Nuclear and plastid markers reveal the persistence of genetic identity: A new perspective on the evolutionary history of Petunia exserta

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ABSTRACT
Recently divergent species that can hybridize are ideal models for investigating the genetic exchanges that can occur while preserving the species boundaries. Petunia exserta is an endemic species from a very limited and specific area that grows exclusively in rocky shelters. These shaded spots are an inhospitable habitat for all other Petunia species, including the closely related and widely distributed species P. axillaris. Individuals with intermediate morphologic characteristics have been found near the rocky shelters and were believed to be putative hybrids between P. exserta and P. axillaris, suggesting a situation where Petunia exserta is losing its genetic identity. In the current study, we analyzed the plastid intergenic spacers trnS/trnG and trnH/psbA and six nuclear CAPS markers in a large sampling design of both species to understand the evolutionary process occurring in this biological system. Bayesian clustering methods, cpDNA haplotype networks, genetic diversity statistics, and coalescence-based analyses support a scenario where hybridization occurs while two genetic clusters corresponding to two species are maintained. Our results reinforce the importance of coupling differentially inherited markers with an extensive geographic sample to assess the evolutionary dynamics of recently diverged species that can hybridize.

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1. Introduction

Young lineages with structured populations and potential for hybridization and introgression provide excellent models to study the evolutionary forces that drive speciation (Cristescu et al., 2012). During the first stages of species establishment, the genomes of diverging lineages are genetically complex, with some parts indicating differentiation while others do not; thus, polymorphisms are shared, at least for some time (Wu, 2001; Nosil et al., 2009; Mims et al., 2010). For plant speciation, specialized relationships between particular plant and animal species may act in concert with ecological and geographic factors to produce isolation (Grant, 1992). However, in a scenario where each component is a key to reproductive isolation, disruption or instability of these factors might allow closely related species to hybridize (Kay and Sargent, 2009). One of the most intriguing challenges for studying closely related species that maintain their hybridization ability is determining how to distinguish between introgression or shared ancestral polymorphisms. Besides that, it is difficult to determine the introgression levels in different parts of the genome because a small number of genes may be responsible for phenotypic differentiation (McCracken and Sorensen, 2005; Lexer and Widmer, 2008; Abbott et al., 2013).

The genus Petunia Juss. is known worldwide, especially through the ornamental Petunia hybrida, which is an artificial hybrid between Petunia integrifolia (Hook.) Schinz & Thell and Petunia axillaris (Lam.) Britton, Sterns & Poggenb (Nakamura et al., 2006). The 14 wild species of Petunia are exclusively from South America, and most of them are found in southern and southeastern Brazil (Stehmann et al., 2009). Studies on the evolutionary history of the genus using different types of molecular markers have found little genetic differentiation among the species (Ando et al., 2005; Kulcheski et al., 2006; Lorenz-Lemke et al., 2010), and these species have preserved their intercrossing ability, at least in experimental conditions (Watanabe et al., 2001). Interestingly, hybrids are rare in nature despite the lack of intrinsic barriers to crossing in most of these species (Ando et al., 2001; Dell’Olivio et al., 2011). The speciation process within the genus may have been driven by adaptive radiation of floral syndromes and geographical isolation (Stehmann et al., 2009; Fregonezi et al., 2013). Petunia exserta Stehmann and P. axillaris are closely related species (Lorenz-Lemke et al., 2008; Abbott et al., 2013).
et al., 2006) that share several morphological characteristics, such as a long and salverform (hypocrateriform) corolla tube, an erect growth habit, and yellow pollen (Fig. 1a–e). The major differences between these two species are associated with different floral syndromes. *Petunia exserta* has red and non-fragrant flowers with exserted styles and anthers (characteristics associated with hummingbird pollination syndrome), whereas *P. axillaris* has white flowers that are strongly fragrant after dusk (typical of hawkmoth pollination syndrome). *Petunia axillaris* grows in open and sunny habitats, such as the Andean slopes, in Argentina to Uruguay and southern Brazil, and *P. exserta* may only be found in shady cracks (the shelters) of sandstone towers, where it is protected from direct rain and sunlight (Fig. 1a and c), and is endemic to specific rock formations in the Serra do Sudeste region of southern Brazil (Fig. 1e). In this region, both species are sympatric, but *P. axillaris* is found only outside the shelters where *P. exserta* occurs (Fig. 1b). The Serra do Sudeste is characterized by the presence of sandstone towers with altitudes ranging between 200 and 500 m above the sea level, and by a great vegetal and landscape diversity (Overbeck et al., 2007).

