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## PRELIMINARY STUDY ON GENETIC DIVERSITY OF ENDEMIC AND THREATENED SPECIES OF *PETUNIA* (SOLANACEAE)

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**Abstract.** Franceschinelli, E. V.; J. R. Stehmann, C. Melo-Silva & J. N. Mesquita Neto. 2013. Preliminary study on genetic diversity of endemic and threatened species of *Petunia* (Solanaceae). *Darwiniana*, nueva serie 1(2): 220-226.

Rare and narrowly endemic species are more vulnerable to extinction due to their restricted geographical distribution. the genus *Petunia* has 14 species and most of them are endemic to a restricted area. Four *Petunia* species that grow in Southern Brazil were studied: *P. bonjardinensis*, *P. reitzii*, *P. integrifolia* subsp. *depauperata*, and *P. exserta*. We presented a preliminary study on the genetic diversity of five populations of *Petunia*, using allozyme markers. For the genetic analyses, allozyme electrophoresis was carried out and eight enzyme systems were reliably scored. Standard measures of allozyme diversity were calculated. The highest genetic diversity and the highest fixation index was found in the two studied populations of *P. bonjardinensis* ( $P = 90$ ,  $\hat{A}_p = 2.2$  and  $1.9$ ,  $\hat{H}_e = 0.33$  and  $0.30$ ,  $F = 0.31$  and  $0.27$ ); this species occurs in small isolated patches, that may restrict gene flow among populations. *Petunia reitzii* showed the second highest genetic diversity values ( $P = 78$ ,  $\hat{A}_p = 1.78$ ,  $\hat{H}_e = 0.28$ ), and *Petunia integrifolia* subsp. *depauperata* showed moderate values ( $P = 40$ ,  $\hat{A}_p = 1.4$  and  $\hat{H}_e = 0.11$ ). *Petunia exserta* did not show any polymorphic locus. Our results show the urgency of preserving the studied population of *P. exserta* that did not show any allozyme variation and occurs in a very particular and threatened habitat.

**Keywords.** Allozyme; rare species; tropical plants.

**Resumen.** Franceschinelli, E. V.; J. R. Stehmann, C. Melo-Silva & J. N. Mesquita Neto. 2013. Estudio preliminar sobre la diversidad genética de las especies endémicas y amenazadas de *Petunia* (Solanaceae). *Darwiniana*, nueva serie 1(2): 220-226.

Las especies raras y estrictamente endémicas son más vulnerables a la extinción debido a su distribución geográfica restringida. El género *Petunia* tiene 14 especies y la mayoría de ellas son endémicas de áreas restringidas. Se estudiaron cuatro taxones de *Petunia* que crecen en el sur de Brasil: *P. bonjardinensis*, *P. reitzii*, *P. integrifolia* subsp. *depauperata* y *P. exserta*. Se presenta un estudio preliminar de la diversidad genética de cinco poblaciones de *Petunia* utilizando marcadores de alozimas. Para los análisis de electroforesis de alozimas se llevaron a cabo ocho sistemas enzimáticos. Se calcularon las medidas estándar de diversidad. La mayor diversidad genética y también el índice de fijación más alto se encontró en las dos poblaciones estudiadas de *P. bonjardinensis* ( $P = 90$ ,  $\hat{A}_p = 2.2$  y  $1.9$ ,  $\hat{H}_e = 0.33$  y  $0.30$ ,  $F = 0.31$  y  $0.27$ ); esta especie se encuentra en pequeñas zonas aisladas entre sí, lo que puede dificultar el flujo génico interpoblacional. *Petunia reitzii* mostró los segundos valores más altos de diversidad genética ( $P = 78$ ,  $\hat{A}_p = 1.78$ ,  $\hat{H}_e = 0.28$ ) y *P. integrifolia* mostró valores moderados ( $P = 40$ ,  $\hat{A}_p = 1.4$  y  $\hat{H}_e = 0.11$ ). *Petunia exserta* no mostró loci polimórficos. Nuestros resultados muestran la urgencia de preservar la población estudiada de *P. exserta*, que no mostró ninguna variación alozímica y se encuentra en un hábitat muy particular y amenazado.

