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Multilocus phylogeny reconstruction: New insights into the evolutionary history of the genus *Petunia*

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ABSTRACT

The phylogeny of *Petunia* species has been difficult to resolve, primarily due to the recent diversification of the genus. Several studies have included molecular data in phylogenetic reconstructions of this genus, but all of them have failed to include all taxa and/or analyzed few genetic markers. In the present study, we employed the most inclusive genetic and taxonomic datasets for the genus, aiming to reconstruct the evolutionary history of *Petunia* based on molecular phylogeny, biogeographic distribution, and character evolution. We included all 20 *Petunia* morphological species or subspecies in these analyses. Based on nine nuclear and five plastid DNA markers, our phylogenetic analysis reinforces the monophyly of the genus *Petunia* and supports the hypothesis that the basal divergence is more related to the differentiation of corolla tube length, whereas the geographic distribution of species is more related to divergences within these main clades. Ancestral area reconstructions suggest the Pampas region as the area of origin and earliest divergence in *Petunia*. The state reconstructions suggest that the ancestor of *Petunia* might have had a short corolla tube and a bee pollination floral syndrome.

1. Introduction

Phylogenetic analysis is frequently used as a preliminary investigation of the evolutionary diversity of groups, especially those that have proven taxonomically challenging using traditional taxonomic methods (Moritz, 1994). This approach is demonstrably effective in discovering cryptic and difficult to distinguish species (Bickford et al., 2006; Dasmahapatra et al., 2010). Compared with traditional morphological characters, genetic data facilitate the delimitation of species that are morphologically indistinguishable, providing valuable information about processes related to speciation (Hey, 2010), recent or ancient gene flow, and the relationships between potential species (Nielsen and Wakeley, 2001; Hey and Nielsen, 2007; Hey, 2010). However, ancestral polymorphism and processes such as incomplete lineage sorting or horizontal gene transfer between species can hamper the phylogenetic reconstruction of recent lineages (Avise and Wollenberg, 1997; Maddison, 1997; Funk and Omland, 2003; Knowles and Carstens, 2007).

The use of a large number of DNA fragments can provide better phylogenetic resolution, allowing the determination of previously unresolved relationships (López-Fernández et al., 2010; Rowe et al., 2011). This occurs because the inclusion of multiple loci allows the differentiation of forces that have affected all loci from those that have acted on individual loci (e.g., natural selection) (Hilton and Hey, 1997). Therefore, the use of multigenic data has been suggested to produce strongly supported phylogenetic estimates (Chen and Li, 2001; Rokas et al., 2003; Gadagkar et al., 2005; Rokas and Carroll, 2005; Smith et al., 2009; Robertson et al., 2011).

Adaptive radiation has been proposed as an explanation for the high diversification presented by several plant species in some regions, especially on islands (e.g., Hou et al., 2011; Rowe et al., 2011), but has also likely occurred in areas that have experienced rapid climatic or geologic changes (Hughes and Eastwood, 2006). The species complexes that originated from these instable areas are of particular interest for evolutionary studies, as they represent ongoing speciation and often include rare taxa.

Petunia Juss. (Solanaceae) is an endemic genus from South America that is suggested to have undergone a rapid diversification process during the Pleistocene climatic changes (Lorenz-Lemke et al., 2010). These species are known worldwide through the

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commercial garden petunia, an artificial interspecific hybrid cultivated since the nineteenth century (Stout, 1952; Sink, 1975). The distribution of *Petunia* species is delimited into three main areas according to altitudinal zones: the lowlands between 0 and 500 m above sea level (m a.s.l.), which form the Pampas region located in Uruguay, some provinces in western Argentina, and part of Rio Grande do Sul, Brazil; the southern Brazilian Plateau, between 500 and 900 m a.s.l. in the Brazilian states of Rio Grande do Sul and Santa Catarina; and the subtropical highland grasslands, at elevations higher than 900 m a.s.l. in the southern Brazilian Plateau located in the Brazilian states of Rio Grande do Sul, Santa Catarina, and Paraná. Additionally, three isolated taxa are distributed in disjunct areas: *P. axillaris* ssp. *subandina*, distributed in the Sub-Andean region of Argentina; *P. occidentalis*, occurring in the Sub-Andean regions of Bolivia and Argentina; and *P. mantiqueirensis*, occurring in tropical highland grasslands in Atlantic Rainforest in Minas Gerais, southeast Brazil (Stehmann et al., 2009).

The morphological circumscription of species within the genus is not easy, and there is no agreement about the number of *Petunia* taxa. Over time, differences in habitat, geographic distribution, and minor details in floral and vegetative structures have led to many changes in the genus's taxonomy, ranging from 19 (Ando et al., 2005) to 18 (Stehmann et al., 2009) taxa but with different synonyms. Table 1 presents taxa names with authorities considering all different morphological species or subspecies.

The species may be classified into two groups according to corolla tube length: a short tube group that includes purple-flowered and bee-pollinated species and a long tube group that comprises three species: *P. exserta*, which presents red flowers and an ornithophilous floral syndrome (Stehmann, 1987; Lorenz-Lemke et al., 2006); *P. axillaris*, with white flowers pollinated by hawkmoths (Galetto and Bernardello, 1993; Ando et al., 2001); and *P. secreta*, a bee-pollinated pinkish-flowered species (Stehmann and Semir, 2005).

