



Can the reproductive system of a rare and narrowly endemic plant species explain its high genetic diversity?

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ABSTRACT

The reproductive system of flowering plants can be highly variable, affecting their biology, gene flow and genetic variability among populations. *Petunia secreta* is a rare annual endemic species of Pedra do Segredo, located in the municipality of Caçapava do Sul, state of Rio Grande do Sul, Brazil. Although rare, the species possesses a high level of genetic variability. We investigated the reproductive system of *P. secreta*, including fruit production and seed germinability, in order to determine if its reproductive system can explain its genetic diversity. We sampled five populations and conducted five greenhouse hand-pollination treatments: 1) autonomous apomixis; 2) self-pollination; 3) hand self-pollination; 4) geitonogamy; and 5) cross-pollination. We analysed a total of 40 plants, 468 flowers, and 6,500 seeds. Only autonomous apomixis and self-pollination did not produce fruit. No differences in fruit weight were observed among pollination treatments ($P > 0.05$). Seeds of two colours were produced, with no differences in germinability. Considering all plants, populations, and treatments, the average germinability was 73 % (range 9 % to 100 %). These results, along with other previous studies, indicate that the reproductive systems of *P. secreta*, and its large effective population size, can explain its high genetic diversity.

Keywords: endogamy, Pampas, *Petunia*, reproductive success, reproductive system, seed germinability

Introduction

The genetic structure of a plant species is shaped by gene flow within and between populations. A key factor influencing patterns of seed and pollen dispersal is the species' mode of reproduction (Ghazoul 2005). Physical changes, including habitat degradation or fragmentation, can affect both gene flow and reproduction (Aguilar *et al.* 2008) by reducing the abundance and density of plant individuals, thus resulting in a decrease in population size and an increase in spatial isolation between populations (Blambert *et al.* 2016).

Plant reproductive systems vary greatly. At one extreme, obligate cross-fertilization in self-incompatible species

requires a mate and a pollen vector. At the other end of the spectrum, obligate self-fertilization in self-compatible species is less dependent on mates and vectors. As a consequence, a species' reproductive system may influence its genetic diversity (Charlesworth 2006). In the context of worldwide biodiversity loss, the management of a threatened plant species requires an understanding of its life history and reproductive system as key factors determining the species' abundance, distribution, genetic diversity, and persistence. Therefore, management efforts to preserve endangered species should build on both ecological and genetic studies.

Here, we investigated the reproductive system of *Petunia secreta* (Solanaceae), a rare annual herbaceous

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species that is endemic to a small area in the municipality of Caçapava do Sul, Serra do Sudeste, southern Brazil (Fig. 1; Stehmann & Semir 2005). According to the criteria adopted by the International Union for the Conservation of Nature, *P. secreta* is classified as critically endangered (Turchetto *et al.* 2016). Despite this, it is not included on the Brazilian red list of plant species. Within the phylogeny of the genus, the species belongs to a clade characterised by long corolla tubes (Reck-Kortmann *et al.* 2014). It can be subdivided into two different genetic lineages, which correspond to its sites of occurrence (lineage 1, from Pedra do Segredo – see below, and lineage 2, from a roadside location approximately 20 km away; Turchetto *et al.* 2016). Based on its pink corolla, *Petunia secreta* has been described as bee-pollinated (Stehmann & Semir 2005). We have confirmed through field experiments conducted at Pedra do Segredo (DM Rodrigues unpubl. res.). Plants from the roadside location possess the same morphological characteristics, suggesting they are also bee-pollinated, but to date, their pollinators have not been investigated. Although *P. secreta* is a rare and narrow endemic species, which generally grows in small fragmented populations, its genetic variability is high and of ancient origin, especially when compared to other *Petunia* species. Analyses of nuclear genetic markers confirm that the extant populations of *P. secreta* are descended from a single large and stable founder population (Turchetto *et al.* 2016).

Here, we address the following questions: (1) what is the reproductive system of *P. secreta*? (2) Is *P. secreta* totally or partially self-incompatible? (3) Do artificial crossings produce seeds, and how viable are these seeds? (4) Are there any constraints to crosses of plants from different populations or evolutionary lineages of *P. secreta*? The obtained results were analysed in the context of the high genetic diversity previously observed in this rare species.