**Palabras clave.** Alozimas; especies raras; plantas tropicales.

## INTRODUCTION

Rare and narrowly endemic species has been the main target of conservation initiatives, because they are more vulnerable to extinction due to their restricted geographical distribution. Frequently, these species occur only in specific and vulnerable habitats threatened by human activity. The dispersion pattern of organism affects the genetic diversity and gene flow among and within populations. Species with restricted geographic distribution usually have low genetic diversity (Loveless & Hamrick, 1984; Hamrick & Godt, 1989; 1996), specially compared to related species with wider geographic distribution (Gitzendanner & Soltis, 2000).

Ecological and evolutionary processes may equally interfere in the genetic diversity and structure of natural populations. Ecological factors, such as pollination and seed dispersal are important processes responsible for the gene flow within and among plant populations. Gene flow in plants may be restricted when pollen and seeds are dispersed to short distances, decreasing neighbourhood size and area (Wright, 1943; Levin & Kerster, 1974; Crawford, 1984; Franceschinelli & Kesseli, 1999).

Levels of genetic diversity may also depend on population size and plant density in the area (Barret & Kohn, 1991; Ellstram & Elam, 1993). For example, rare species with large populations may show high levels of genetic diversity (Ellstram & Elam, 1993; Ægisdóttir et al., 2009). However, endemic species with a particular microhabitat requirement would be prone to have a disjunctive distribution and thus be affected by founder effect and selective pressure (Godt et al., 1997; Franceschinelli et al., 2006). Gitzendanner & Soltis (2000) suggested that genetic data for a rare species are more informative if compared with data for a widespread related species.

The genus *Petunia* Juss. has 14 species that are exclusively from South America, and 13 of them are found in southern and south-eastern Brazil (Stehmann et al., 2009). Intrinsic reproductive barriers among the species of *Petunia* are weak and genetic isolation is a consequence mainly of geographical separation and ecological diversification (Stehmann et al., 2009). Most *Petunia* species are

endemic, and occur in very restricted areas. They are annual or biannual herbs or shrubs, mostly pollinated by bees, and seed are dispersed close to their mother plants (Lorenz-Lemke et al., 2006). Eight endemic taxa of *Petunia* occur in southern Brazil (States of Santa Catarina and Rio Grande do Sul); four of them were selected for this study: *P. bonjardinensis* T. Ando & Hashim, *P. reitzii* L. B. Sm. & Downs, *P. exserta* Stehmann, and *P. integrifolia* (Hook.) Schinz & Thell. subsp. *depauperata* (R. E. Fr.) Stehmann. The three first species have restricted distributions.

*Petunia bonjardinensis* and *P. reitzii* occur in the State of Santa Catarina and their geographical distributions are restricted to high elevation fields of the municipalities of Bom Jardim da Serra and Bom Retiro, respectively (Lorenz-Lemke et al., 2006); both are self-incompatible and are visited by small bees (J. R. Stehmann, unpublished data). *Petunia exserta* is endemic of Serra do Sudeste, in the State of Rio Grande do Sul, and grows in sandstone towers in shady cracks within the rock, which is a very restricted and specific habitat (Stehmann, 1987); this species is self-compatible (Watanabe et al., 1996), has red flowers, and it is pollinated by hummingbirds (Lorenz-Lemke et al., 2006). *Petunia integrifolia* subsp. *depauperata* grows in sandy soils of beaches along the coast from Rio Grande do Sul to the island of Florianópolis, Santa Catarina (Stehmann et al., 2009), its area of distribution is larger compared to the rest of the species; this subspecies is self-incompatible and it is pollinated by bees (J. R. Stehmann, unpublished data). The natural habitats in which these four species grow are not protected by law.

All four species are herbs, with a short life cycle. They flower generally from October to December, and die after fruit production (monocarpic), except for *P. integrifolia* subsp. *depauperata* that has a thick subterranean system (Stehmann et al., 2009). *Petunia exserta* can flower twice a year, in May and from September to December (Stehmann, 1987), and generally, it occurs in small patches of few individuals. Plants of *P. bonjardinensis* and *P. reitzii* occur isolated or in small patches of two to eight individuals. These patches may be considered demes or subpopulations. On the other hand, *Petunia integrifolia* subsp. *depauperata* has larger population size (from 10 to 100 plants).

We undertake a preliminary study using allozyme markers to evaluate the population genetic diversity of four *Petunia* species in southern Brazil.