Lorenz-Lemke et al. (2006) have described the likely occurrence of natural hybrids between *P. exserta* and *P. axillaris* that are characterized morphologically by pinkish corolla lobes and weakly exserted stamens and styles, and these plants were found exclusively inside the shelters with *P. exserta* individuals (Fig. 1d). These authors attributed the sharing of chloroplast haplotypes to hybridization between these species, suggesting a scenario of extensive hybridization and introgression. Different studies using plastid markers have reported high ancestral polymorphism-sharing in *Petunia* species and a lack of genetic structure among species, which could mask the true introgression level between them (Kulcheski et al., 2006; Lorenz-Lemke et al., 2010).

To estimate the content of hybridization in this *Petunia* species system, it is important to verify whether the same pattern appears when nuclear markers are studied as was done by other authors (Fuchs et al., 2013; Kashiwagi et al., 2012; Zielinski et al., 2013). The hypothesis of recent gene flow could be reinforced if a congruent pattern of genetic sharing between nuclear and plastid markers were found. An important prerequisite for disentangle the genetic sharing in this system is to analyze more populations of *P. exserta* and *P. axillaris* from different parts of its total distribution. If a widespread pattern of genetic sharing were being found, it could be counted as an evidence of ancestral polymorphism. In the other hand, if only pattern of local sharing coinciding with sympatric regions, it could be considered in favor of recent hybridization between the species (Mims et al., 2010).

In this work, we sampled a large number of individuals of *P. exserta* and *P. axillaris*, as well as their putative natural hybrids,
combining plastid and nuclear markers, aiming to examine the genetic differentiation between these divergent morphologies, to quantify the genetic sharing in both species, to compare the genetic variability distribution assessed through nuclear and plastid markers, to clarify the hybridization scenario between these two species, and to estimate the loss of genetic identity in *P. exserta* due to interspecific hybridization.

2. Materials and methods

2.1. Sample collection and DNA extraction

We collected 374 individuals of *P. exserta* from 39 natural collection sites (hereafter, referred to as populations) in the Serra do Sudeste (the populations were located in 17 distinct towers) and 197 individuals of *P. axillaris* (126 individuals from 41 populations in Brazil, 39 from 20 populations in Uruguay and 32 from 16 populations in Argentina) (Fig. 1e and Supplementary Table S1). The samples covered the entire distribution of *P. axillaris* and included individuals of the three subspecies recognized by *Ando* (1996). Populations of *P. axillaris* were classified as sympatric to *P. exserta* populations if they were located in the Serra do Sudeste region. *Petunia axillaris* subsp. *axillaris* (Steere) Cabrera is the subspecies that occurs in Serra do Sudeste. Allopatric populations were collected outside the Serra do Sudeste, including individuals of the three subspecies (*P. axillaris* subsp. *axillaris*, *P. axillaris* subsp. *parodi* (Steere) Cabrera and *P. axillaris* subsp. *subandina* T. Ando). We classified all individuals that were found inside the shelters as *P. exserta* irrespective of flower color and all individuals that were found outside the shelters as *P. axillaris*. This classification was adopted due to the great color range found inside the shelters, making it impossible to accurately differentiate between pure *Petunia exserta* individuals and putative hybrids of the two species. The geographic coordinates were obtained using the Global Positioning System (GPS), and one plant of each population was deposited at BHCN Herbarium (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) or ICN Herbarium (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil). Leaves were dried in silica gel and pulverized in liquid nitrogen to allow DNA extraction with cetyltrimethylammonium bromide (CTAB) as described in Roy et al. (1992).

