P. axillaris*. Our results also indicated that admixture events between the species were rare as the scenarios that included migration were poorly supported and interspecific gene flow estimates were also reduced. For CO2, we observed introgression from *P. axillaris* to *P. exserta* more so than in CO1. In a hybrid swarm among species of *Mandevilla* Lindl., hybridization events have been described as rare but, once they occur, the differences in scent signals emission can facilitate unidirectional interbreeding, increasing the introgression in one direction with hummingbirds as main flower visitors in hybrids and one of the parental species (Pisani *et al.*, 2019). There are few studies about bird-pollinated plants in Neotropics, and general conclusions are not possible, with pre-zygotic barriers being more frequently described (Cuevas, Espino & Marques, 2018) than post-zygotic ones (Nunes *et al.*, 2016). The behaviour of the pollinators and different microenvironments could play an important role in the reproductive isolation between *P. exserta* and *P. axillaris* and could have given rise to PE2 lineage.

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, Lexer & Cozzolino, 2009) that act in a hierarchical order (Dell'Olivo & Kuhlemeier, 2013). *Petunia* is a young group of species endemic to South America that show low genetic differentiation 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; Barros *et al.*, 2015). Microenvironmental isolation should reduce fitness in heterospecific habitats, whereas pollinator specialization might decrease interspecific pollen movement (Baak *et al.*, 2015).

Several investigations have been conducted to aid understanding of the role that ecological divergence plays in the origin of new species (Coyne & Orr, 2004; Nosil, Funk & Ortiz-Barrientos, 2009; Butlin *et al.*, 2014), but it is also clear that, even with gene exchange, reproductive barriers can evolve as a by-product of ecologically based divergent selection (Feder *et al.*, 2005; Cruickshank & Hahn, 2014). In nature, atypical phenotypes, an intermediate morph between *P. axillaris* and *P. exserta* canonical morphologies, are observed only inside shelters sharing the microenvironment with *P. exserta*, which suggests local adaptation to the same environmental conditions. In addition, microsatellite profiles and evolutionary models for these individuals indicated that interspecific hybridization played an important role in the variability of these species.

Studies have suggested that reproductive isolation during the early stages of divergence took place because shifts in pollinator preferences are related to changes in a few extensive genomic regions, whereas the isolation induced by changes in the mating system would be connected with several minor genetic changes (Rieseberg & Willis, 2007). *Petunia axillaris* subsp. *axillaris*, initially described as self-incompatible (Ando, 1996), in the Guaritas region is preferentially inbred, probably as a result of its patchy distribution (isolated populations in sandstone towers) and limited seed dispersal (Turchetto *et al.*, 2015a). Similarly, *P. exserta* shows a high degree of selfing, biparental inbreeding and significantly correlated paternity (Turchetto *et al.*, unpublished data). The mating system as a barrier to interspecific gene exchange has been demonstrated in many taxa from different families (Palma-Silva *et al.*, 2011, 2015; Brys, van Cauwenberghe & Jacquemyn, 2016) as opposed to the common belief. Frequently, self-incompatibility is considered as evolutionarily advantageous because, by preventing selfing, it can increase genetic variability (Streher *et al.*, 2018). In this region, *P. axillaris* and *P. exserta* could use selfing as a strategy to avoid interspecific gene exchange despite being able to cross in co-occurring sites, probably owing to the short spatial distance among individuals of

the two species in these specific towers (CO1 and CO2). Changes in floral biology within species or populations may be part of the polymorphism in a specific trait in a diversified and widely distributed species as *P. axillaris*, but they may also trigger a speciation process (Oliveira *et al.*, 2017).

The PE2 lineage seems to persist over time, even at sites considered isolated and where individuals show morphological traits canonical to *P. exserta*. Seeds obtained from individuals with intermediate corolla colour from CO sites and belonging to the PE2 genetic lineage germinated and produced viable seedlings (Teixeira, UFRGS, pers. comm.), which suggests viability for these individuals at least under controlled conditions. Furthermore, in nature, we observed individuals with intermediate flower colour for more than a decade at the same locations (Turchetto *et al.*, 2015b). This lineage has probably originated from hybridization between *P. axillaris* and *P. exserta* and can persist through inbreeding among CO individuals, which could explain the range in their floral morphology at these sites, and/or through sporadic interspecific crossings followed by multiple generations of backcrosses, especially with *P. exserta* because of physical proximity between intermediate and canonical individuals. As discussed above, the presence of hybrid plants can favour hybridization and introgression through shifts in pollinator biology (Ippolito, Fernandez & Holtsford, 2004) and this could be the case in the contact zones between *P. exserta* and *P. axillaris*.

CONCLUSIONS

In this study, we show that genetic and morphological integrity of *P. exserta* and *P. axillaris* are maintained despite natural hybridization and that introgression can explain, at least in part, the floral morphological polymorphism observed in *P. exserta*. Morphology has played an important role in the species isolation between *P. axillaris* and *P. exserta* because the preferences associated with their specific pollinators, and adaptations to divergent microenvironments have contributed to species differentiation. However, in a number of locations, such as those described herein, the interspecific gene flow and hybridization can form stable hybrid zones. These findings shed light on the role of hybridization between well-established taxa that present divergent floral syndromes and on the evolutionary processes that drive species diversification.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

Table S1. Morphological measurements and flower pictures per individual.

Table S2. Summary statistics used in ABC analyses.

Figure S1. PCA of each coalescent model.

Figure S2. The principal component of the best-supported model in ABC analysis.

Figure S3. Comparisons between observed and simulated summary statistics.

Table S3. Genetic information based on nuclear microsatellites.