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Pollen dispersal and breeding structure in a hawkmoth-pollinated Pampa grasslands species *Petunia axillaris* (Solanaceae)

Caroline Turchetto[1], Jacqueline S. Lima[2], Daniele M. Rodrigues[1], Sandro L. Bonatto[3] and Loreta B. Freitas[1,*]

[1]Laboratory of Molecular Evolution, Department of Genetics, Universidade Federal do Rio Grande do Sul, PO Box 15053, Porto Alegre, Brazil, [2]Laboratory of Genetic Diversity, Universidade Federal de Goiás, PO Box 131, 74001-970 Goiania, GO, Brazil and [3]Laboratory of Genomic and Molecular Biology, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610-001 Porto Alegre, RS, Brazil
** For correspondence. E-mail loreta.freitas@ufrgs.br*

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• **Background and Aims** The evolution of selfing is one of the most common transitions in flowering plants, and this change in mating pattern has important systematic and ecological consequences because it often initiates reproductive isolation and speciation. *Petunia axillaris* (Solanaceae) includes three allopatric subspecies widely distributed in temperate South America that present different degrees of self-compatibity and incompatibility. One of these subspecies is co-distributed with *P. exserta* in a restricted area and presents a complex, not well-understood mating system. Artificial crossing experiments suggest a complex system of mating in this sympatric area. The main aims of this study were to estimate the pollen dispersal distance and to evaluate the breeding structure of *P. axillaris* subsp. *axillaris*, a hawkmoth-pollinated taxon from this sympatric zone.
• **Methods** Pollen dispersal distance was compared with nearest-neighbours distance, and the differentiation in the pollen pool among mother plants was estimated. In addition, the correlation between genetic differentiation and spatial distance among plants was tested. All adult individuals (252) within a space of 2800 m^2 and 15 open-pollinated progeny (285 seedlings) were analysed. Genetic analyses were based on 12 polymorphic microsatellite loci.
• **Key Results** A high proportion of self-pollination was found, indicating a mixed-mating system. The maximum pollen dispersal distance was 1013 m, but most pollination events (96 %) occurred at a distance of 0 m, predominantly in an inbreeding system. Both parents among sampled individuals could be identifed in 60–85 % of the progeny.
• **Conclusions** The results show that most pollen dispersal in the hawkmoth-pollinated *P. axillaris* subsp. *axillaris* occurs within populations and there is a high proportion of inbreeding. This mating system appears to favour species integrity in a secondary contact zone with the congener species *P. exserta*.

Key words: Pollen dispersal, breeding structure, *Petunia axillaris*, *P. exserta*, Solanaceae, Pampas, selfing, inbreeding, genetic structure, microsatellites, hawkmoth pollination, gene flow.

INTRODUCTION

Among the numerous traits that influence life history, those that govern reproduction are particularly influential in facilitating adaptive radiation. Indeed, mating patterns affect key evolutionary processes, including genetic transmission, selection response, speciation and the evolutionary diversification of lineages (Barrett, 2013).

The evolution of selfing is one of the most common transitions in flowering plants (Stebbins, 1957; Baker, 1959); this change in mating pattern has importance in systematics and ecological consequences because the evolution of selfing often initiates the reproductive isolation and speciation (Baker, 1961; Barrett, 1989). The importance of mating system evolution in contributing to reproductive isolation between co-occurring species has recently received considerable attention (e.g. Fishman and Wyatt, 1999; Fishman, 2000; Lowe and Abbott, 2004; Martin and Willis, 2007; Ruhsam *et al*., 2011; Ruhsam, 2013; Brys *et al*., 2014).

Petunia axillaris (Solanaceae) is a historical parent of garden petunias (Sink, 1984), and this species is of particular interest in mating system studies because not all of its allopatric infraspecific taxa have the same status of self (in)-compatibility. Currently, three allopatric subspecies, *P. axillaris* subsp. *axillaris*, *P. axillaris* subsp. *parodii* (Cabrera, 1977) and *P. axillaris* subsp. *subandina* (Ando, 1996), are recognized (hereafter referred to only as *axillaris*, *parodii* and *subandina* subspecies). These subspecies occupy nearly adjacent territories (Ando, 1996) but are morphologically distinguishable from each other based on the size of the corolla limb, length of the corolla tube and condition of the stamen (Ando, 1996; Kokubun *et al*., 2006). These subspecies also present genetic differences (Turchetto *et al*., 2014*a*), and diversification between them can be associated with adaptive selection to ecological factors, such as pollinators and soil composition (Turchetto *et al*., 2014*b*). Although some *Petunia* species are narrowly endemic and are associated with specific phytoecological regions, the

