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Evolutionary Genetics

Shades of white: The *Petunia* long corolla tube clade evolutionary history

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Abstract

Delimiting species is challenging in recently diverged species, and adaptive radiation is fundamental to understanding the evolutionary processes because it requires multiple ecological opportunities associated with adaptation to biotic and abiotic environments. The young *Petunia* genus (Solanaceae) is an excellent opportunity to study speciation because of its association with pollinators and unique microenvironments. This study evaluated the phylogenetic relationships among a *Petunia* clade species with different floral syndromes that inhabit several environments. We based our work on multiple individuals per lineage and employed nuclear and plastid phylogenetic markers and nuclear microsatellites. The phylogenetic tree revealed two main groups regarding the elevation of the distribution range, whereas microsatellites showed high polymorphism-sharing splitting lineages into three clusters. Isolation by distance, migration followed by new environment colonization, and shifts in floral syndrome were the motors for lineage differentiation, including infraspecific structuring, which suggests the need for taxonomic revision in the genus.

Keywords: Solanaceae, genetic variability, speciation, evolutionary relationships.

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Introduction

Adaptive radiation plays a fundamental role in our understanding of the evolutionary process, and it is frequently accepted that adaptive radiation requires multiple ecological opportunities associated with adaptation to biotic and abiotic environments (Gillespie *et al.*, 2020). Criteria such as common ancestry, phenotype-driver selector, and rapid speciation have been proposed to identify adaptive radiation (Schluter, 2009). However, some authors consider it challenging to prove for most studies (Gillespie *et al.*, 2020). Delimiting species is difficult in recently derived species because of the short time interval since speciation could not be enough to accumulate genetic differentiation (e.g., Knowles and Carstens, 2007).

The genus *Petunia* (Solanaceae) encompasses 17 wild species distributed in southern South America (Greppi *et al.*, 2019) and one of the most important ornamental plants, *P. hybrida.* Divided into two main clades based on molecular phylogenetic analysis (Reck-Kortmann *et al.*, 2014), the genus has 14 bee-pollinated species that share several morphological traits, especially the corolla tube length, which is short, and the bluish pollen. Three other species display long corolla tubes and yellow pollen and are more variable in attracting different pollinators (Stehmann *et al.*, 2009; Fregonezi *et al.*, 2013). The ornamental species *P. hybrida* is considered a perfect supermodel for genetic and physiological studies (Vandenbussche *et al.*, 2016), and the wild species might be excellent models for understanding the evolutionary process for young groups. The clades diverged ca. 2.8 Mya (Särkinen *et al.*, 2013), and species in the short corolla clade colonized highland grasslands, diversifying ca. 1.0 Mya (Lorenz-Lemke *et al.*, 2010).

The topology of *Petunia* phylogenetic trees profoundly changes when different molecular markers are considered. When based only on plastid markers, species are preferentially grouped according to their distribution in highlands (elevation up to 500 m above the sea level – a.s.l.) or lowlands (below 500 m high), respectively (Ando *et al.*, 2005; Lorenz-Lemke *et al.*, 2010). When the relationships are recovered based on only nuclear markers or combining nuclear and plastid sequences, the clades' composition is supported by the corolla tube length, with the terminals' position varying among gene trees (Chen *et al.*, 2007; Kriedt *et al.*, 2014; Reck-Kortmann *et al.*, 2014; Segatto *et al.*, 2016).

The species in the short corolla tube clade (ST) share several morphological and ecological traits, and often it is difficult to distinguish them based only on morphology (Longo *et al.*, 2014). The extensive genetic polymorphism sharing and some variable traits have promoted changes in the taxonomic classification of this group over time (Segatto *et al.*, 2017). In the long corolla tube group (LT), the species are identified based on the corolla color (Stehmann *et al.*, 2009), and no doubt has been put on their identity.