2.2. Amplification of CAPS markers, restriction cleavage, and allele sequencing

The nuclear CAPS (Cleaved Amplified Polymorphic Sequences) markers were previously developed for *Petunia* species (available online at http://www.botany.unibe.ch/dev/caps/index.html). The CAPS markers are codominant and have been used to study genetic diversity in several plant species (Tsumura et al., 1999; Barth et al., 2002; Kaundun and Matsumoto, 2003). Screening these markers provides a rapid and convenient way to detect polymorphisms in large numbers of individuals. In this work, we used six CAPS markers (HF1, EPF1, MYB75, MYB60, MYB8X, and PAL2B), which were amplified in accordance with the protocols described on the website. To confirm that the different patterns of bands that were detected by gel electrophoresis corresponded to different DNA sequences, one PCR product of each pattern was purified using 20% polyethylene glycol (PEG) (Dunn and Blattner, 1987) and sequenced automatically in a MegaBACE 1000 DNA Analysis System (GE HealthCare) in accordance with the manufacturer’s instructions. The sequences for the different restriction band patterns (which we considered to be different alleles for a given marker) were deposited in GenBank (Supplementary Table S2). Allelic frequencies and the number of alleles per locus (Supplementary Table S2) were estimated using the program GENEPOP 4.1 (Rousset, 2008). The alleles present in each sample are available in Supplementary Table S3.

2.3. Amplification and sequencing of chloroplast markers

The plastid intergenic spacers *trnH-psbA* and *trnS-trnG* were amplified by PCR using the primers described by Sang et al. (1997) and Hamilton (1999), respectively. PCR amplification was performed as described in Lorenz-Lemke et al. (2006), and the resulting products were purified and sequenced as described in the previous section. The sequences were deposited in GenBank (Supplementary Table S1).

2.4. Genetic diversity – CAPS markers

The most likely number of clusters of individuals and the membership of the inferred clusters for each individual were assessed using the Bayesian model-based clustering method implemented in the program STRUCTURE 2.3.3 (Pritchard et al., 2000). To estimate the value of *K*, we conducted ten replicate runs for each cluster value *K* (one to six) under the admixture model with no information regarding the population origin or species, and corre- lated allelic frequencies (Pritchard et al., 2000; Falush et al., 2003), using a burn-in of 250,000 Markov Chain Monte Carlo (MCMC) followed by 1,000,000 chains. The optimal number of genetic clusters was determined using the ad hoc statistic *ΔK*, which favors the model with the greatest second-order rate of change in the log probability of data between successive *K* values (Evanno et al., 2005). The results of the best *K* value were summarized using the CLUMPP 1.1.2 program, on the basis of the average pairwise similarity of individual assignments across runs using the Full-Search method weighted by the number of individuals in each population and statistics *G* (Jakobsson and Rosenberg, 2007). The proportion of membership to the inferred clusters was assessed for each individual, and DISTRUCT 1.1 (Rosenberg, 2004) was used to visualize the STRUCTURE results after processing with CLUMPP. As an alternative approach to represent the genetic sharing between the two species, a Principal Component Analysis (PCA) was also applied using the allelic frequencies. The frequency of one allele per locus was removed, and the loci were considered independent variables. The two principal components were plotted to evaluate the dispersion of the data. The PCA was conducted with the STATISTICA 9.0 program (available at http://www.statsoft.com/). We performed locus-by-locus analysis of molecular variance (AMOVA), and the overall *F*<sub>ST</sub> with the CAPS data to evaluate the partition of the genetic variation among populations of the two species, within and between species, and to compare the genetic variation based on nuclear and plastid data. In our study, *F*<sub>ST</sub> values indicate the proportion of variation explained among species, *F*<sub>SC</sub> values indicate the proportion of variation explained among populations within species, and *F*<sub>ST</sub> values indicate the proportion of variation explained within populations. The AMOVA analysis was performed in ARLEQUIN 3.5.1.2 using 10,000 permutations (Excoffier et al., 1992; Excoffier and Lischer, 2010). All the populations of *P. exserta* are sympatric to populations of *P. axillaris*, making it impossible to compare the genetic variability among allopatric populations of both species. Taking this into account, we did three independent comparisons using the same samples of *P. exserta*: (a) comparing values of *F*<sub>ST</sub> among the sympatric species (both from Serra do Sudeste); (b) comparing the values of *F*<sub>ST</sub> among allopatric populations of *P. axillaris* subsp. *axillaris* and *P. exserta*; and (c) comparing the values of *F*<sub>ST</sub> among allopatric populations of *P. axil- laris* (all subspecies from regions other than Serra do Sudeste) and *P. exserta*. The same comparisons were done using the cpDNA markers.
2.5. Genetic diversity – Chloroplast markers