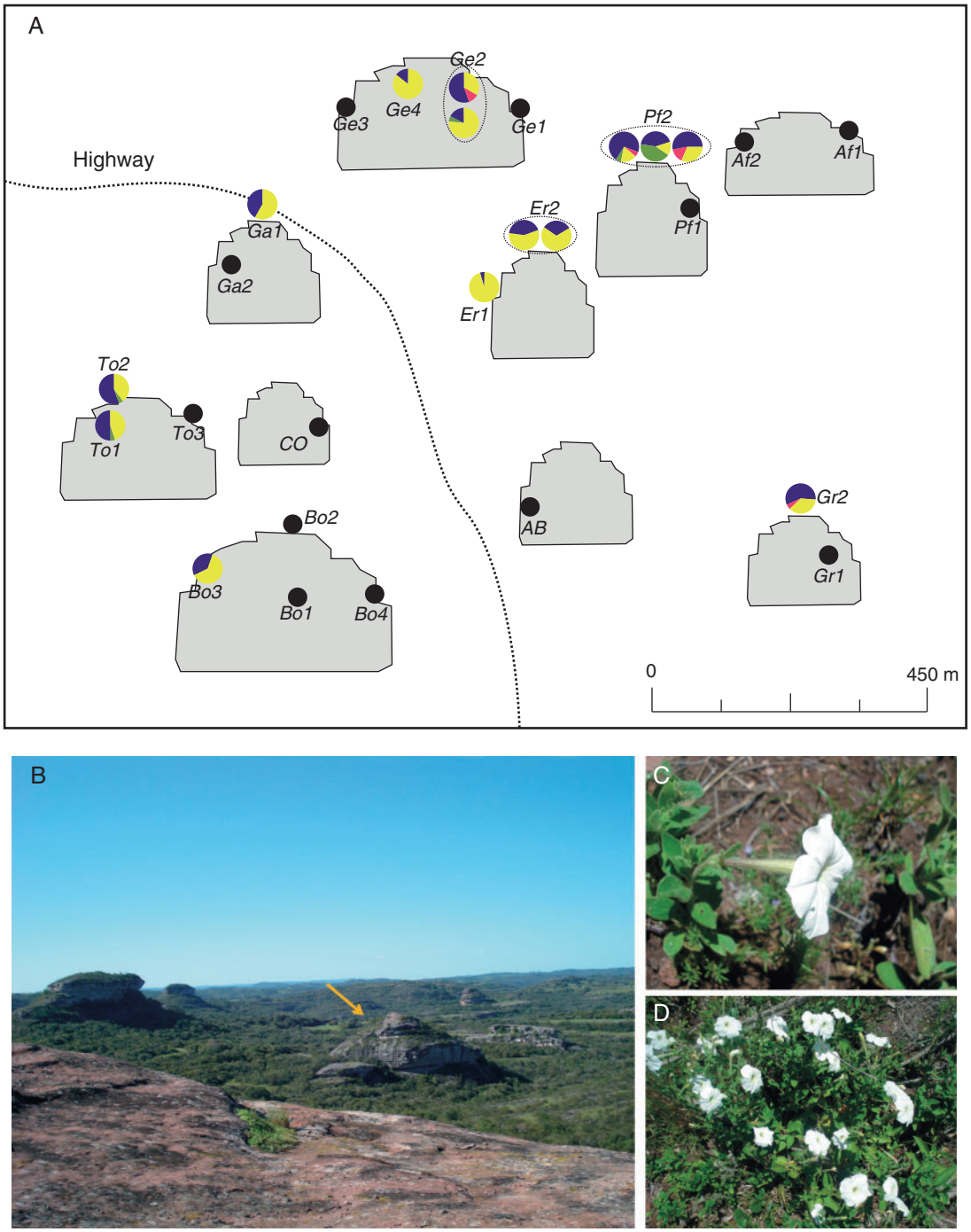

FIG. 1. (A) Schematic representation of the area studied showing ten towers and 23 populations. Each circle represents one population (number of individuals per population according to Table 1). Black circles are populations from which only adult individuals were collected. Coloured circles represent mother plants with the respective percentage of: selfing events (yellow); cross-pollination between individuals from the same population (green); cross-pollination between individuals from different populations (red); and non-attributed paternity considering a 95 % confidence interval (blue). (B) Landscape of the Serra do Sudeste/Guaritas region highlighting sandstone towers. The arrow represents the AB tower in (A). (C) *Petunia axillaris* subsp. *axillaris* corolla flower. (D) *Petunia axillaris* subsp. *axillaris* individual.

P. axillaris complex is widely distributed in temperate South America, occurring throughout the entire Pampas region. These *P. axillaris* populations usually form spatially distributed local patches in which several individuals grow together in a small area (Stehmann *et al.*, 2009).

This species is the only one in the *Petunia* genus that has white flowers (Fig. 1) and is mainly pollinated by hawkmoths (Ando *et al.*, 1995; Venail *et al.*, 2010; Klahre *et al.*, 2011). Autochory, seeds falling close to the mother plant (van der Pijl, 1982), is the rule in *P. axillaris* as well as in other *Petunia*

species (Stehmann *et al*., 2009), and the plastid genome is maternally inherited (Derepas and Dulieu, 1992). Flowering usually occurs during the spring in the Southern Hemisphere (September–December).

Previous studies have suggested that the *parodii* and *subandina* subspecies are self-compatible (SC) (Ando *et al*., 1998; Kokubun *et al*., 2006), whereas the *axillaris* subspecies presents a more complex and not completely understood reproductive system over its entire range of geographic distribution. Initially, this subspecies was described as self-incompatible (SI) (Ando, 1996), but further studies showed that some individuals are SC in some populations in Uruguay, Brazil and Argentina (Ando *et al*., 1998, 2001; Kokubun *et al*., 2006). Moreover, it is interesting that the few analysed populations in the distribution edges of this subspecies in Uruguay and in a sympatric region with *P. exserta* in Brazil present a high number of SC individuals (Kokubun *et al*., 2006).

The *axillaris* subspecies occurs in sympatry with *P. exserta* in a specific region in Brazil named Guaritas (Serra do Sudeste, Caçapava do Sul municipality, Rio Grande do Sul). The Guaritas region is characterized by the presence of sandstone towers on which the *axillaris* subspecies grows only in sunny patches of grasslands; *P. exserta*, an SC bird-pollinated species that presents red flowers, inhabits small caves inside the towers. Although natural hybridization has been described between these species (Lorenz-Lemke *et al*., 2006; Segatto *et al*., 2014), putative hybrids were observed only inside the caves, sharing the microenvironment with *P. exserta*.