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The diversification in each clade has been attributed to different main drivers. For species in the ST, especially those occupying higher elevations (ca. 900 m a.s.l. or more), it has been proposed an allopatric speciation, strongly influenced by climate changes during the late Pleistocene (Lorenz-Lemke *et al.*, 2010; Barros *et al.*, 2015, 2020). Pleistocene effects were also implicated in the intraspecific diversification of some species (Backes *et al.*, 2019; Souza *et al.*, 2022; Soares *et al.*, 2023). Additionally, for ST lowland species (elevation < 500 m), ecological factors and geomorphology were the most important features, even when the species are parapatric (Ramos-Fregonezi *et al.*, 2015; Segatto *et al.*, 2017). The LT species show morphological traits associated with distinct floral syndromes, and the interaction with different pollinators is described as the main driver for diversification (Fregonezi *et al.*, 2013).

The LT clade encompasses the species *P. axillaris*, divided into three subspecies [*P. axillaris* subsp. *axillaris*; *P. axillaris* subsp. *parodii,* and *P. axillaris* subsp. *subandina* – (hereafter shortly *P. axillaris*, *P. parodii*, and *P. subandina*, respectively)], *P. exserta*, *P. secreta*, and *P. occidentalis*. The *P. axillaris* subspecies display white flowers that are moth-pollinated (Ando *et al.*, 1995; Venail *et al.*, 2010); the bright red color and flower morphology of *P. exserta* attract hummingbirds (Stehmann *et al.*, 2009); *P. secreta* shows pink corollas and is a bee-pollinated species (Rodrigues *et al.*, 2018). The morphology of *P. occidentalis* corresponds to the melitophilous floral syndrome. However, no systematic pollination studies have been conducted with this taxon, and its effective pollinator is still unknown.

Each taxon in LT shows different patterns of genetic structure throughout the geographic range (Segatto *et al.*, 2014; Turchetto *et al.,* 2014a,b, 2016; Giudicelli *et al.*, 2022) and a complex process of intraspecific diversification emerges: *P. parodii* shows three main lineages, geographically structured (Chaco, Pampa-Brazil, and Pampa-Uruguay; Giudicelli *et al.*, 2022); *P. exserta* revealed two lineages with slight morphological variation and distribution (*P. exserta* E1 and *P. exserta* E2), each one occurring in a different rock formation in Serra do Sudeste; and *P. secreta* that would have two main genetic lineages (Turchetto *et al.*, 2016), more distinct from each other than canonical *P. secreta* is from *P. axillaris* (here treated as *P. secreta* and *P.* sp1, respectively). An unnamed taxon (*P.* sp3) occurs close to *P. secreta* and *P. exserta* E1.

All taxa in LT have high levels of genetic polymorphism sharing (Kulcheski *et al.*, 2006; Fregonezi *et al.*, 2013; Reck-Kortmann *et al.*, 2014; Turchetto *et al.*, 2016), and interspecific hybridization has been observed among them (Lorenz-Lemke *et al.*, 2006; Segatto *et al.*, 2014; Turchetto *et al.*, 2015, 2019a, b; Giudicelli *et al.*, 2019; Teixeira *et al.*, 2019; Schnitzler *et al.*, 2020; Caballero-Villalobos *et al.*, 2021). Intraspecific morphological diversity was also observed (Turchetto *et al.*, 2016; Giudicelli *et al.*, 2019; Teixeira *et al.*, 2020), even in taxa that did not display differentiated genetic lineages as *P. axillaris* (Turchetto *et al.*, 2014b), which has a morphotype from coastal (A1) and another from inland (A2) distribution.

Except for phylogenetic analyses, the LT taxa were not evaluated together based on their intra and interspecific genetic diversity. Thus, we aimed to (i) determine the phylogenetic relationships among taxa and intraspecific lineages in the long corolla tube clade of *Petunia* based on phylogenetic informative markers; (ii) compare the intraspecific genetic diversity among the LT taxa based on nuclear microsatellites; and (iii) identify any diversification process in course among LT lineages. We based our study on the cohesive species concept proposed by Templeton (1989) and as treated in Haselhorst *et al.* (2019).