Materials and methods

Sampling and site of study

The floral morphology of *Petunia secreta* Stehmann & Semir is similar to that of its sister species *P. axillaris* (Stehmann *et al.* 2009), and they can only be distinguished by the colour of their corolla (pink in *P. secreta*; Fig. 2A). The distribution of *P. secreta* is restricted to two isolated locations (Fig. 1) at the northern edge of the Pampas region in Rio Grande do Sul, Brazil. The species was first described at Pedra do Segredo (30°32'S 53°33'W), a sandstone tower complex set amidst savannah vegetation at an altitude of ~300–400 m (RADAMBRASIL Project 2013). At this site, *P. secreta* grows in isolated patches of few individuals. Outside this area, only three groups of individuals have been found,

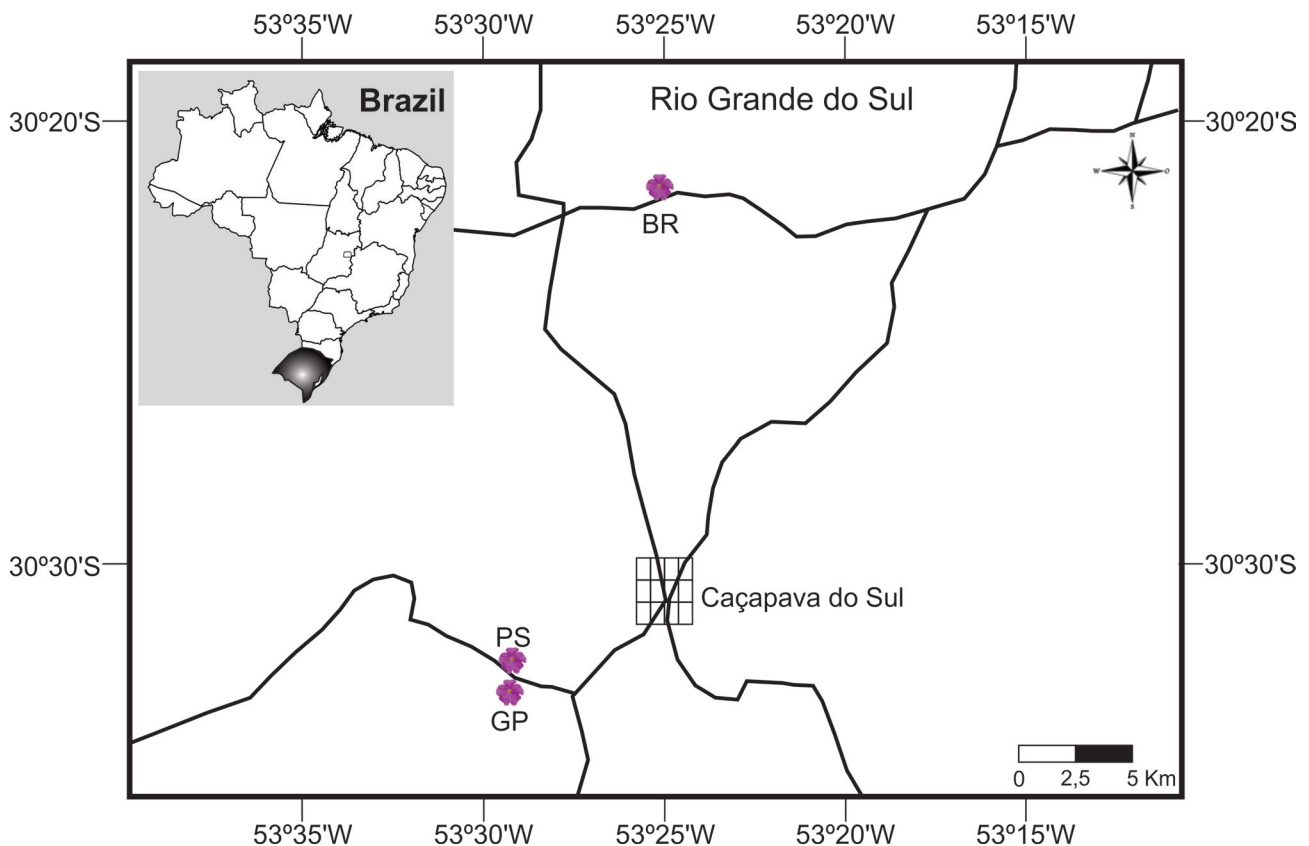


Figure 1. Geographical location and distribution of the studied populations of *Petunia secreta* in the municipality of Caçapava do Sul, Rio Grande do Sul, Brazil.



all in a flat roadside area of open vegetation, ca. 20-30 km from Pedra do Segredo, in the adjacent municipality of Caçapava do Sul (30°21'S 53°28'W). The habitat of the roadside population of *P. secreta* is similar to that of *P. axillaris*, although we did not observe these two species near each other.