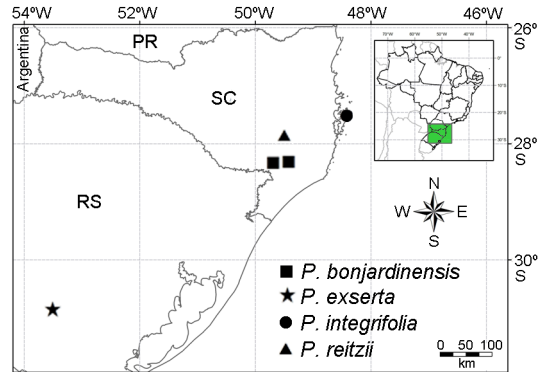
## MATERIALS AND METHODS

### Studied species

For the genetic analyses, we collected leaves of two populations (20 and 12 plants of each population) for *P. bonjardinensis*, 40 plants of one population of *P. exserta*, 30 plants of one population for *P. integrifolia* subsp. *depauperata*, 20 plants of one population for *P. reitzii* (Table 1). The small sample size is due to species rareness. The plants were numbered and tagged in the field. The location of the studied populations are shown in Fig. 1.

### Allozyme analyses

The leaf material was preserved in liquid nitrogen ( $-196^{\circ}\text{C}$ ) from the moment of field collection and transported to the Universidade Federal de Minas Gerais, where it was stored at  $-80^{\circ}\text{C}$  until enzyme extraction. A small piece of each leaf was grounded in liquid nitrogen and the powdered tissue was mixed with extraction buffer number 1 of Alfenas et al. (1998) and absorbed onto filter paper wicks. Allozyme electrophoresis was carried out on horizontal 13% starch-gel. Twenty enzyme systems were screened with three electrophoretic buffers. Eight enzyme systems showed simple banding patterns and could be reliably scored: shikimate dehydrogenase (SKDH, EC 1.1.1.25), 6-phosphogluconic dehydrogenase (6PG, EC 1.1.1.44), phosphoglucomutase (PGM, EC 5.4.2.2), uridine diphosphoglucose pyrophosphorylase (UGPP, EC 2.7.7.9), isocitrate dehydrogenase (IDH, EC 1.1.1.41), glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49), phosphoglucose isomerase (PGI, EC 5.3.1.9), and aspartate aminotransferase (GOT, EC 2.6.1.1). UGPP, 6PG, and PGM were resolved on a morpholine citrate buffer system at pH 6.5 (Alfenas et al., 1998). IDH, SKDH, and G6PDH were resolved on a histidine buffer at pH 7.0 (Alfenas et al., 1998). GOT and PGI were assayed on a lithium borate buffer at pH 8.3 (Soltis et al., 1983). Locus banding patterns were consistent with typical subunit structures. Different loci and alleles for a given system were



**Fig. 1.** Location of the studied populations of *Petunia* species at Santa Catarina (SC), and Rio Grande do Sul (RS).

designated sequentially, with the lowest number corresponding to the most anodally migrating locus or allele.

### Data analyses

Standard measures of allozyme diversity were calculated: the percentage of polymorphic loci following the 0.99 criterion ( $P_{0.99}$ ), the mean number of alleles per locus ( $\hat{A}$ ) and per polymorphic locus ( $\hat{A}_p$ ), the observed heterozygosity ( $\hat{H}_o$ ), the expected heterozygosity ( $\hat{H}_e = 1 - \sum p_i^2$ ), and Wright's fixation index ( $F = 1 - \hat{H}_o / \hat{H}_e$ ). Significant deviations from Hardy-Weinberg expectations were verified through chi-square tests within populations. These parameters were calculated using BIOSYS-2 (original version of Swofford & Selander, 1989; modified by Black, 1997).

## RESULTS

For *Petunia bonjardinensis*, seven enzyme systems showed simple banding patterns and were reliably scored: UGPP, PGM, IDH, SKDH, GOT (loci 1 and 2), PGI (loci 1 and 2) and G6PDH. For *P. reitzii*, six enzyme systems were scored: UGPP (loci 1 and 2), G6PDH, IDH, SKDH, GOT (loci 1 and 2), PGI (loci 1 and 2). Thus, nine loci were used to analyze the genetic diversity of *P. bonjardinensis* and *P. reitzii*. For *P. integrifolia* subsp. *depauperata*, seven enzyme systems were reliably

**Table 1.** Number of individuals, localities, geographical coordinates, and voucher number of the sampled populations of *Petunia* species.