Material and Methods

Phylogenetic approach

We collected young and healthy leaves from multiple individuals of each LT lineage (Figure 1), except for *P. occidentalis,* for which we used an herbarium-derived sample (Table S1). We extracted the total DNA using the CTAB (cetyl-trimethyl ammonium bromide)-based method (Roy *et al.*, 1992), evaluated DNA quality in a NanoDrop DN 1000 spectrophotometer (Thermo Fischer Scientific Co., Waltham, USA), and estimated the quantity using a Qubit fluorometer (Thermo Fischer).

We amplified seven nuclear regions and five plastid DNA markers through PCR reactions using previously described primers and protocols (Table S2). We included once-obtained sequences (Reck-Kortmann *et al.*, 2014) for some samples. We used two *Calibrachoa* species (Mäder and Freitas, 2019) and *P. integrifolia* representing the ST (Reck-Kortmann *et al.*, 2014) as outgroups. Amplicons were purified using a polyethylene glycol method (Dunn and Blattner, 1987) and sequenced in an ABI 3730XL (Thermo Fischer Sci.) sequencer.

We assembled and edited sequences using Chromas v.2.0 software (Technelysium, Helensvale, Australia) and prepared alignments per DNA marker using Muscle in MEGA X (Kumar *et al.*, 2018) and concatenated them to the phylogenetic analyses. We manually edited the alignments when necessary and coded contiguous insertion/deletion (indels) events involving more than one base pair (bp) as one mutational event (Simmons and Ochoterena, 2000). We did not include ambiguous sites (more than one pick in the chromatogram) from nuclear markers in the final matrix (Mäder *et al.*, 2010). One representative of each different sequence was deposited at GenBank (Table S3). We also used MEGA to estimate genetic diversity per marker (Table 1).

To estimate the evolutionary relationships among taxa and lineages, we used a Bayesian inference (BI) as implemented in BEAST v.1.10 (Suchard *et al.*, 2018), assessing the tree support with posterior probability (PP) with 10^7 chains. We estimated the best substitution model and gamma rate heterogeneity using jModelTest v.3.06 (Darriba *et al.*, 2012) based on the Akaike information criterion (AIC) for each nuclear marker, *matK* gene, and combined intergenic plastid spacers, respectively (Table 1). We conducted BI analysis under the Yule process and two independent runs of 10 million generations, sampling every 1000 generations. We assessed Markov chain Monte Carlo (MCMC) convergence by examining effective sample size values (ESS > 200) and likelihood plots in Tracer v.1.7 (Rambaut *et al.*, 2018). We discarded the initial 25% of trees as burn-in and summarized the remaining trees to generate a maximum clade credibility tree using TreeAnnotator v.1.7.5 (Suchard *et al.*, 2018) visualized with FigTree v.1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/). PP ≥ 0.90 values were considered to represent strong support.