To estimate the reproductive parameters in *P. secreta*, we sampled five populations (Tab. 1) at sites representing the species' main habitats (sandstone tower complex and roadside). These five populations were chosen to cover the larger area at the core of the native distribution of this species, as well as both evolutionary lineages (Turchetto *et al.* 2016). Within each of the two main sites, the distance between sampled populations ranged from around 0.2 km (BR163 and BR573) to 1.2 km (PS59 and GP62). The Pedra do Segredo and roadside assemblies are separated by around 20-30 km. For the hand pollination experiments,

we collected mature seeds from five individuals per population in nature during the flowering season of 2014 (September-December). The seeds were then germinated in a growth chamber at 22 °C in a 12-h light:12-h dark cycle. To maximize germination, the seeds were pre-treated with a 100 µM solution of gibberellic acid (GA_4 ; Sigma-Aldrich Co., St. Louis, MO, USA), dissolved in 1 mL DMSO (dimethyl sulfoxide; Sigma-Aldrich) and then diluted in water (Ali-Rachedi *et al.* 2004). Treatment was applied in a dark chamber at 4 °C for 24 h. Germination began four days after planting, and the initial germination success was high ($88.4 \pm 4.6\%$, population mean \pm SE, $N = 150$ seeds in the first seven days). Eight seedlings per population were randomly chosen for transfer to a greenhouse, where they were cultivated until bloom in accordance with the standard practice for garden petunias, for a total of 40 plants.