Species	N	Locality	Geographical coordinates	Voucher number (BHCb)
<i>P. bonjardinensis</i> 1	20	Bom Jardim da Serra, SC	28°19'54.5" S, 49°41'22.4" W	Stehmann 3344
<i>P. bonjardinensis</i> 2	12	Bom Jardim da Serra, SC	28°20'24.4" S, 49°37'50.5" W	Stehmann 3346
<i>P. exserta</i>	40	Caçapava do Sul, RS	30°50'11.9" S, 53°30'18.2" W	Stehmann 3293
<i>P. integrifolia</i> subsp. <i>depauperata</i>	30	Florianópolis, SC	27°31'22.2" S, 48°25'1.8" W	Stehmann 3368
<i>P. reitzii</i>	20	Bom Retiro, SC	27°51'2.1" S, 49°29'44.1" W	Stehmann 3332

scored: UGPP (loci 1 and 2), PGM, IDH, 6PG, SKDH, GOT (loci 1 and 2), PGI (loci 1 and 2). For *P. exserta*, seven enzyme systems were scored: UGPP (loci 1 and 2), PGM, IDH, 6PG, SKDH, GOT (loci 1 and 2), PGI (loci 1 and 2). Thus, 10 loci were used to analyze the genetic diversity of *P. exserta* and *P. integrifolia* subsp. *depauperata*. Significant deviations from Hardy–Weinberg expectations were detected in UGPP and PGI-2 for the *P. bonjardinensis* population 1, and in GOT-1 and PGI-2 for the population 2. Only one locus (UGPP) showed significant deviation from the genotypic proportion expected by the Hardy–Weinberg equilibrium for *P. reitzii* population. *Petunia integrifolia* subsp. *depauperata* did not show any Hardy–Weinberg equilibrium deviation.

The highest genetic diversity was found in the two populations of *P. bonjardinensis* (Table 2). They both have a high percentage of polymorphic loci ( $P = 90$ ) and a high mean number of alleles per polymorphic locus ( $\bar{A}_p$  2.2 and 1.9). Both populations showed high values of expected heterozygosity (0.33 and 0.30) and a low value of observed heterozygosity (0.23), resulting in high but not significant fixation indices ( $F = 0.31$  and 0.27).

The population of *P. reitzii* also showed high percentage of polymorphic loci ( $P = 78$ ) and  $\bar{A}_p = 1.78$ . The expected heterozygosity (0.28) was slightly lower than the value found for *P. bonjardinensis*. *Petunia reitzii* population showed  $\hat{H}_o < \hat{H}_e$ , and a positive but not significant fixation index ( $F = 0.13$ ). The population of *P. integrifolia* subsp. *depauperata* showed only 40% of polymorphic loci

and  $\hat{A}_p = 1.4$ . The expected and observed heterozygosity were low (0.11 and 0.09, respectively). The fixation index was positive but not high ( $F = 0.12$ ). *Petunia exserta* did not show any polymorphic locus. All analysed loci showed only one allele and did not show heterozygosity.

## DISCUSSION

According to Hamrick & Godt (1996), short-living plants show on average between 30 to 53.6% of polymorphic loci and 0.08–0.17 of expected heterozygosity. These authors found that endemic to regional distributed species have on average between 13.5 to 60% of polymorphic loci and 0.03 to 0.21 of expected heterozygosity. The analysed populations of *Petunia bonjardinensis* and *P. reitzii* showed higher values of genetic diversity parameters than the means found by those authors, suggesting that they may be well established species. However, both populations of *P. bonjardinensis* showed lower values of observed heterozygosity than of expected heterozygosity and high fixation indices. This species occur in fields located at the edges of Serra Geral in the State of Santa Catarina. The main economical activity in this area is cattle grazing, which may have contributed to the low population number and size and consequently to the high fixation indices of *P. bonjardinensis*. The isolated occurrence of these plants in small isolated patches may restrict gene flow among them. The main pollinators of *Petunia* species are bees

**Table 2.** Genetic diversity parameters for the studied populations of *Petunia* species. N, average sampled size; P, percentage of polymorphic loci ( $P_{0.99}$ );  $\hat{A}_n$ , mean number of alleles per polymorphic locus;  $\hat{A}$ , mean number of alleles per locus;  $\hat{H}_o$ , observed heterozygosity;  $\hat{H}_e$ , expected heterozygosity; F, Wright's fixation index. F was not significant for any species.