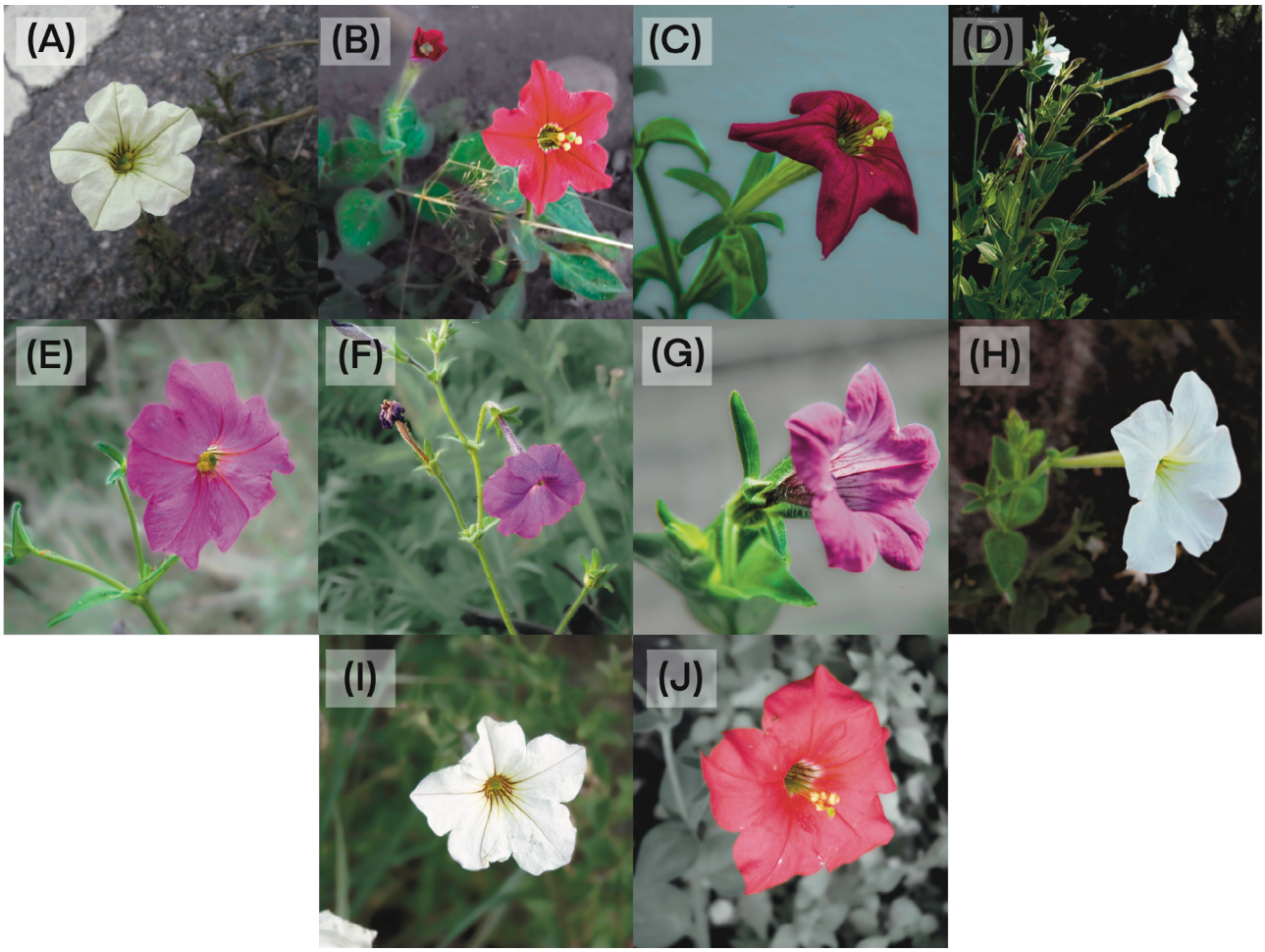

Figure 1 – Representative individuals of each analyzed *Petunia* lineage. (A) *P. subandina*; (B) *P. exserta* E2; (C) *P.* sp3; (D) *P. axillaris* A2; (E) *P.* sp1; (F) *P. secreta*; (G) *P. occidentalis*; (H) *P. axillaris* A1; (I) *P. parodii*; (J) *P. exserta* E1.

Table 1 – Genetic diversity per marker used to obtain the phylogenetic tree for *Petunia* long corolla tube clade.

Genetic Marker	Alignment length	Variable sites (%)	PI sites (%)	Evolutionary Model*
trnH-psbA[1]	424	20 (4.7)	9 (2.1)	HKY+G
trnS-trnG[1]	652	15 (2.3)	9 (1.4)	HKY+G
rps12-rpl20[1]	777	26 (3.4)	13 (1.7)	HKY+G
trnL-rpl32[1]	976	25 (2.6)	13 (1.3)	HKY+G
matK	857	21 (2.5)	13 (1.5)	GTR+I
cpDNA Total	3,686	107 (2.9)	57 (1.6)	–
ITS	536	55 (10.3)	29 (5.4)	GTR+G
Hf1	1,395	50 (3.6)	24 (1.7)	GTR+G
PolA1	987	25 (2.5)	9 (0.9)	HKY+G
G3pdh	544	28 (5.2)	15 (2.8)	HKY+G
PID3C4	209	10 (4.8)	6 (2.9)	HKY+G
WOX4	231	43 (18.6)	32 (13.9)	HKY+G
WUS	206	31 (15.1)	18 (8.7)	HKY
nuDNA Total	4,108	242 (5.9)	133 (3.2)	–
Total	**7,794**	**349 (4.5)**	**190 (2.4)**	