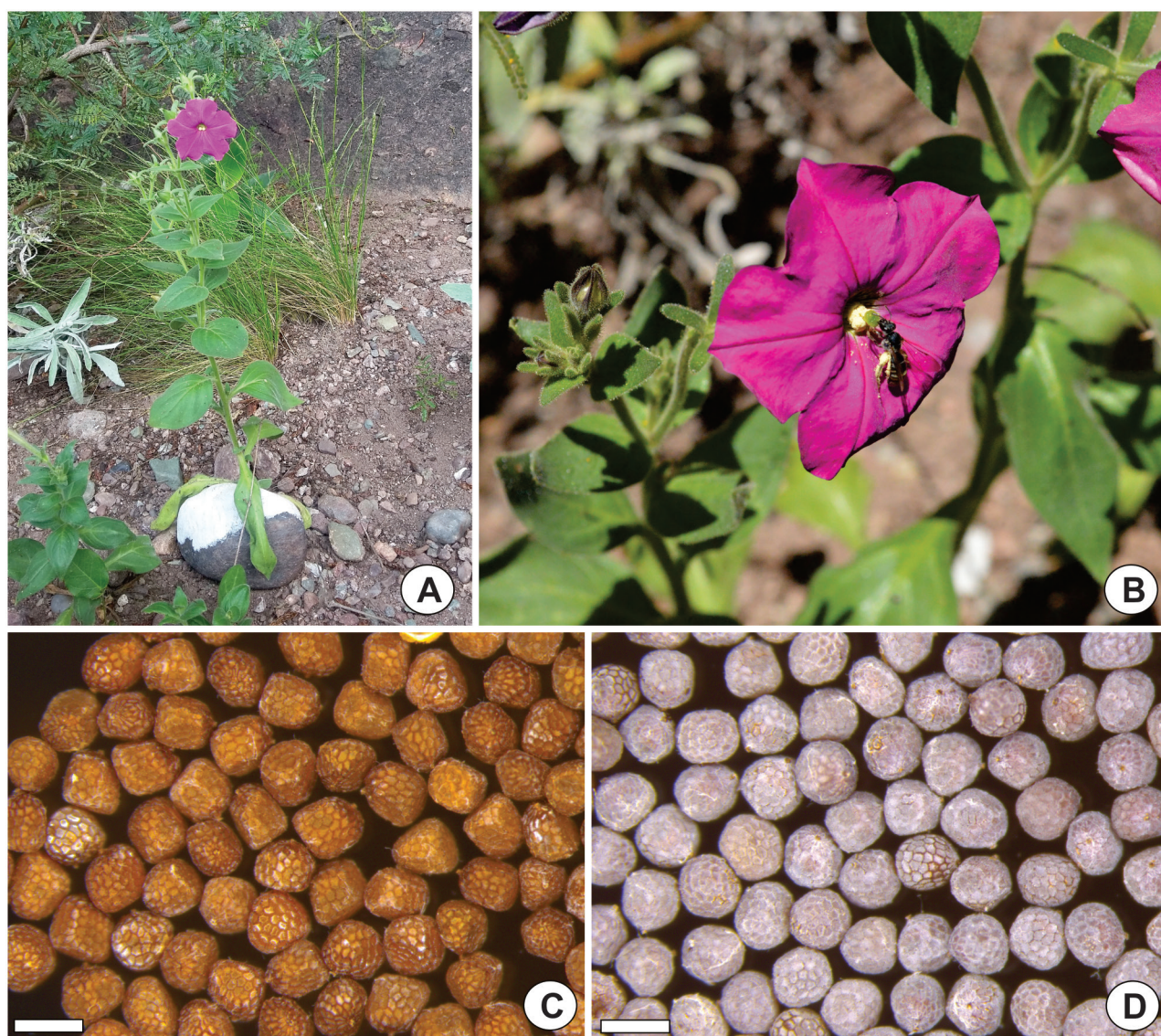


Figure 2. **A.** General view of the *Petunia secreta* plant. **B.** *Pseudagapostemon* sp. collecting pollen on *Petunia secreta* in Pedra do Segredo. **C-D.** Seeds of different colours under the stereomicroscope. Bars = 2 mm.

Table 1. Sampling information for plants collected in nature.

Population	Location	Evolutionary lineage*	Coordinates
PS59	Pedra do Segredo	1	30°32'09.56"S 53°33'06.82"W
GP62	Pedra do Segredo	1	30°32'45.90"S 53°33'00.97"W
BR142	Roadside	2	30°21'32.79"S 53°28'33.73"W
BR163	Roadside	2	30°21'30.80"S 53°28'45.38"W
BR573	Roadside	2	30°21'31.70"S 53°28'52.60"W

*According Turchetto *et al.* (2016)

Reproductive experiments

To determine the reproductive system of *P. secreta*, five pollination treatments were applied in the greenhouse: (1) autonomous apomixis: buds were isolated in a mesh bag, and anthers were removed before dehiscence; (2) autonomous self-pollination: buds were bagged throughout their anthesis; (3) hand self-pollination: previously bagged flowers were hand pollinated with pollen from the same flower; (4) hand geitonogamy: bagged flowers were hand pollinated with pollen from a different flower from the same plant; (5) hand cross-pollination: bagged flowers were emasculated before anthesis and pollinated with pollen from plants obtained from other populations. For all experiments, bags were kept until fruit spontaneously opened. The number of fruits from each treatment was recorded after three weeks, and the fruit set was estimated as the proportion of flowers setting fruits. Mature fruits were collected and their seeds kept at 4° C. Overall, we analysed 468 flowers across all plants, populations, and pollination treatments (Tab. 2). We also recorded several morphological characters of the plants, including flowers, fruits, and seeds, to characterise the two lineages of *P. secreta*.

Seed production and germinability

For each pollination treatment, we collected the seeds of three randomly selected fruits in a 0.2 µL tube. The seeds were weighed, and their weight served as a representative estimate of seed production. Up to 100 randomly selected seeds per fruit were germinated in a growth chamber at 22 °C in a 12-h light:12-h dark cycle,

as described previously. The total number of seeds was 6,500. Upon emergence of the seedlings, the temperature was increased to 25 °C. Seedlings with open cotyledons were counted every seven days for seven weeks after planting. A comparison of germinability among seeds from different treatments and of different colours (see results) was carried out based on 200 randomly chosen seeds covering all colours and treatments.

Data analysis

Seedlings were observed until the cotyledons were fully open. Germinability was expressed as the cumulative percentage of seeds that had germinated by the end of the experiment. The statistical evaluation of seed germination data is not straightforward (McNair *et al.* 2012). Nevertheless, similar to a seed technology test, our intention here was to compare possible ways of crossing to provide an estimate of new plants potentially available in nature, and the chosen statistical tests are sufficient for this purpose (ISTA 1985). We used the Chi-square and Kruskal-Wallis tests implemented in the WinPEPI software suite (Abramson 2011) and the PASW/SPSS software to compare germinability across treatments and populations.