Here, we evaluate the pollen dispersal distance and breeding structure in subspecies *axillaris* distributed in the Serra do Sudeste region. Our working hypothesis is that pollen dispersal follows the distribution of nearest neighbours due to vegetation patch distribution. We also hypothesize that *P. axillaris* has a high capacity for autonomous selfing that may function as a protective mechanism against heterospecific pollen deposition and hybrid seed formation with its sister species.

MATERIALS AND METHODS

Study site and sampling

We analysed 23 patches (hereafter called populations) of the *Petunia axillaris* subsp. *axillaris* from the Guaritas region distributed among ten towers (Fig. 1). Most of the towers present more than two different *axillaris* populations in different rock faces. We used a Global Positioning System (GPS) to obtain geographic co-ordinates for each population. The number of adult individuals in each population varied from one to 45 (Table 1).

All adult individuals (252) of *axillaris* were mapped, and leaves were collected for DNA extraction. Fifteen individuals were randomly selected (Fig. 1) for sampling open-pollinated progeny arrays. We collected 1–3 fruits per mother plant and cultivated the seeds in a growth chamber with controlled temperature and luminosity. We collected 16–21 seedlings per mother plant, for a total of 285 seedlings. We used the CTAB (cetyltrimethylammonium bromide) protocol (Roy *et al*., 1992) to extract genomic DNA from all individuals (537 individuals including adults and seedlings).

Table 1. *Origin of the* Petunia axillaris *subsp*. axillaris *populations analysed in this study*

Population	Tower	*n*	Geographic co-ordinate
CO1	CO	1	30·83664115 °S, 53·50501430 °W
TO1 (2)	TO	12	30·83749722 °S, 53·50689722 °W
TO2	TO	8	30·83765833 °S, 53·50696944 °W
TO3	TO	14	30·8372222 °S, 53·50666666 °W
GA1 (1)	GA	8	30·83425414 °S, 53·50482285 °W
GA2	GA	29	30·83439161 °S, 53·50526207 °W
BO1	BO	8	30·83861181 °S, 53·50449571 °W
BO2	BO	5	30·83897022 °S, 53·50325862 °W
BO3 (1)	BO	18	30·83972652 °S, 53·50529165 °W
BO4	BO	4	30·83823588 °S, 53·50266862 °W
AF1	AF	3	30·83102912 °S, 53·49563537 °W
AF2	AF	1	30·83228498 °S, 53·49622042 °W
PF1	PF	1	30·8328853 °S, 53·49840659 °W
PF2 (3)	PF	25	30·83286132 °S, 53·49832789 °W
GR1	GR	8	30·83822624 °S, 53·49508392 °W
GR2 (1)	GR	27	30·83837133 °S, 53·49511493 °W
ER1 (1)	ER	4	30·83427677 °S, 53·50045136 °W
ER2 (3)	ER	45	30·83429396 °S, 53·50020350 °W
GE1	GE	7	30·83037424 °S 53·50211609 °W
GE2 (2)	GE	7	30·83078823 °S, 53·50228255 °W
GE3	GE	7	30·83218909 °S, 53·50368635 °W
GE4 (1)	GE	5	30·83125305 °S, 53·50327682 °W
AB	AB	5	30·83726434 °S, 53·49937655 °W
Total	10	252	

Populations in bold are those from which mother plants originated, with the number of mother plants collected per population in parentheses.

Characterization of microsatellite loci

The adults and progeny were genotyped using 12 microsatellite loci named PM188, PM8, PM21, PM195, PM177, PM167, PM192, PM173, PM88, PM191, PM101 and PM184 (Bossolini *et al*., 2011). The polymerase chain reactions (PCRs) were conducted in a final volume of 10 µL containing approx. 10 ng of genomic DNA as template, 200 µM of each dNTP (Invitrogen, Carlsbad, CA, USA), 1·7 pmol of each fluorescently labelled M13(–21) primer, 3·5 pmol of reverse primer, 0·35 pmol of forward primer with a 5′-M13(–21) tail, 2·0 mM $MgCl_2$ (Invitrogen), 0·5 U of Platinum Taq DNA polymerase (Invitrogen) and 1× Platinum Taq reaction buffer (Invitrogen). The PCR conditions were as follow: an initial denaturation at 96 °C for 3 min; 32 cycles of 96 °C for 15 s, 50–52 °C for 30 s and 72 °C for 1 min; and a final extension cycle at 72 °C for 7 min. The forward primers were FAM, NED, or HEX labelled. The DNA fragments were denatured and size-fractionated using capillary electrophoresis on a MegaBACE 1000 automated sequencer (GE Healthcare Biosciences, Pittsburgh, PA, USA) with a GeneTab-500 internal size ladder (GE Healthcare). The manufacturer's software was used to determine the alleles. The primer sequences, repeat motif, fragment size range and chromosome localization are described in Bossolini *et al*. (2011) and are available at the website http://www.botany.unibe.ch/deve/caps/ssrlist.html.

We used Micro-Checker software (Oosterhout *et al*., 2004; http://www.microchecker.hull.ac.uk/) to estimate genotyping errors due to stutter bands, allele dropout and null alleles, and FSTAT 2.9.3.2 software (Goudet, 2002; http://www2.unil.ch/popgen/softwares/fstat.htm) to obtain the number of alleles per

locus and inbreeding coefficient (F_{IS}). We obtained the observed (H_O) and expected (H_E) heterozygosity under Hardy–Weinberg equilibrium (after Bonferroni's correction), as performed in ARLEQUIN 3.5.1.2 software (Excoffier and Lischer, 2010), and also tested for deviation from linkage equilibrium for all loci (Goudet *et al*., 1996) with Bonferroni's correction. All these analyses and estimates were performed considering all 252 adult individuals. In addition, these same summary statistics were also performed in ARLEQUIN and FSTAT for each one of the 23 sampled populations.