PI – parsimoniously informative sites; *Best substitution model estimated with jModelTest based on Akaike information criterion; [1]concatenated sequences

Intraspecific variability

To estimate the intraspecific diversity, we amplified seven nuclear microsatellite loci (Table S4) for all taxa (except *P. occidentalis*), including individuals throughout the entire geographic distribution of each lineage, proportional to population density. We genotyped 10 *P. axillaris* A1, 63 *P. axillaris* A2, 13 *P. exserta* E1, 82 *P. exserta* E2, 50 *P. secreta*, 39 *P. parodii*, 23 *P. subandina*, 23 *P.* sp1, and 11 *P.* sp3. We visualized and scored the alleles with GeneMarker v.1.97 software (Softgenetics LLC, State College, USA) and used Micro-Checker (van Oosterhout *et al.*, 2004) software to identify possible null alleles, significant allele dropout, and scoring errors due to stutter peaks.

We used the FSTAT v.2.9.3.2 software (Goudet, 1995) to evaluate the number of alleles per locus (A) and Nei's unbiased gene diversity (GD; Nei, 1987). Additionally, we used AZDE (Szpiech *et al.*, 2008) to estimate allelic richness (AR) and number of private alleles (PA) through rarefaction, as sample sizes vary among lineages.

We conducted a discriminant analysis of principal components (DAPC; Jombart *et al.*, 2010) employed in the R program for Statistical Computing v.3.3.2 (R Core Team, 2020) to explore genetic groups. The lowest Bayesian information criterion (BIC) in DAPC was used to assess the best number of groups, and we did not include taxonomic and geographic prior information.

Results

Evolutionary relationships

We obtained a data matrix with 7,794 characters based on the DNA markers, from which ~5% were variable, and ~3% were parsimoniously informative. Nuclear regions were more variable and informative than plastid markers (Table 1). The BI analysis (Figure 2A) split the species with long corolla tubes in two main clades, mainly based on elevation: clade I, species distributed in elevations higher than 700 m a.s.l (*P. subandina* and *P. occidentalis*), and clade II, species found at less than 700 m a.s.l (remain lineages). Clade II also could be divided into two subclades, IIA encompassing *P. secreta, P.* sp1, *P.* sp3, and the inland lineage of *P. axillaris*. In subclade IIB, we found coastal *P. axillaris* lineage, two *P. exserta* lineages, and *P. parodii.* These ten lineages were well supported (PP ≥ 0.90), except for the *P. parodii* positioning (PP < 0.90). The separation between *Petunia* LT and ST clades was confirmed.

Intraspecific variability

Considering the seven SSR loci, all individuals exhibited a maximum of two alleles per locus, as expected for diploid species, and the sizes of the alleles were compatible with the repetition for each locus. All loci were polymorphic among lineages. The most variable lineage was *P. axillaris* A1, considering AR and GD indices, whereas the least variable was *P.* sp1. The highest number of private alleles (PA) was observed in *P. subandina*, whereas *P. exserta* E1 has the lowest (Table 2).