Results

Vegetative growth and number and size of flowers

During the initial developmental stages, plants from different populations and lineages presented identical vegetative morphology, with a rosette arrangement for all

Table 2. Reproductive success obtained per pollination treatment used in *Petunia secreta*.

	PS59			GP62			BR142			BR163			BR573		
	n plant	n flower	n fruit	n plant	n flower	n fruit	n plant	n flower	n fruit	n plant	n flower	n fruit	n plant	n flower	n fruit
Autonomous apomixes	8	16	0	8	11	0	8	21	0	4	15	0	7	14	0
Autonomous self-pollination	8	19	0	8	13	0	8	26	0	4	15	0	7	17	0
Hand self-pollination	8	18	18	8	12	12	8	20	20	4	15	15	7	17	17
Hand geitonogamy	8	15	15	8	9	9	8	15	15	4	14	14	7	16	16
Hand cross-pollination	8	25	25	8	13	13	8	23	23	4	49	49	7	40	40



leaves. Just before bloom, the rosette-type shoots elongated in plants from PS59 and GP62 populations, from the Pedra do Segredo site. In plants from BR142, BR163, and BR573 populations, from the roadside collection site, four or five lateral shoots emerged before the elongation of the central rosette-type shoot. Plants from lineage 2 hence seemed to have invested more in vegetative growth than plants from lineage 1. Nevertheless, no differences in flower numbers and in numbers of individual plants producing more than 20 flowers during the flowering season were observed among populations or lineages. As observed in nature, all individuals were able to maintain flowers and fruits simultaneously. Plants survived in the greenhouse for eleven months.

Timeline of flower opening and senescence and fruit production

As observed in nature (DM Rodrigues unpub. res.), flowers of *P. secreta* opened during the daytime and remained open for four days if not pollinated. Senescence began around four days after flower aperture and was characterised by changes in the colour of the corolla, followed by the gradual wilting of the petals. In pollinated flowers, senescence started two days after pollination; otherwise, it resembled that of non-pollinated flowers. Anther dehiscence occurred simultaneously with the opening of the flower (within around 30 minutes), and the anthers were always positioned below the stigma. No differences were observed among plants or populations. In unfertilised flowers, the wilted corolla remained attached to the calix; in fertilised flowers, it fell as fruits were developing.

Among the five pollination treatments applied here, only autonomous apomixis and autonomous self-pollination did not produce any fruits (Tab. 2), indicating that *P. secreta* depends on a pollinator to set fruits, while being, at least partially, self-compatible.

Seed quantity estimates

In all plants, the fruit-stalk was erect and contained hundreds of seeds. No differences in estimated seed content

were observed between pollination treatments that did not produce fruit (Kruskal-Wallis test; $P > 0.05$). The weight of 0.2 mL of seeds (mean of three fruits \pm SE) was: hand self-pollination, 0.193 ± 0.01 g; hand geitonogamy, 0.198 ± 0.01 g; hand cross-pollination, 0.188 ± 0.01 g. The results for cross-pollination are based on crosses both within and between lineages.

Seed germinability

Considering all plants, populations, and treatments, the average rate of germinability was 73 % (9 % to 100 %; Tab. 3). Pollination treatment germinability results differed between populations (data not shown) and between lineages (Fig. 3A). There were no marked differences in germinability between pollination treatments within lineage 2, whereas the highest germinability was observed after hand geitonogamy in lineage 1.

For hand self-pollination, hand geitonogamy, and hand cross-pollination within and between lineages, all tested flowers produced fruits full of seeds. The obtained seeds were of two different colours, brown and grey (Fig. 2C, D). These colours were observed for all modes of pollination, individuals, and populations, suggesting natural polymorphism. Seed colour proportions were similar for all treatments and ranged around 50 %. No correlation was observed between seed colour and germinability (Kruskal-Wallis test; $P > 0.6$). Considering all pollination treatments and plants, a mean of 68 % of seeds germinated for each colour, with a range of 0 % to 100 %.