Species	N	P	$\hat{A}_p$	$\hat{A}$	$\hat{H}_e$	$\hat{H}_o$	F
<i>P. bonjardinensis</i> 1	18.2	90	2.20 ± 0.20	2.33	0.33 ± 0.07	0.23 ± 0.05	0.31
<i>P. bonjardinensis</i> 2	11.2	90	1.90 ± 0.10	2.00	0.30 ± 0.06	0.23 ± 0.07	0.27
<i>P. reitzii</i>	28.0	78	1.78 ± 0.10	2.00	0.28 ± 0.07	0.25 ± 0.05	0.13
<i>P. integrifolia</i> subsp. <i>depauperata</i>	28.2	40	1.40 ± 0.16	2.00	0.11 ± 0.05	0.09 ± 0.04	0.12
<i>P. exserta</i>	40	0	0	1	0	0	0

that may fly short distances (Stehmann, 1999), making more difficult the exchange of pollen among far apart patches. Besides, *Petunia* species have restricted seed dispersal, which may cause genetic substructure of their populations. The studied populations of *P. bonjardinensis* may be going through a process of endogamy, which is most probably biparental since this species is self-incompatible. Crossings among genetically similar plants are common in small populations due to genetic drift. Apart from cattle grazing, the increments of agricultural activities, such as fruit-producing orchards, may threaten these populations.

Although the distribution range of *P. integrifolia* subsp. *depauperata* is the largest among the species studied (35000 km<sup>2</sup>), the population analysed showed low values of genetic diversity parameters compared to *P. bonjardinensis* and *P. reitzii* (with a distribution area of 8000 and 6000 km<sup>2</sup>, respectively). *Petunia integrifolia* subsp. *depauperata* occurs in the beaches and has larger population size. Probably, the population analysed may have undergone through a bottleneck process, followed by a recent expansion.

*Petunia exserta* is endemic of a very small area (about 500 km<sup>2</sup>) of the Serra do Sudeste region. It grows at a very specific habitat (Stehmann, 1987) on sandstone towers in shady cracks within the rock (shelters). Despite of being the only ornithophilous species of the genus (Lorenz-Lemke et al., 2006), it did not show any allozyme diversity.

A very low allozyme diversity (or not at all) has been found in other endemic herbaceous species such as *Harperocallis flava* McDaniel (Godt et al., 1997) and even for long lived trees such as *Pilgerodendron uviferum* Florin (Premoli et al., 2001). In a review on plant allozyme literature, Hamrick & Godt (1989) found several widespread to narrowly distributed plant taxa that lacked allozyme diversity. Lorenz-Lemke et al. (2006) also found low nucleotide diversity in *P. exserta* and suggested that this species may have experienced a recent process of population bottleneck followed by expansion. Besides, they found low diversity within populations, which may have been caused by low gene flow between populations due to the autochoric seed dispersal system. These features and the specific habitat where this species occurs may explain the complete lack of allozyme diversity found in this species.

Our results suggest the urgency of the re-evaluation of the conservation status of *Petunia exserta* in order to preserve the only known population of this species. *Petunia exserta* was classified as threatened species by the Brazilian scientific community (<http://www.biodiversitas.org.br/boletim/EAO>). Moreover, the Brazilian government excluded it from the list of threatened species in the country, considering it only as a species with deficient data (Normative Instruction No. 6/2008 of the Brazilian Ministry of Environment). This species did not show any allozyme variation and probably it is



not able to growth in any habitat other than the shady cracks within rocks. The transformation of the area of distribution of *P. exserta* in a conservation unit would also preserve other 30 endemic species (Guadagnin et al., 2000).

*Petunia bonjardinensis*, *P. reitzii* and *P. integrifolia* subsp. *depauperata* showed higher genetic diversity indices although a higher number of populations should be studied to get better conclusions on the genetic variability of these species.

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