The DAPC analysis (Figure 2B), including all individuals and microsatellite loci, revealed the most probable K = 3 groups. Individuals of most lineages were distributed in two or three groups, except *P.* sp1, from which all individuals belonged to the first cluster. Approximately 50% of *P. subandina, P. parodii*, and *P. axillaris* A2 samples composed the first cluster. The second cluster encompassed most *P. exserta* E1 and E2, and *P.* sp3 individuals, whereas all lineages had representatives in group 3, except *P.* sp1. Most *P. secreta* and *P. axillaris* A1 belonged to the third group (Table S5). The polymorphism sharing based on microsatellite alleles did not replicate the evolutionary relationships among species. Groups were homogeneous with low superimposition in the Cartesian plane.

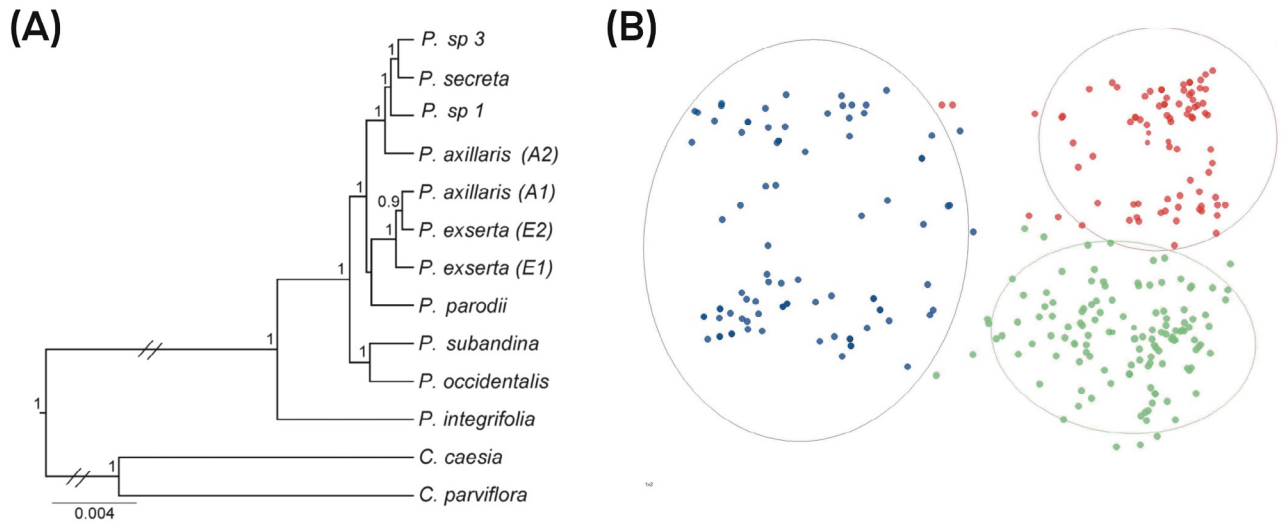

Figure 2 – Evolutionary relationships among *Petunia* long corolla tube clade. (A) Bayesian inference phylogenetic tree including plastid and nuclear sequences. Each branch represents collapsed individuals with identical sequences. (B) Cartesian plane obtained in DAPC analysis based on nuclear microsatellites (best K = 3). Colors indicate clusters: red, cluster 1; green, cluster 2; and blue, cluster 3. Cluster composition in lineages and individual numbers follow Table S5.

Table 2 – Median values for genetic diversity indices observed in *Petunia* long corolla tube lineages based on seven nuclear microsatellites

Lineages	N	A	AR	PA	GD
P. axillaris A1	10	6.3	6.3	0.68	0.74
P. axillaris A2	63	8.1	5.5	0.25	0.70
P. exserta E1	13	3.7	3.5	0.06	0.54
P. exserta E2	82	6.7	4.5	0.59	0.57
P. secreta	50	7.6	5.2	0.43	0.62
P. parodii	39	6.3	4.7	0.37	0.64
P. subandina	23	4.9	4.3	0.70	0.69
P. sp1	23	4.1	3.2	0.38	0.43
P. sp3	11	4.7	4.7	0.11	0.67

N – number of analyzed individuals; A – total number of alleles per species; AR – allele richness; PA – number of private alleles; GD – gene diversity.