Genetic identity (*I*; Chakravarati and Li, 1983) and paternity exclusion (*Q*; Weir, 1996) probabilities were estimated for each locus, and paternity exclusion {*QC* 1/4 1[*P*(1*Q i*)]} and genetic identity (*IC* 1/4 *PI i*) combined probabilities were estimated for the overall loci using the IDENTITY 1.0 software (Wagner and Sefc, 1999).

Population structure

We evaluated the population structure based on the 252 adult individuals sampled in Guaritas. We implemented analyses of molecular variance (AMOVA; Excoffier *et al*., 1992) in ARLEQUIN among the 23 populations and among the ten towers, and computed the pairwise estimators of F_{ST} between the pairs of population or towers to measure the level of differentiation between them. The significance was tested using 10 000 permutations. We also used a Bayesian clustering approach, as implemented in STRUCTURE 2.3 (Pritchard *et al*., 2000), to infer the population structure of adult individuals. The number of groups (*K*) was evaluated from 1 to 26, with ten independent runs per *K*-value. Each run was performed using $2{\cdot}5 \times 10^5$ burn-in periods and 10^6 Markov chain Monte Carlo (MCMC) repetitions after burn-in was used for population clustering without prior information under an admixture model and assuming correlated allele frequencies (Falush *et al*., 2003). The optimal *K*-value was identified using the maximum value of ΔK (Evanno *et al*., 2005), as implemented in STRUCTURE HARVESTER 0.6.93 (Earl and von Holdt, 2012). We used CLUMPP 1.1.2 to summarize the results of the optimal *K*-value based on the pairwise similarity average of individual assignments across runs using Greedy's method and the G' statistic (Jakobsson and Rosenberg, 2007). We used the DISTRUCT 1.1 program (Rosenberg, 2004) to visualize the STRUCTURE results after processing with CLUMPP.

To verify whether there are significant correlations between F_{ST} and geographical distance, we performed a Mantel test between the pairwise F_{ST} and spatial distance among the 23 populations as well among as the ten towers.

Breeding structure and pollen dispersal

Fifteen open-pollinated progenies were analysed, with 16–21 individuals per mother plant, for a total of 285 seedlings. Before performing paternity analyses, mismatching between the mother plant and each offspring was visually inspected. All genotyped individuals were included because we did not find exclusion of any mother.

We used the genotypes of the progeny arrays to estimate mating system parameters in the software MLTR 3.4 (Ritland, 2002, 2004). We used all sampled families under the mixed mating model of Ritland and Jain (1981) and Ritland (1989) and all 12 loci in the analysis. We also calculated multilocus (t_m) and single locus (t_s) outcrossing rates. The difference $t_m - t_s$ can be used to estimate biparental inbreeding (Ritland, 2002); under biparental inbreeding, the magnitude of the difference $t_m - t_s$ should be positive, as single locus estimates of outcrossing rates will include apparent selfing due to mating between relatives. We also calculated the correlation of paternity estimated for a single locus (r_{ps}) or multiple loci (r_{pm}); the difference $r_{ps} - r_{pm}$ can be employed to indicate whether outcrossed mating within a progeny array occurs between related males. The correlation of selfing among families (r_s) was also estimated. For instance, a positive $r_{ps} - r_{pm}$ may occur when the population sub-structure results in genetic similarity among male parents, whereas a negative value indicates biparental inbreeding (Ritland, 2002). All parameters of the model were estimated via the Expectation–Maximization method, and the pollen allele frequencies were assumed to be equivalent to those of the ovules. The results were subjected to 1000 bootstraps using families as the resampling unit to assess significance using 95 % confidence intervals.

To identify the most likely pollen donor and to determine the mating structure and pollen dispersal distance, we used the paternity assignment test approach (Marshall *et al*., 1998). The assignment test was performed using the maximum likelihood-based method implemented in CERVUS 3.0.6 software (Kalinowski *et al*., 2007; http://helios.bto.ed.ac.uk/evolgen). The results were based on the multilocus genotypes of 285 seedlings and 252 reproductive individuals of the 23 populations. The most likely parents and parent pairs were determined by the Δ statistic (Marshall *et al*., 1998) using the allele frequencies of the adults as a reference. The significance of Δ (critical Δ) was determined through paternity tests simulated in CERVUS. An individual with the highest calculated Δ value was accepted as the father of a seed if the difference between its LOD score (logarithm of likelihood ratios) and the second most likely candidate's LOD score was greater than the critical Δ. We used the following parameters for simulations: 10 000 repetitions; 0·9262 proportion of loci; 95 % (strict) and 85 % (relaxed) confidence levels; and 252 sampled individuals (all adult individuals in the plot) as pollen donor candidates for each mother plant. We considered 90 % of the parents in the area to be sampled and 1 % genotyping error. The pollen dispersal distance was obtained based on the pairwise distance between mother plant and pollen donator estimated using the geographic co-ordinates of each patch. The mean and variance of distance of pollen dispersal were generated, and the effective distance of pollen dispersal was compared with the distance among all adult individuals using the Kolmogorov–Smirnov test (Sokal and Rohlf, 1995).

In addition, to estimate the differentiation of allele frequencies among the sampled pollen pool by mother plants in the populations (Φ_{FT}; Austerlitz and Smouse, 2001, 2002) and the effective pollen donor density (d_e), we used TWOGENER software (Austerlitz and Smouse, 2001, 2002) as part of the POLDIST package (Robledo-Arnuncio *et al*., 2007). Due to missing data for the mother plants, only ten loci were used in these analyses. A Mantel test was performed between the pairwise Φ_{FT} and spatial distance matrices to test the hypothesis of

differentiation increasing with distance. All Mantel tests were performed using the software SAM 4.0 (Rangel *et al*., 2010).