Germinability tests showed greater differences between plants and pollination treatments than fruit production. Each population presented a different pattern of germinability in relation to pollination treatments (Tab. 3). The only population that did not show differences in germinability between pollination treatments was BR573, where all capsules showed a similar mean of germinability (Tab. 3). In general, germinability indices were lower in plants from PS59 and GP62 (lineage 1) than in plants from BR142, BR163, and BR573 (lineage 2). The lowest values were observed for plants from GP62 that received pollen

Table 3. Seed germinability expressed as percentage¹ of seedlings with open cotyledons per population per treatment in *Petunia secreta* considering eight plants per population.

	PS59			GP62			BR142			BR163			BR573		
	Mean ¹	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max	Mean	Min	Max
Self ²	45	30	87	48	10	83	68	42	94	96	95	96	92	88	95
Geito ³	59	21	100	30	9	98	83	76	89	93	91	94	96	95	96
Out (within) ⁴	36	9	100	9	9	10	87	78	93	95	92	98	95	93	97
Out (between) ⁵	57	9	95	48	26	92	83	56	99	67	22	92	96	92	100

1 % estimates were based on overall 6,500 seeds cultivated in growth chamber and controlled conditions during seven weeks; 2hand pollination using pollen from the same flower; 3hand pollination using pollen from different flower from the same individual; 4hand outcrossing using pollen from individuals from the same evolutionary lineage; 5hand outcrossing using pollen from individuals from different evolutionary lineage; Mean – considering at least 100 seeds per plant and eight plants per population; Min – minimum number of seedlings; Max – maximum number of seedlings. All differences were statistically significant, with $P < 0.001$.

from PS59 (Fig. 3A). In plants from populations PS59, GP62, and BR142, capsules obtained from hand geitonogamy had higher seed germinability than those obtained through hand self-pollination (Fig. 3B, C). In plants from population BR142, no differences were observed between hand geitonogamy and hand outcrosses, independently of the pollen donor, whereas for plants from BR163, a lower germinability was observed for capsules resulting from outcrosses with plants from populations PS59 or GP62 (Fig. 3C). Independent of the mode of pollination, plants from PS59 had a higher germinability than plants from GP62 (Fig. 3B), whereas in lineage 2 (Fig. 3C), plants from BR142 had the lowest germinability (except when these plants received pollen from plants from lineage 1). All differences were statistically significant, with $P < 0.001$.

Discussion

We investigated the reproductive system of *P. secreta* through a range of hand pollination experiments. The geographic range of the species is fragmented, with small populations at Pedra do Segredo, and slightly larger populations at a roadside location ~20 km from Pedra do Segredo. Our experiments showed that the species as a whole is self-compatible but non-autogamous, and there were no marked barriers for pollination between lineages and populations. The results are discussed in detail below.

According to Charlesworth (2006), it is useful to distinguish two types of plant reproductive systems, which can both contribute to our understanding of genetic diversity and population structure in plant species: (1) “sex systems”, which consider whether the plant is monoecious, dioecious, or other, and (2) the mating systems of monoecious plants, including inbreeding, outcrossing, and intermediate mixed systems. *Petunia secreta* is a hermaphrodite plant, in which the stigma is located slightly above the anthers (Stehmann & Semir 2005; Fig. 2B). No significant differences are found in morphology across populations (Turchetto *et al.* 2016). Additionally, *P. secreta* displays several characteristics to attract bees (DM Rodrigues unpubl. res.; Fig. 2B), suggesting that the species is dependent on a pollinator. Our results fully confirm this, as neither autonomous apomixis nor autonomous selfing led to the production of fruit.

Although dependent on pollinators, *P. secreta* does not seem to have any genetic mechanisms to prevent endogamy, since hand selfing and geitonogamy produced fruits in all studied populations. In addition, seed germinability was higher in several of these fruit than in fruit produced through other modes of pollination, suggesting that *P. secreta* is, at least in part, self-compatible (Figs. 2, 3). The loss of self-incompatibility and increase of endogamy have been seen in *P. axillaris* populations occurring in sympatry with *P. exserta* (Turchetto *et al.* 2015). *Petunia secreta* grows in the same area as *P. axillaris* and *P. exserta*, two species