Discussion

Here, we investigated the evolutionary relationships among the *Petunia* long corolla tube species employing a phylogenetic approach and intraspecific genetic variability. The taxa in the LT clade display marked differentiation in floral traits associated with pollinator attraction (Stehmann *et al.*, 2009), and plant-pollinator interaction was proposed as the main speciation driver in the group (Fregonezi *et al.*, 2013). Despite attracting different pollinators, several hybrid populations are found (e.g., Turchetto *et al.*, 2019b; Giudicelli *et al.*, 2019).

Our results revealed unexpected relationships regarding previous studies (e.g., Reck-Kortmann *et al.*, 2014). On the other hand, the present work is the first to include multiple samples and intraspecific lineages throughout their entire geographic distribution. In the *Petunia* genus, geographic isolation is often implicated in population structure and reproductive isolation (e.g., Giudicelli *et al.*, 2022; Guzmán *et al.*, 2022). Moreover, local adaptation and microenvironmental conditions keep species limits (e.g., Segatto *et al.*, 2017; Caballero-Villalobos *et al.*, 2021), contributing to differentiation (Fregonezi *et al.*, 2013; Pezzi *et al.*, 2022).

The phylogenetic tree and SSR-based analyses were not entirely congruent. Phylogenetic markers indicated with full support the split between high elevation-distributed species (*P. occidentalis* and *P. subandina*) and the lowland species (remaining lineages, all distributed at < 500 m a.s.l.), whereas SSR profiles formed three groups that did not reflect phylogenetic clades and subclades. SSR-based group 2 encompassed all *P. exserta* individuals, independently of their occurrence area, most *P.* sp3, one *P. secreta* from the same region than *P.* sp3, and one *P. axillaris* A2 sampled close to *P. exserta. Petunia exserta* occupies the subclade IIB in the tree, whereas the remaining lineages from group 2 form the subclade IIA. In turn, groups 1 and 3 clustered individuals of all lineages in different proportions (except for *P.* sp1, which integrates only group 1): *P. axillaris* A2, *P. parodii*, and *P. subandina* were equally distributed between groups 1 and 3, whereas *P. axillaris* A1 and *P. secreta* mainly integrated the group 3. The lineages *P. axillaris* A1 and *P. secreta* were not closely related in the phylogenetic tree, occupying different subclades despite the high similarity in their SSR profiles. The geographic distribution of *P. axillaris* A1 is on the southern Atlantic coast, predominantly in Uruguay (Turchetto *et al.*, 2014a), whereas *P. secreta* is endemic to Serra do Sudeste in Rio Grande do Sul (Stehmann and Semir, 2005).

Almost all phylogenetic analyses including the LT taxa (Ando *et al.*, 2005; Kriedt *et al.*, 2014; Reck-Kortmann *et al.*, 2014; Segatto *et al.*, 2016) placed *P. subandina* and *P. occidentalis* as sister species (but also see Chen *et al.*, 2007), despite the first displays long corolla tube and yellow pollen as the remaining species in the LT, whereas *P. occidentalis* shows a short corolla tube and bluish pollen as all species in the ST. Regarding the geographical distribution, *P. occidentalis* is restricted to the sub-Andean region, in elevation up to 900 m, and isolated from the other *Petunia* species by the Chaco (Tsukamoto *et al.*, 1998); the remaining species in LT are found in grasslands in Chaco or Pampa (Stehmann *et al.*, 2009), in open rocky ground areas and roadside slopes, except for *P. subandina,* which occurs only in the sub-Andean mountains (Ando, 1996). The taxa *P. axillaris*, *P. exserta*, and *P. secreta* occur in sympatry in Brazil. However, *P. axillaris* is widely distributed in the Uruguayan Pampa, whereas the other two species are narrowly endemic to rocky formations in southern Brazil. The *P. parodii* can be found in Chaco (Argentina) and Pampa (southern Brazil, Uruguay, and Argentina), where the plants grow disjunct from *P. axillaris*. Except for *P. subandina* and *P. occidentalis*, the species in LT are distributed from zero to less than 500 m a.s.l., occupying areas proposed as ancestral for the *Petunia* genus (Reck-Kortmann *et al.*, 2014).