Spatial genetic structure

Spatial genetic structure in *P. axillaris* populations was evaluated by a spatial autocorrelation analysis performed to verify kinship structure in all adult individuals and estimate seed dispersal using the Nason estimator F_{ij} (Loiselle *et al*., 1995). We computed F_{ij} values between all pairs of adult individuals using SPAGeDI 1.2 (Hardy and Vekemans, 2002), and these values were regressed onto the natural logarithm of the spatial distance between individuals to test for kinship structure. The multilocus F_{ij} average was computed for ten distance classes that were defined to keep the number of pairwise comparisons within each distance interval approximately constant. Permutation tests (10 000 permutations) were used to verify the deviation of the observed kinship for each distance class from the null expectation and of regression. Standard errors (s.e.) over loci were estimated by Jackknife.

The strength of the spatial genetic structure was quantified using the parameter $S_p = b/(F_1 - 1)$, where F_1 is the average kinship coefficient among individuals of the first distance class and b is the slope of the regression (for details, see Vekemans and Hardy, 2004).

RESULTS

Characterization of microsatellite loci

The characterization of loci was based on adult individuals. All pairs of loci were in linkage equilibrium (almost all $P < 0{\cdot}001$, Bonferroni's adjusted value for a nominal level of 5 %), and most of the 12 microsatellite loci displayed high levels of polymorphism and diversity. We detected 119 alleles among the adult individuals (Table 2). For all loci, the observed heterozygosity (H_O) was lower than the expected (H_E), showing a deficit of heterozygotes in relation to the Hardy–Weinberg principle ($P < 0{\cdot}005$). For all loci, the inbreeding coefficient (F_{IS}) was positive but not significant (Table 2). Nevertheless, the high combined paternity exclusion probability ($QC = 0{\cdot}9997$) and the low combined probability of identity ($IC = 1{\cdot}6 \times 10^{-11}$) showed that this locus set is suitable for parentage analyses (Table 2). When we performed this same analysis on the 23 populations, we observed that only some loci and populations were not under Hardy–Weinberg equilibrium, showing a deficit of heterozygotes. Similarly, the inbreeding coefficient results for the populations showed positive but not significant values (see Supplementary Data Table S1).

TABLE 2. *Characterization of 12 microsatellite loci of* Petunia axillaris *subsp*. axillaris *based on 252 adult individuals sampled in the Guaritas region, Serra do Sudeste, Rio Grande do Sul/Brazil*

Locus	A	H_E	H_O	F_{IS}	Q	I
PM188	9	0·787	0·484	0·385	0·602	0·070
PM8	6	0·573	0·312	0·456	0·353	0·220
PM21	7	0·611	0·313	0·487	0·341	0·222
PM195	7	0·543	0·236	0·566	0·270	0·290
PM177	24	0·906	0·402	0·556	0·812	0·015
PM167	11	0·816	0·520	0·363	0·649	0·054
PM192	12	0·767	0·425	0·447	0·579	0·080
PM173	17	0·557	0·321	0·424	0·363	0·219
PM88	10	0·582	0·183	0·686	0·374	0·205
PM191	6	0·590	0·322	0·455	0·360	0·209
PM101	6	0·756	0·394	0·479	0·535	0·098
PM184	4	0·599	0·164	0·726	0·309	0·244
Overall		0·6739		0·496	$QC = 0{\cdot}9997$	$IC = 1{\cdot}564 \times 10^{-11}$

A, number of alleles; F_{IS}, inbreeding coefficient (all values were not significant, $P > 0{\cdot}004$, Bonferroni's adjusted P-value for a nominal level of 5 %); H_E, expected heterozygosity; H_O, observed heterozygosity (all values were significant, $P < 0{\cdot}005$, Bonferroni's adjusted P-value for a nominal level of 5 %); I, probability of genetic identity; IC, combined probability of genetic identity; Q, probability of paternity exclusion; QC, combined probability of paternity exclusion.

Population structure

The AMOVA analysis revealed that 17 % of the genetic variation ($P < 0{\cdot}001$) was distributed among the 23 populations, whereas 83 % ($P < 0{\cdot}001$) was within the populations. When we considered each tower, the AMOVA analysis also showed the highest fraction of genetic variation within populations (82 %; $P < 0{\cdot}001$) rather than among towers (3·5 %; $P < 0{\cdot}001$). We also measured the level of differentiation among the 23 populations by considering adult individuals computing pairwise estimators of F_{ST}. The F_{ST} values showed significant differentiation for most comparisons (Supplementary Data Table S2). Note that the highest F_{ST} value was found in the comparisons between BO3 vs. GA1 (0·376) and BO3 vs. PF2 (0·303); these three populations grow on the top of three different towers (Fig. 1).

A Mantel test based on correlations between F_{ST} values and geographic distances showed no significant values when we considered all 23 populations ($R^2 = 0{\cdot}003$; $P = 0{\cdot}30$) or when each tower was considered as a population ($R^2 = 0{\cdot}022$; $P > 0{\cdot}50$).

In the Bayesian clustering approach to infer the adult individual population structure, we observed that the best inferred number of clusters was $K = 3$ (Fig. 2). We also observed that the majority of individuals of each population and the majority of populations of each tower were grouped preferentially in one or another cluster (e.g. CO, TO and GA towers in the blue cluster; AF, PF and GR towers in the yellow cluster; ER, AB and GE towers in the orange cluster; Figs 1 and 2). However, a few individuals in some populations appeared as migrants from other populations, even in more distant towers.