For each cpDNA marker, both forward and reverse strands were assembled using Chromas (available at http://technelysium.com.au/). DNA sequences were aligned manually using GENEDOC (Nicholas and Nicholas, 1997). All insertion/deletion events (indels) that involved poly-T/A were eliminated from the analyses because their homologies cannot be adequately accessed (Aldrich et al., 1988). All analyses were conducted using a concatenated set of sequences for the two cpDNA markers trnH/psbA and trnS/trnG. Haplotypes were identified using DNASP 5.10.01 (Rozas et al., 2003), and the evolutionary relationships among haplotypes were estimated with the NETWORK 4.600 program (available at http://www.fluxus-engineering.com) using the median-joining network method (Bandelt et al., 1999). Haplotypes and nucleotide diversities, AMOVA, overall FST and the neutrality tests Tajima’s D (Tajima, 1989) and Fu’s F (Fu, 1997) were estimated using ARLEQUIN. The hierarchical analysis of molecular variance (AMOVA) was used to quantify the partitioning of genetic variation among different populations and between the two species. Tajima’s D and Fu’s F, are classical neutrality tests used to assess population demographic history, and both assume that populations have been in mutation–drift balance for a long period of time (Nei and Kumar, 2000); when this is not the case due to sudden expansion, these neutrality tests present negative values. Tajima’s D negative values indicate an excess of low-frequency polymorphisms, suggesting a population expansion or a selective sweep. In contrast, positive values indicate a paucity of low-frequency polymorphisms, suggesting a bottleneck or the influence of balancing selection. Fu’s F, uses information from the haplotype distribution and tests the demographic expansion. It is more sensitive than Tajima’s D, and negative and significant values indicate population growth. The significance of the tests was determined based on 10,000 coalescent simulations, which assume neutrality and equilibrium conditions.

The introgression ratio (IG) and the expected introgression ratio (IGe) were calculated according to Belahbib et al. (2001) to test whether the haplotypes were structured based on geography instead of species delimitation. The IG summarizes the amount of locally shared haplotypes; values near 1.0 indicate less difference between species. The IGe is the value expected if the sharing haplotypes are not geographically structured.

2.6. Demographic history

We used a coalescent-based Bayesian analysis as implemented in LAMARC 2.1.8 to estimate the demographic parameters and to compare these when they are calculated using CAPS information and cpDNA data, independently (Kuhner, 2006). LAMARC has the K-allele mutational model that may be used with CAPS markers. In these estimates, Petunia axillaris was represented by the subspecies Petunia axillaris subsp. axillaris (see Supplementary Table S1), which is the one that is sympatric to Petunia exserta and which appears to be the subspecies most closely related to Petunia exserta (Watanabe et al., 2001). Each species was considered to be one population. In the absence of a mutational rate for the CAPS estimates, we report the LAMARC estimates in terms of parameters scaled by the mutation rate. The estimates of theta (θ) were θ = 4μNe (for CAPS) and θ = 2μNe (for cpDNA); Ne was the estimated population size, and μ was the mutation rate per nucleotide and per generation. The migration rate was calculated as M = m/μ (where m is per generation migration rate). Exponential growth rate (g) was obtained as Θ1 = Θ0exp(-gtμ), comparing two populations with the same mutation rate, where the one that presents a higher g is growing faster. We used 10 initial chains with 5,000 genealogies sampled and two final chains with 30,000 genealogies sampled; 10,000 genealogies were used as burn-in. The priors were kept as the default. We ran the program four times with different random seeds to confirm the consistency of the results. The Effective Sample Size (ESS > 200) and the adequate examination of parameter space were checked in the program Tracer version 1.5 (available at http://tree.bio.ed.ac.uk/software/tracer/).