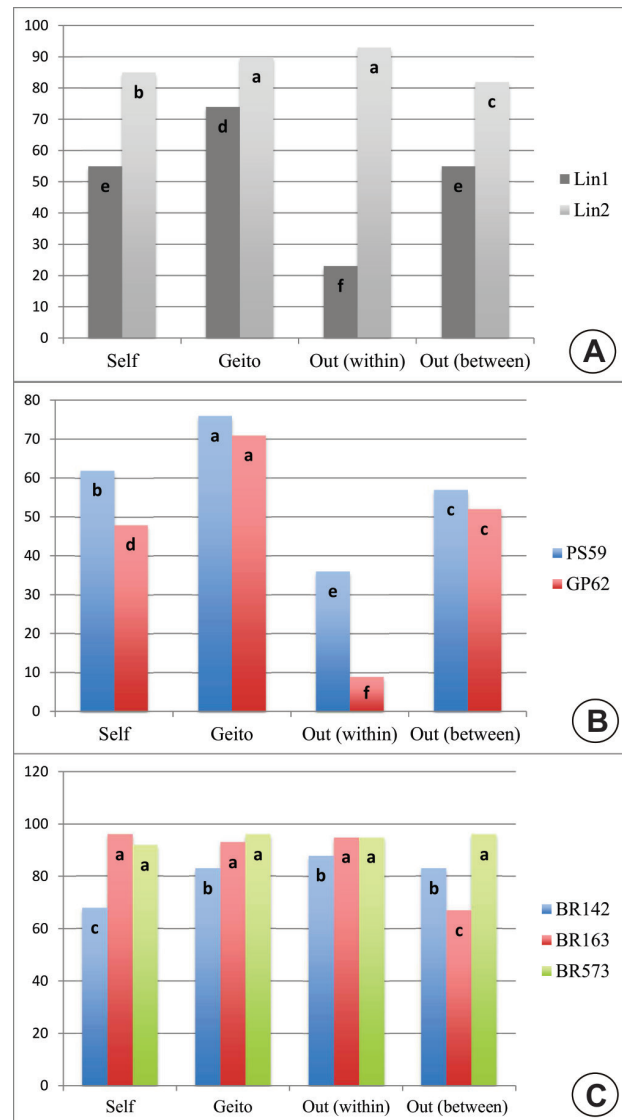


Figure 3. Comparison of the germinability after different pollination treatments in different populations and evolutionary lineages of *Petunia secreta*. **A.** Comparison between the two evolutionary lineages. **B.** Comparison between two populations from Pedra do Segredo locality (lineage 1). **C.** Comparison between three roadside populations (lineage 2). Different letters indicate statistical significance ($P < 0.001$; Kruskal-Wallis test). Seed germinability is shown on the vertical axis (mean of three experiments). Self-pollination - Self; Geitonogamy - Geito; Cross-pollination within lineage or populations - Out within; or between lineages or populations - Out between.

that share a recent common ancestor with *P. secreta* (Reck-Kortmann *et al.* 2014), and as *P. integrifolia*, which is also bee-pollinated.

Reproductive success in stressful or variable environments, in which pollination may be uncertain, is a common problem for many lineages of seed plants. For outcrossing species in disturbed habitats, pollinator limitation and restricted partner availability are commonly encountered problems (Yin *et al.* 2016). *Petunia secreta* has a

fragmented distribution across disturbed areas, where plants are generally found in small populations (Turchetto *et al.* 2016). While these attributes generally suggest a depletion of genetic diversity (Ellstrand & Elam 1993), they are not reliable predictors of genetic dynamics (Premoli *et al.* 2001), as is demonstrated by our findings in *P. secreta*. The rate of seed germinability in endogamous treatments was at least equivalent to that obtained from outcrossing (Fig. 3), suggesting a high tolerance of endogamy in this species. This seems to enable *P. secreta* to overcome the limitations in partner availability and to keep high genetic diversity indices despite the potentially low level of long distance gene flow as a consequence of the distance between individuals.

In this study we observed that non-pollinated *P. secreta* flowers remained open and fresh and kept a receptive stigma over four days, while pollinated flowers entered senescence two days earlier. These findings parallel observations of non-pollinated flowers in nature (DM Rodrigues unpubl. res.).

Plant species have evolved a range of reproductive strategies to cope with unfavourable pollination conditions. For example, plants faced with severe pollinator restriction, and hence reduced possibilities for cross-pollination, can ensure reproductive success by increasing self-compatibility (Larson & Barrett 2000). In *P. secreta*, both hand selfing and geitonogamy resulted in high levels of seed germinability (Tab. 3).