Breeding structure and pollen dispersal

The mating parameters estimated by the MLTR analysis are summarized in Table 3. The multilocus estimates of outcrossing rates (t_m) were 0·619 (± 0·069). However, the single locus estimates of outcrossing rates (t_s) were somewhat lower, 0·222 (± 0·033), and hence the difference between t_m and t_s was 0·397 (± 0·0549). The latter relatively high values of $t_m - t_s$ may have been influenced by consanguineous mating, indicating biparental inbreeding. The negative value of $r_{ps} - r_{pm}$ (Table 3)

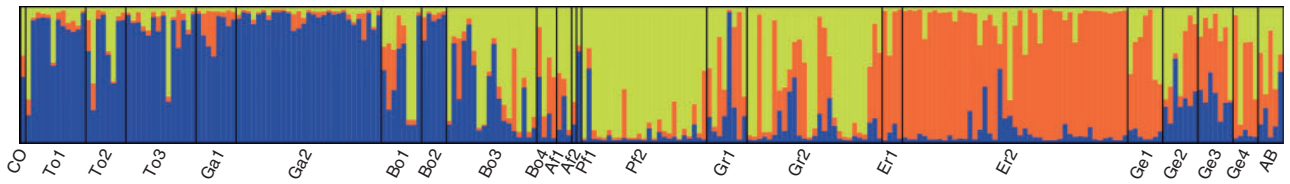

FIG. 2. STRUCTURE bar plot under an admixture coefficients model based on 12 microsatellite loci and 23 populations of *Petunia axillaris* subsp. *axillaris*. Bars represent individuals, and black vertical lines represent each population; different colours indicate $K = 3$ genetic components representing individual membership of one of the genetic clusters.

TABLE 3. *Inbreeding and mating system parameters of* Petunia axillaris *subsp.* axillaris *estimated from 15 families by MLTR analysis*

Parameters	Estimate (s.d.)
Parental F	0·141 (0·049)
Multilocus t (t_m)	0·619 (0·069)
Single locus (t_s)	0·222 (0·033)
Difference ($t_m - t_s$)	0·397 (0·055)
Correlation of paternity singlelocus (r_{ps})	0·237 (0·096)
Correlation of paternity multilocus (r_{pm})	0·907 (0·070)
Difference ($r_{ps} - r_{pm}$)	–1·906 (0·076)
Correlation of selfing among families (r_s)	0·386 (0·067)

also indicated biparental inbreeding. The high multilocus correlation of paternity was in agreement with CERVUS results (60 % of the offspring were assigned with only one father candidate) and indicates that most progeny were full sibs (Table 3). The correlation of selfing among families (r_s) was 0·386 (± 0·067), and this value indicates that the events of fertilization are not structured among families.

In the paternity assignment analysis, when we considered the strict confidence interval (95 %, critical value $\Delta = 2{\cdot}04$), 60 % of the offspring were assigned with only one father candidate from the sampled individuals. This percentage was increased to 85 % when we considered the relaxed confidence interval (85 %, critical value $\Delta = 0{\cdot}18$). However, paternity was still unknown for 15 % of the progeny when considering all adults sampled in Guaritas. The proportion of self-pollination was higher than outcrossing, with only 13 % of progeny assigned as a result of outcrossing with a strict confidence interval and 21 % when the relaxed confidence interval was considered.

The greatest pollen dispersion distance observed was 1013 m between towers BO3 and PF2, with both populations located on the top of the towers.

The distance among the distribution of adult individuals was significantly different from the distribution of the pollen dispersal distance (Kolmogorov–Smirnov test, $P < 0{\cdot}001$). The mean distance among adult individuals was 649·41 m (s.d. = 271·07 m), and most individuals (73 %) were at a distance of <700 m from each other. The mean pollen dispersal distance was 27·46 m (s.d. = 141·34), and most pollen dispersal (96 %) occurred at a distance of 0 m (Fig. 3).

A high differentiation in the pollen pool received by each mother plant was observed: the pairwise Φ_{FT} ranged from 0·136 to 0·681, and the global Φ_{FT} was 0·434 (according to TWOGENER results). However, a Mantel test showed no significant correlation between the pollen pool of mother plants

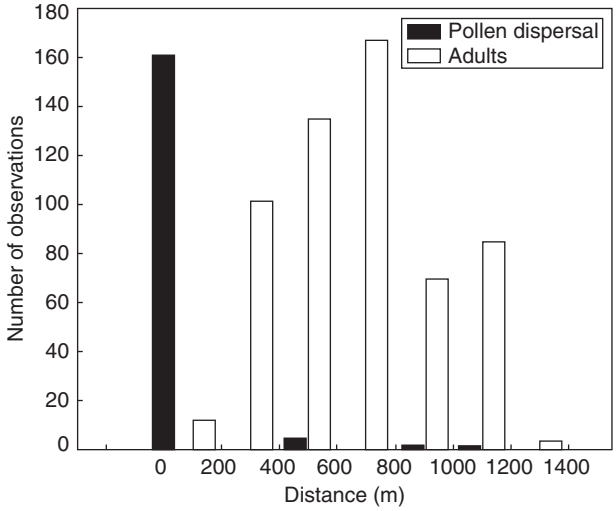

FIG. 3. Pairwise distance distribution of individual adults (white bars, right scale) and pollen dispersal distribution based on the distance between each pollen donor assigned by CERVUS (black bars, left scale).

and the pairwise spatial distance ($R^2 = 0{\cdot}037$; $P = 0{\cdot}296$). The effective pollen donor density (d_e) was 0·00153 m^{-2}.

Spatial genetic structure

An autocorrelation analysis showed a cline pattern with a significant spatial autocorrelation up to 400 m ($P < 0{\cdot}001$). Kinship coefficients were slightly related to the logarithm of the distance class ($b = -0{\cdot}0321$; $R^2 = 0{\cdot}0354$; $P < 0{\cdot}001$; Fig. 4). The spatial genetic structure (S_p) was 0·037.