3. Results

3.1. CAPS markers analyses

We genotyped 571 individuals for the six CAPS markers: 197 Petunia axillaris individuals growing in open habitats and 374 Petunia exserta individuals growing inside the shelters. Four loci were bi-allelic, and two loci (HF1 and MYB75) presented additional alleles with very low frequencies (<1%). These low frequency alleles were observed only in Petunia axillaris (Supplementary Tables S2 and S3). In the Bayesian clustering analysis, K = 2 was found to be the best number of genetic clusters (Fig. 2 and Supplementary Fig. S1), as determined by Evanno et al. (2005) method. In general, the two clusters corresponded to the two species as defined here using the criteria of occurrence inside the shelters (Petunia exserta), and in open habitats (Petunia axillaris). Some individuals of both species had mixed ancestry and presented a higher proportion of membership to a cluster different from their prior assignment, based on the location of occurrence (Fig. 2). Populations of Petunia axillaris from the region of sympathy with Petunia exserta presented higher proportions of individuals with mixed ancestry than allopatric populations. To consider one individual as hybrid in the sympatric populations we assumed that it should have higher level of genetic sharing than individuals from allopatric populations (more than 0.25% of shared ancestry). Taking this classification into account there were only 29 individuals classified as Petunia exserta (all found inside the shelters) and 13 individuals of Petunia axillaris (from sympatric zone, found outside the shelters) that could be considered as hybrids (Fig. 2).

The PCA analysis (Fig. 3) showed two well-structured groups; only one population of Petunia axillaris from Serra do Sudeste was positioned inside the Petunia exserta dispersion points in the graph. Petunia axillaris had a more dispersed distribution in the PCA, demonstrating its greater genetic variability.

Fig. 2. Population structure based on CAPS markers. Genomic constitution is inferred by the program STRUCTURE on the basis of the six nuclear CAPS markers. Each individual is represented as a vertical line partitioned into K = 2 colored components that represent each individual’s proportional assignment to one of the genetic clusters. Populations are separated with black vertical lines and numbered according to Table S1. A horizontal black line indicates the 0.25 and 0.75 proportions of genetic sharing.
3.2. Chloroplast haplotypes

The trnH-psbA and trnS/trnG cpDNA concatenated markers resulted in an alignment of 1069 base pairs (bp). Twenty-two haplotypes were found in the 498 samples of the two species (Supplementary Table S4). One haplotype was exclusive for *P. exserta* (H1), five haplotypes (H2, H3, H4, H5, and H6) were found in both species, and the remaining sixteen haplotypes were exclusive to *P. axillaris*. In general, adjacent populations presented the same haplotype, independent of the species classification. All haplotypes in the network diverged by one or a few mutations from the shared, most frequent and central haplotype H2. Only one population of *P. exserta* presented the haplotype H6, which was shared with the nearest population of *P. axillaris*. In general, adjacent populations presented the same haplotype, independent of the species classification. All haplotypes in the network diverged by one or a few mutations from the shared, most frequent and central haplotypes H2 and H5 (Fig. 4b–c). No phylogenetic structuring of the species was observed, and a large number of individuals from both species presented the same haplotype (Fig. 4b–c). Each population, in general, presented only one haplotype, demonstrating population structuring confirmed by the AMOVA tests (see below).

### Table 1

| AMOVA results for *Petunia exserta* and *Petunia axillaris*, sympatric and allopatric populations and different markers. |
|---------------------------------|-------|--------|--------|--------|
| Percentage of variation         | *P. exserta* | *P. axillaris* sympatric | *P. axillaris* allopatric | *P. axillaris* total |
| **CAPS**                        |       |       |       |       |
| Among populations               | 20.7  | 11.3  | 62.5  | 45.3  |
| **cpDNA**                       |       |       |       |       |
| Among populations               | 98.3  | 62.8  | 84.00 | 77.70 |

3.3. Population differentiation and neutrality tests

The AMOVA showed less pronounced population structure for CAPS than for cpDNA markers in both species (Table 1). The populations of *P. exserta* were more strongly structured than those of *P. axillaris*. For CAPS, the AMOVA results indicated a markedly higher...
proportion of variation between species (69.91, Table 2), while in relation to cpDNA, the variation among populations within species was higher (76.91). All these values were statistically significant.

For CAPS, the $F_{ST}$ was (a) 0.64 among sympatric and (b) 0.67 among allopatric populations of $P. axillaris$ subsp. $axillaris$ and $P. exserta$ and (c) 0.68 among allopatric populations of three subspecies of $P. axillaris$ and $P. exserta$. For cpDNA, the $F_{ST}$ values were 0.06, 0.16 and 0.17 for the same comparisons, respectively. Note that there were no differences in comparisons b and c, and when we refer to $P. axillaris$ (with the exception of the LAMARC analysis), we are referring to all individuals sampled (of the three subspecies). The genetic differentiation was similar among the $F_{ST}$ comparisons when CAPS markers were considered. Unlike the CAPS markers, when cpDNA was considered, the $F_{ST}$ between sympatric populations was lower than for allopatric populations of $P. axillaris$ and $P. exserta$, suggesting a high level of gene flow between species in the sympatry zone.