Likewise, prolonged floral longevity can compensate for a low rate of pollinator visitation (Barrett 2003) and can be seen as an evolutionary strategy to overcome a sparse or unpredictable pollinator service (Steinacher & Wagner 2010). In line with this, Darling & Barrett (2011) showed that flowers with longer exposure times had greater chances to be visited by pollinators, as well as a higher reproductive success. Unpredictability in the availability of pollinators or partners can lead to selection for a combination of self-compatibility and improved outcrossing pollination strategies (Kalisz *et al.* 2004). This seems to be the case in *P. secreta*, since the differences in germinability were greater between populations than between pollination treatments (Figs. 2, 3).

In small populations, several evolutionary processes, including genetic drift, can lead to a reduction in genetic variation and increase the rate of endogamy with all its negative consequences. Mechanisms preventing self-fertilization might act in very small populations to avoid genetic load and inbreeding depression. In line with this, an increase in the proportion of heterozygotes has been observed in sexual species (Cole 2003). When a species is divided into a series of small populations, which are so isolated from each other that dispersal rarely carries genes from one population to another, genetic drift can lead to divergent allele frequencies in the isolated populations, increasing the levels of several diversity indicators (Ellstrand 1992). As previously shown through the analysis of microsatellites (Turchetto *et al.* 2016), F_{ST} values are higher

in *P. secreta* lineages ($F_{ST} = 0.358$) than in other *Petunia* species with larger geographic distribution and/or bigger populations (*P. secreta* and *P. exserta* - $F_{ST} = 0.195$; *P. secreta* and *P. axillaris* - $F_{ST} = 0.080$; *P. axillaris* and *P. exserta* - $F_{ST} = 0.113$). Thus, endogamy, coupled with occasional gene flow due to pollen or seed dispersal between populations, could satisfactorily explain the genetic structure found in *P. secreta*.

The high levels of genetic diversity and population scattering in *P. secreta* may be better explained by the species' herkogamic flowers than by the action of a molecular self-incompatibility mechanism (see the results for hand self-pollination, Fig. 3). Herkogamy has been described to affect selfing and outcrossing proportions (Palma-Silva *et al.* 2015; Neri *et al.* 2017) and may contribute to maintain genetic diversity levels within populations by reinforcing outcrossing strategies to face potential endogamy.

Various factors affecting the reproductive system, including the mode of pollination, pollinator viability, and the presence of molecular or structural self-incompatibility systems (Karron *et al.* 2012), might contribute to an explanation of our findings. Moreover, when pollinators move only over short distances, as in the case of the solitary bees found to visit *P. secreta* (DM Rodrigues unpubl. res.; Fig. 2B), and when pollen carryover is limited, as it might happen in the small populations of *P. secreta* (Turchetto *et al.* 2016), a large fraction of the pollen deposited on a stigma might come from closely related individuals (Mitchell *et al.* 2009). Alternatively, it might constitute a mix of self-pollen, pollen of relatives, and pollen from distant donors. If all pollen, independent of its origin, has the same ability to fertilize and develop viable seeds, as we observed here for all pollination treatments (Figs. 2, 3), high diversity levels would be obtained, both for the population and for the species in general.

Clearly, the genetic dynamics of a species should not be predicted based on its geographical range alone: widely distributed species are not always characterised by high levels of genetic diversity (Paggi *et al.* 2015); conversely, genetic diversity levels are not necessarily low in species with a narrow distribution (Fernández-Mazuecos *et al.* 2014; Jiménez-Mejías *et al.* 2015; Forrest *et al.* 2017). A great number of factors can influence diversity levels within and between populations. These include the nature of the reproductive system (Coppi *et al.* 2014), which affects several aspects of the natural biology of plants, such as population homozygosity and genetic variability, as well as large effective population sizes, a common explanation for the maintenance of diversity in rare species (Ellstrand & Elam 1993) such as *P. secreta*.

Based on the present results and on the genetic microsatellite variability described in a previous paper (Turchetto *et al.* 2016), we conclude that the reproductive systems in place in *P. secreta*, coupled with a large ancestral effective population size, may be responsible for the high diversity observed in this species. We observed that *P. secreta*



does not show autonomous self-pollination and, although we do not know the rates of cross-pollination in nature, the elevated levels of homozygosity observed previously (Turchetto *et al.* 2016) suggest that *P. secreta* might show some degree of inbreeding. We conclude that the greatest risk to the species lies in the small and fragmented nature of its populations, highlighting the importance of including *P. secreta* in the Brazilian red list of plant species.

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