DISCUSSION

The genetic diversity structure and distribution found within and among populations is primarily affected by gene flow (Wright, 1940; Slatkin, 1985). In animal-pollinated species, pollen presentation and behavioural differences of pollinators and their geographic dispersion capacity determine pollen carryover and thus the distances over which the pollen is dispersed (Barrett and Harder, 1996; Barrett, 2003). Ecological factors such as population spatial distribution, density and flowering phenology may also affect the foraging behaviour of pollinators and, in turn, the distance of pollen dispersal (Handel, 1983; Ghazoul, 2005). In mass-flowering species, with a high

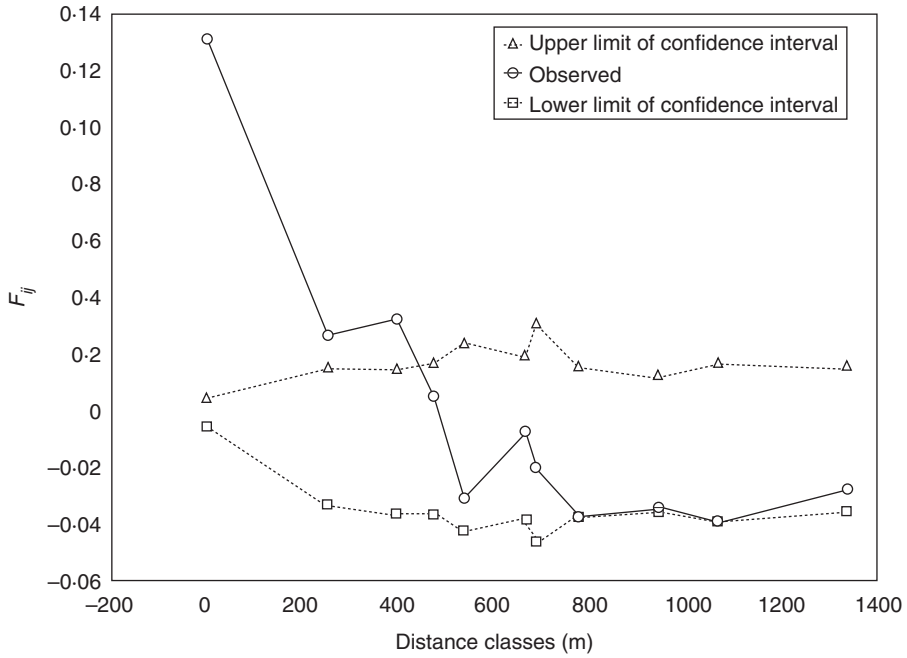

FIG. 4. Relationship between kinship (F_{ij}) and distance classes of adult individuals of *Petunia axillaris* subsp. *axillaris* with the respective 95 % confidence intervals.

synchrony of flowering in neighbouring plants, the pollen dispersal distance may be shorter because of the high proportion of pollination among neighbours, but the asynchrony of flowering of neighbouring plants may result in a longer distance of pollen dispersal (Augspurger, 1980; White and Boshier, 2000). Furthermore, species with limited dispersal and spatially structured populations may have reduced genetic neighbourhoods, implying an increase in mating between relatives (e.g. Carrillo-Angeles *et al*., 2011).

This study was conducted in 23 populations of the *P. axillaris* subsp. *axillaris* from Serra do Sudeste, belonging to the Pampas region. The vegetation structure in the Pampas is very diverse as a consequence of several factors such as climate, soil and topography, which are variable across the region, and due to anthropic vegetation management (Boldrini, 2009).

Rambo (1956) stated that it was not be possible to make a detailed description of all landscapes from Serra do Sudeste, which often diverge from the rest of the Pampa by presenting a savannah vegetation (trees and shrubs) on shallow soils. The region has undulations ranging from 150 to 500 m, and it is characterized by the presence of sandstone towers in an approx. 30 km^2 region called Guaritas. Interestingly, in this region, the *axillaris* subspecies only grows in patches of grasslands on the towers and does not occur among towers (Fig. 1). The individuals are found in relatively large spots made up of several individuals, and this spatial arrangement could be responsible for the major proportion of cross-pollination that has been found between individuals from the same population, with an overall low pollen dispersal distance (96 %). Selfing individuals often live in different environments compared with their outcrossing progenitors. This observation is usually interpreted as being a function of invasion demography. As only a few individuals establish in new habitats, selfing or biparental inbreeding is initially a necessity (Kamran-Disfani and Agrawal, 2014). In the case of the *axillaris* subspecies, this could be due to a combination of the patchy occurrence of suitable habitats and limited seed dispersal, resulting in the clustering of related individuals because Serra do Sudeste may be considered as a secondary colonization event in the *Petunia* genus (Reck-Kortmann *et al*., 2014).

Our results showed a mixed mating system for the *axillaris* subspecies, with a high proportion of SC in all analysed populations. Moreover, we observed that most cross-pollination occurs within patches ($t_m - t_s = 0{\cdot}397$), suggesting biparental inbreeding, a result in accordance with the population genetic structure observed in adult individuals (Fig. 2). This preferential selfing status suggests a more complex or flexible mating system for this subspecies than previously proposed (Ando *et al*., 1998; Kokubun *et al*., 2006). When pollen dispersal occurs between patches, it can be at long distances (at least 1063 m). However, most pollination events occurred at distances of 0 m. Gleiser *et al*. (2014) analysed one population of the *axillaris* subspecies from Uruguay, revealing that individuals were strictly outcrossing, with the most successful pollination occurring preferentially among neighbours.