Table 3 shows the diversity indices and neutrality tests for the cpDNA markers. As expected for a widely distributed species, $P. axillaris$ had higher levels of haplotype and nucleotide diversity than the endemic $P. exserta$. Concerning the neutrality tests, Fu's $F_s$ showed significant negative values for $P. axillaris$, both across the full range and among sympatric populations, which suggests population expansion. For $P. exserta$, the neutrality tests were slightly positive but not significant. The value of $I_G$, the value expected if the shared haplotypes were not genetically structured was 0.9, indicating that the sharing of haplotypes occurs preferentially in the sympatric area.

### 3.4. Demographic history

Based on the LAMARC analyses, $\Theta$ for CAPS was more than 100 times higher compared to cpDNA, especially for $P. axillaris$, while migration (m) was more than 10 times smaller for CAPS than for cpDNA markers and was slightly higher in $P. axillaris$. Growth (g) was more than 10 times higher for CAPS than cpDNA in $P. exserta$ and three times smaller for CAPS than cpDNA in $P. axillaris$ (Table 4).

### 4. Discussion

To distinguish the origin of the genetic sharing between close species is difficult and the evidence that speciation can occur despite interspecific-gene flow is increasing. It has been accepted that some parts of the genome can be affected by introgression and/or ancestral polymorphisms without affecting the divergent selection in other genomic regions (Wu, 2001; Mallet, 2005; Lexer and Widmer, 2008). The pattern of genetic sharing and genetic isolation observed in genetic markers depends on many factors including the kind of genetic inheritance, demographic and adaptive disparities, and sex-biased asymmetries (Chan and Levin, 2005; Petit and Excoffier, 2009; Zielinski et al., 2013). These phenomena may be interfering in the divergence pattern seen through different molecular markers between $P. axillaris$ and $P. exserta$.

Our results clearly show that $P. axillaris$ and $P. exserta$ are closely related species, as proposed previously (Ando et al., 2005; Kulchieski et al., 2006; Lorenz-Lemke et al., 2006). However, whereas Lorenz-Lemke et al. (2006) interpreted the sharing of cpDNA haplotypes between the two species as a consequence of recent hybridization, our results suggest a more complex history. Here, we included nuclear markers and a more extensive sampling to evaluate these species in sympatric and allopatric occurrence zones.

The nuclear markers studied here showed that $P. axillaris$ and $P. exserta$ represent distinct evolutionary groups, as indicated by the two distinct genetic clusters obtained in the STRUCTURE and PCA analyses (Fig. 2 and 3). However, individuals displaying hybridization (sharing genetic components) were present in both species classifications according to the occurrence positioning in the sympatric region (Fig. 2). Haplotype sharing was more pronounced than nuclear sharing and more frequently observed in sympatric populations, where individuals with intermediate floral morphologies are found (Fig. 4). Moreover, the nearest populations of each species frequently presented the same cpDNA haplotype and no phylogenetic structure concerning the species was found (Fig. 4). Several examples are found among plants and animal that present different evolutionary scenarios when nuclear and organelle markers are compared (Avise, 1994; Curtu et al., 2007; Mir et al., 2009; A.L.A. Segatto et al. / Molecular Phylogenetics and Evolution 70 (2014) 504–512).

### Table 2

AMOVA results between the species for the different markers.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Percentage of variation</th>
<th>$F_{statistics}$</th>
<th>$P$-value</th>
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<tr>
<td>CAPS</td>
<td></td>
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<tr>
<td>Between species</td>
<td>69.91</td>
<td>$F_{ST} = 0.699$</td>
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<td>Among pops within species</td>
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<td>cpDNA</td>
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<td>Between species</td>
<td>14.56</td>
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<td>Among pops within species</td>
<td>8.53</td>
<td>$F_{ST} = 0.9$</td>
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### Table 3

Diversity indices and neutrality tests for the cpDNA markers.