Recently, several studies have included molecular data in phylogenetic reconstructions of *Petunia*, but all of these studies have failed to include all taxa (Ando et al., 2005; Kulcheski et al., 2006; Chen et al., 2007) and/or have analyzed few genetic markers (Chen et al., 2007; Lorenz-Lemke et al., 2010). A common result in these analyses is short genetic distances observed between taxa and, consequently, poorly resolved phylogenies, indicating recent diversification of the genus. The genetic variability in both plastid and nuclear markers is low, and several markers have failed to differentiate species (Kulcheski et al., 2006) or individuals within species (Lorenz-Lemke et al., 2010).

Nevertheless, when based on plastid markers (Ando et al., 2005; Lorenz-Lemke et al., 2010), phylogenetic analysis detected two major groups: one corresponding to species that occur in areas more than 500 m a.s.l. and another composed of species that live in areas up to 500 m a.s.l. On the other hand, based on the nuclear marker *Hf1* gene, Chen et al. (2007) also found two major clusters that were not necessarily associated with the elevation of their geographic distributions. The *Tnt1*-related mobile elements (Kriedt et al., 2014) found in *Petunia* species present an evolutionary history compatible with the *Hf1* gene tree obtained by Chen et al. (2007), and the two main clades of elements correspond to species that present short and long (+*P. occidentalis*) corolla tubes.

Despite several phylogenetic studies of the genus *Petunia*, the relationships among many of the species remain unclear. Phylogeographic approaches used in particular comparisons have obtained partial success. Lorenz-Lemke et al. (2006) studied *P. exserta* and *P. axillaris* ssp. *axillaris*, considering three plastid intergenic spacers, and established the closeness of these taxa despite the differences in their morphologies and pollination syndromes. Segatto et al. (2014a) improved the sample sizes for the same species and included nuclear markers, confirming the genetic proximity of taxa and identifying natural hybrids by their morphological and genetic traits. Lorenz-Lemke et al. (2010) evaluated two combined plastid sequences in seven species from highland open fields, and the principal result obtained was an ancestral polymorphism shared among the species. Longo et al. (2014) studied plastid markers and the internal transcribed spacers of the nuclear ribosomal DNA (ITS) in five taxa of the *P. integrifolia* group, which could be considered ochlospecies (Ando et al., 2005), and were able to confirm only three evolutionary lineages. Natural interspecific hybrids have not been described for *Petunia* except between *P. exserta* and *P. axillaris* ssp. *axillaris*, based on molecular and morphological data (Lorenz-Lemke et al., 2006; Segatto et al., 2014a), and between two subspecies of *P. axillaris*, based on morphological traits (Kokubun et al., 1997).

In this study, we employed the most inclusive genetic and taxonomic datasets for the genus, aiming to reconstruct the evolutionary history of *Petunia* based on molecular phylogeny, biogeographic distribution, and character evolution.

2. Materials and methods

2.1. Sample collection and DNA extraction

We included all 20 *Petunia* morphological species or subspecies in these analyses. Samples were preferably collected from the type localities or at least from nearby places, and all exhibited the canonical morphology reported in their original descriptions. The geographic coordinates of samples were obtained using the Global Positioning System (GPS), and one plant of each taxon was deposited at the BHCB (Universidade Federal de Minas Gerais, Belo Horizonte, Brazil) herbarium (acronyms according to Thiers, 2010). We extracted the total genomic DNA from silica-dried leaves following the basic procedures of the CTAB (cetyl-trimethyl ammonium bromide)-based method described by Roy et al. (1992). Additionally, we included samples of three *Calibrachoa* species representing two subgenera (Fregonezi et al., 2012) as outgroups as well as sequences of *Petunia* from the literature. Table 1 provides voucher information and GenBank accession numbers for each taxon analyzed.

2.2. Polymerase chain reaction (PCR) amplification and sequencing

New sequences were obtained for six nuclear regions: glyceraldehyde 3-phosphate dehydrogenase gene (*G3PDH*); microsatellite-flanking regions PID1D6 and PID3C4; and nuclear introns of genes *WOX1*, *WOX4*, and *WUS*. Three nuclear and five plastid DNA markers were obtained from the literature and included in this study: ITS (Kulcheski et al., 2006); the *Hf1* gene (Chen et al., 2007); the *PolA1* gene (Zhang et al., 2008); plastid gene spacers *trnH-psbA* (Kulcheski et al., 2006; Lorenz-Lemke et al., 2006, 2010), *trnS-trnG* (Lorenz-Lemke et al., 2006, 2010), and *trnL-trnF* (Kulcheski et al., 2006); the *trnL* intron (Kulcheski et al., 2006); and the *matK* gene (Chen et al., 2007). The collection and molecular criteria used here were the same as in Kulcheski et al. (2006) and Lorenz-Lemke et al. (2006, 2010). Primers and PCR conditions are cited in Table 2. Where previous studies did not include all morphological species or subspecies of the genus, we completed the matrix, amplifying the missing taxa using the same protocols and primers previously described, except for the *Hf1* gene, for which a new primer set developed from sequences available in GenBank (AB242220–AB242238) was used (Pet_Hf1a and Pet_Hf1b).

All PCR products were purified using 20% polyethyleneglycol (Dunn and Blattner, 1987) followed by amplification with a DYEnamic ET Terminator Sequencing Premix Kit (GE Healthcare Bio-Sciences Corp., Piscataway, NY, USA), which employs dideoxy chain