In our study, when cross-pollination involved individuals from different populations, it occurred in populations at the top of the towers. On the top of towers, we found the majority of the 15 % individuals to which we could not assign a father among the sampled adult individuals. Due to the longer distance pollen dispersal found among these towers and their spatial distribution (in the periphery of the sampled area), a plausible explanation for this lack of assignment is the occurrence of gene flow at a greater distance than expected in this study. This is congruent with the MLTR results, which showed

a higher proportion of cross-pollination (60 %) than paternity assignment estimated in the CERVUS analysis.

The flowers of *P. axillaris* present characteristics that suggest that they are preferentially hawkmoth pollinated (Ando *et al.*, 2001; Hoballah *et al.*, 2005), though several other floral visitors were found that could also act as effective pollinators for this species (Hoballah *et al.*, 2007; Dell'Olivo *et al.*, 2011). Moreover, hawkmoths are powerful flyers and have the ability to disperse broadly and visit widely spaced plants, with some being recaptured in mark–release studies (Powell and Brown, 1990) at several kilometres from the site of origin. Combined with their learning behaviour, hawkmoth pollinators can enhance gene flow between fragmented populations of specialized plants (Martins and Johnson, 2007, 2009, 2013).

The *axillaris* subspecies is primarily SI. Ando *et al.* (1998) surveyed >100 natural populations from Uruguay and found that most of them were composed of virtually all SI individuals. Only a few populations encompassed some SC individuals in addition to the prevalent SI ones, and these populations were referred to as mixed populations. Tsukamoto *et al.* (1999) studied the cause of breakdown of self-incompatibility in these individuals from Uruguay and showed that an S-haplotype carried by three SC individuals was not functional in the style but was functional in the pollen. Tsukamoto *et al.* (2003) later suggested that a modifier locus, unlinked to the S-locus, specifically suppresses the expression of an S-RNase gene in the same three SC plants, explaining the reduced self-incompatibility.

The evolution of self-compatibility due to the loss of self-incompatibility is regarded as one of the most frequent transitions in flowering plants and is influenced by biotic or abiotic factors that affect seed and pollen dispersal, resulting in the rise of subpopulation structures (Griffin and Eckert, 2003). In this study, we found high self-pollination in all the *axillaris* subspecies populations. Our findings may also be related to the scenarios of speciation between *P. axillaris* and its sister species *P. exserta*, for which natural hybridization events have been suggested (Lorenz-Lemke *et al.*, 2006; Segatto *et al.*, 2014). The transition to selfing in *P. axillaris* in the Guaritas region may constitute a reproductive barrier that is primarily responsible for preventing hybridization (pre-zygotic barrier) with *P. exserta*. Moreover, the characteristics of *P. axillaris* floral display (positional proximity between anthers and stigma) can result in a higher capacity for autonomous selfing, which could function as an efficient barrier to counterbalance the higher risk for interspecific hybrid mating.

Transitions in plant reproductive systems involve changes in reproductive traits driven largely by natural selection. The traits initially appear within populations and, if adaptive, can spread to survive numerous speciation events and ultimately characterize entire lineages, as with wind pollination evolution (Friedman and Barrett, 2008). Alternatively, some transitions appear repeatedly but are ephemeral (Igic and Busch, 2013). Populations with mixed mating often possess standing genetic variation for traits promoting outcrossing, for example herkogamy, the spatial separation of anthers and stigmas (Shore and Barrett, 1990); however, there is no evidence that they exhibit reduced inbreeding depression compared with predominantly outcrossing species (Winn *et al.*, 2011).

Differences in mating systems between co-occurring plant species can be expected to shape pre-zygotic barriers and therefore have important effects on the direction of heterospecific pollen flow and the extent of hybridization (Brys *et al.*, 2014). For instance, Lowe and Abbott (2004) reported in two recently divergent species of *Senecio* that the predominant self-fertilization in *S. eboracensis* contributed to strong reproductive isolation and ecological differentiation. Martin and Willis (2007) also showed that in *Mimulus nasutus* almost exclusive autogamy offered nearly complete isolation from its close relative *M. guttatus* when growing in sympatry. However, Coyne and Orr (2004) argued that exclusive autogamy differs profoundly from other isolating barriers and is in fact not an isolating barrier because gene flow between individuals of different taxa is as much impeded as gene flow between individuals of the same taxon. In many cases, however, plants are neither exclusive selfers nor obligate outcrossers but show a breeding system that represents an intermediate form between both extremes, so-called mixed-mating species (Goodwillie *et al.*, 2005). This may be the situation for *P. axillaris* from Serra do Sudeste because a previous study demonstrated under experimental conditions that all *Petunia* species preserve intercrossing ability (Watanabe *et al.*, 2001). However, hybrids in nature are rare, despite the lack of intrinsic barriers to crossing in most of these species (Ando *et al.*, 2001; Dell'Olivio *et al.*, 2011).

The finding of low autocorrelation values confirmed previous observations that seed dispersal is restricted and that most siblings grow near the mother plant, with Φ_{FT} showing positive and significant values of kinship in the first distance class quickly decreasing after 0 m. Similar values of genetic spatial structure, measured by the statistic S_p, have been observed for other herbaceous species and for species with gravity-dispersed seeds (Vekemans and Hardy, 2004).

In conclusion, our results showed that the hawkmoth-pollinated *axillaris* subspecies is capable of long-distance pollen dispersal, but most dispersal occurs within populations and a high proportion of inbreeding is observed. A high proportion of self-fertilization occurs in the Serra do Sudeste region sympatric area where the *axillaris* subspecies occurs with its sister species *P. exserta*.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxfordjournals.org and consist of the following. Table S1: parameters of genetic diversity per locus per population. Table S2: pairwise F_{ST} among 23 populations analysed in this study.

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