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<th>Parameters</th>
<th>$P. exserta$</th>
<th>$P. axillaris$</th>
<th>$P. axillaris$ allopatric</th>
<th>$P. axillaris$ total</th>
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<td>6</td>
<td>19</td>
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<td>0.002 (0.001)</td>
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<td>Haplotype diversity h</td>
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<td>0.700 (0.055)</td>
<td>0.678 (0.049)</td>
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<td>Fu's $F_s$</td>
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<td>0.918</td>
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</table>

* Significant values.

### Table 4

LAMARC estimates of demographic parameters.

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<th>$\theta 2$</th>
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<th>$m_2$</th>
<th>$g_1$</th>
<th>$g_2$</th>
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<td>CAPS</td>
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<tr>
<td></td>
<td>(0.0001–0.001)</td>
<td>(0.0012–0.0053)</td>
<td>(36.7565–100.0560)</td>
<td>(21.8809–99.8934)</td>
<td>(–465.1919–965.3965)</td>
<td>(–395.9692–984.8015)</td>
</tr>
</tbody>
</table>

The 95% support interval is shown in parentheses, $\theta 1$ – Petunia exserta, $\theta 2$ – Petunia axillaris, $m_1$ – migration rate into Petunia exserta from Petunia axillaris, $m_2$ – migration rate into Petunia axillaris from Petunia exserta, $g_1$ – growth for Petunia exserta, $g_2$ – growth for Petunia axillaris.
Neophytou et al., 2011; Fuchs et al., 2013). In most instances, the plastid and mitochondrial markers are more locally structured and shared by different species, although hybridization levels were low (Belahbib et al., 2001; Chan and Levin, 2005; Mir et al., 2009). This incongruence may be attributed to the influence of marker inheritance kind, demographic and selective pressures after hybridization or even been a consequence of post-divergence gene flow occurred in the past (Chan and Levin, 2005; Petit and Excoffier, 2009). Conversely, species that only share cpDNA haplotypes as a result of ancestral polymorphism present different patterns of geographic distribution of genetic variability (Palmé et al., 2004).

The literature available states there are no intrinsic reproductive barriers between P. exserta and P. axillaris in greenhouse experiments (Watanabe et al., 2001) and we found individuals with intermediary morphology, suggesting that recent hybridization may be a feasible phenomenon. In relation to ancestral polymorphism sharing, allopatic populations of both species share low frequency cpDNA haplotypes, indicating that this hypothesis must not be discarded. Additionally, the CAPS markers showed low levels of genetic admixture outside the sympatric region, indicating that the proximity of the species and ancestral polymorphism sharing play a role in the evolutionary development of these species. Furthermore, the genus Petunia is considered relatively young, and the species display low genetic differentiation (Ando et al., 2005; Kulcheski et al., 2006), with high genetic similarity and polymorphism sharing (Lorenz-Lemke et al., 2006, 2010), especially when considering the plastid genome.

The genome inheritance type and the seed and pollen dispersion systems may influence the genetic variability distribution seen in the CAPS and cpDNA markers. In Petunia species, the cpDNA is maternally inherited (Derepas and Dulieu, 1992; Stehmann et al., 2009), and these species also present autochoric seed dispersion systems (Van der Pijl, 1982; Stehmann et al., 2009), where dispersion is entirely by free fall or explosive propulsion by a fruit that opens suddenly or by a trip lever. Both the cpDNA inheritance mode and the seed dispersal system may explain the relative isolation among populations observed for plastid haplotypes. Together and coupled with possible incomplete lineage sorting and/or genetic drift, the factors cited above might contribute in the pattern observed, where specific haplotypes were restricted to particular geographic regions, and high among-population structure is observed in the cpDNA marker analyses. This structure is more pronounced in P. exserta, most likely because of the exclusive occurrence inside the shelters that are not physically connected. The fact that the shelters are small and spatially separated one from each other could represent a reduced opportunity to gene flow between and favor genetic drift within each local population. Other factors could be considered besides hybridization that may have contributed to the scenario described here based on the cpDNA results.

Based on the genetic variability shown by CAPS, the population structure was less pronounced than that obtained when cpDNA information was considered. Genetic variability was distributed most between species when CAPS were considered that may be a consequence of the pollen being dispersed through longer distances than seeds. Empirical studies predict that markers showing lower intraspecific gene flow introgress more readily than others (Currat et al., 2008; Petit and Excoffier, 2009). Our data substantially agree with this, once the gene flow seen in cpDNA markers is less pronounced than that presented by CAPS markers; although selection against hybrids may have contributed as well (Toews and Breilsford, 2012).

Hummingbirds pollinate P. exserta and field observations reported that these birds prefer its red flowers but often visit the white-flowered P. axillaris and putative hybrids between these species (Lorenz-Lemke et al., 2006). Hummingbirds were also observed visiting a population of P. axillaris in Uruguay (Gübitz et al., 2009). The foraging behavior of hummingbirds was evaluated in other groups of plants and it was proposed that they can make exploratory visits to a wide range of flowers and learn which are satisfactory sources of nectar (Grant, 1992; Grant and Temes, 1992; Schemske and Bradshaw, 1999), and thereafter, the hummingbirds visit these plants daily in the same order (Feinsinger et al., 1988). Hummingbirds are territorial and, in general, individuals do not have overlapping foraging areas (Aigner and Scott, 2002). Therefore, it is possible that these birds have been the agents mediating pollen transfer between the petunia species and their putative hybrids. As the individuals presenting intermediate morphology were found only inside the shelters and no putative hybrids were found outside the shelters, these findings could be interpreted as evidence for selection against genetic components from one species in the habitat of the other, which may be caused by biotic or abiotic factors. The shelters where P. exserta grow do not receive direct sunlight or rain, appear to have poor soil and seem to be unsuitable habitats for other species of Petunia. Phenotypic composition in hybrid zones may be influenced by pollinator behavior, which can change in response to the phenotypes distributions causing a complex pattern of variation over the years (Tastard et al., 2012).

The migration estimates obtained by the LAMARC analyses were higher for P. axillaris, which is surprising because intermediary phenotypes were found exclusively inside the shelters. The absence of morphological putative hybrids outside the shelter does not mean there is not introgression in this direction, which was demonstrated by STRUCTURE results, it may represent just another example of ecological adaptation and differentiation as reviewed in Wu and Ting (2004). Theta values were higher in P. axillaris, which may be explained by the wide distribution of this species. There is no indication of recent population expansion when cpDNA markers were analyzed, and the q values were positive for CAPS markers and higher in P. exserta. The difference between cpDNA and nuclear estimates obtained from LAMARC could be explained by the fact that coalescence time is different between the markers used. This difference could also be explained by the species constraints commented on above, as pollen is dispersed for longer distances than seeds, with seeds generally falling near the mother plant, resulting in a high level of shared ancestral polymorphisms for cpDNA in Petunia species.

Here, we are proposing a complex scenario to explain the genetic variability distribution and ancestry to these species of Petunia. It is plausible that both hybridization and ancestral polymorphism sharing could play important roles in building the evolutionary pattern currently observed. Given the sharing of cpDNA haplotypes, the admixture content presented by CAPS markers and the fact that there are no intrinsic reproductive barriers between these two species (Watanabe et al., 2001; Lorenz-Lemke et al., 2006), hybridization cannot be discarded as an evolutionary driving force, at least in Serra do Sudeste, where both species are found in sympatry. Nevertheless, introgression seems to be less pronounced that previously thought (Lorenz-Lemke et al., 2006), and these independent taxonomic units present different gene repertoires. It is possible that the remarkable differences in habitat between the species might have created a situation where the species boundaries are maintained in spite of gene flow because the hybrids may have lower levels of fitness and the amount of gene flow may never have been sufficiently high to disrupt the speciation process. In our work, the importance of extensive sampling and the use of different markers were demonstrated with a complex evolutionary scenario. Empirical studies of pollinator behavior and more nuclear marker characterization should help to precisely identify the intensity of hybridization between these species. Our results do not allow us to ignore the differences either in coalescence time or the
adaptive role of the CAPS markers to explain the genetic variability distribution pattern proposed. Although we showed that the integrity of P. exserta is maintained in spite of gene flow, contrary to the results reported by Lorenz-Lemke et al. (2006), more analyses must be employed with both species to better understand their complete evolutionary process.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ympev.2013.10.011.

References