The most surprising result was the divergence between *P. axillaris* interspecific lineages A1 and A2. According to the phylogenetic markers, this taxon was paraphyletic. Previous works (Turchetto *et al.*, 2014a, b) support the separation found here among *P. axillaris*, *P. parodii,* and *P. subandina,* indicating they should be treated as independent evolutionary units and not only as subspecies. Although *P. axillaris*, *P. parodii,* and *P. subandina* shared several plastid haplotypes and no genetic-based intraspecific groups have been found (Turchetto *et al.*, 2014a), morphologic floral traits revealed that *P. axillaris* can be divided in two groups that correspond to coastal (A1 in the present work) and inland (A2) populations. In the same way, ecological features pointed to the same *P.*

axillaris subgroups and three groups in *P. parodii* (Chaco, Pampa-Brazil, and Pampa-Uruguay), which were not perceived based on morphologic analysis. The *P. parodii* subdivision was not confirmed here in the phylogenetic tree and SSR, but it was also identified using a sizeable genomic evaluation (Giudicelli *et al.*, 2022).

It is widely accepted that ecological divergence due to habitat differences plays an essential role in lineage differentiation (e.g., Foster *et al.*, 2007), notably regarding to adaptation to extreme environments such as coastal areas (e.g., Lowry *et al.*, 2008) that are often reflected in morphological traits in addition to genetic markers. Significant morphologic differences were already observed comparing *P. axillaris* inland populations in Brazil with coastal populations from Uruguay, whereas *P. parodii* Brazilian populations were not different from those collected in Uruguay (Kokubun *et al.*, 2006). Such differences or their absence followed taxa's self(in)-compatibility system.

The polymorphism sharing between some lineages in the *Petunia* LT can be explained by introgression due to hybrid populations' high frequency and stability (e.g., Schnitzler *et al.*, 2020), whereas others are based on shared ancestry. Hybridization could be discarded because of the long distance between populations, such as *P. exserta* and *P. axillaris* A1 or *P. subandina* and all others, as the distance between populations exceeds 1 km, which is the maximum estimated distance for pollen dispersal (e.g., Turchetto *et al.*, 2015, 2022; Rodrigues *et al.*, 2019). Moreover, seed dispersal in *Petunia* is very limited, with seeds falling close to the mother plant by autochory (Stehmann *et al.*, 2009).

The evolutionary relationships and polymorphism-sharing in the *Petunia* long corolla tube clade could be explained based on the migration routes (Figure 3) from an albino ancestor (Wijsman, 1983), which originated in lowland (Reck-Kortmann *et al.*, 2014), ca. 2.8 Mya (Särkinen *et al.*, 2013), with subsequent diversification after colonized new environments or under pollinator selection (Fregonezi *et al.*, 2013). The albino lineage arose from the anthocyanin 2 (*AN2*) gene inactivation. The *AN2* is active in the species of the ST clade and responsible for the pink color (Quattrocchio *et al.*, 1999), the critical morphologic trait to attract bees. The ST species probably represent the genus ancestor, which appeared in lowlands in southern South America, likely in the Pampa (Reck-Kortmann *et al.*, 2014). The genus diverged from the sister group ca. 8.0 Mya (Särkinen *et al.*, 2013).

The first step in LT clade differentiation was the highlands' colonization, which also explains the presence of *P. occidentalis* in the clade despite its several morphological traits in common with ST species. *Petunia occidentalis* could represent an incomplete lineage sorting in the highland LT clade, sister of the albino *P. subandina*. The albino lineage would expand its distribution towards the southern South American grasslands as the Pleistocene climate changes allowed. The albino lineage colonized the Chaco, migrating to the north, and Pampa, growing to the south and east, nowadays represented by *P. parodii* (Giudicelli *et al.*, 2022) and its parapatric lineage *P. axillaris* A2.

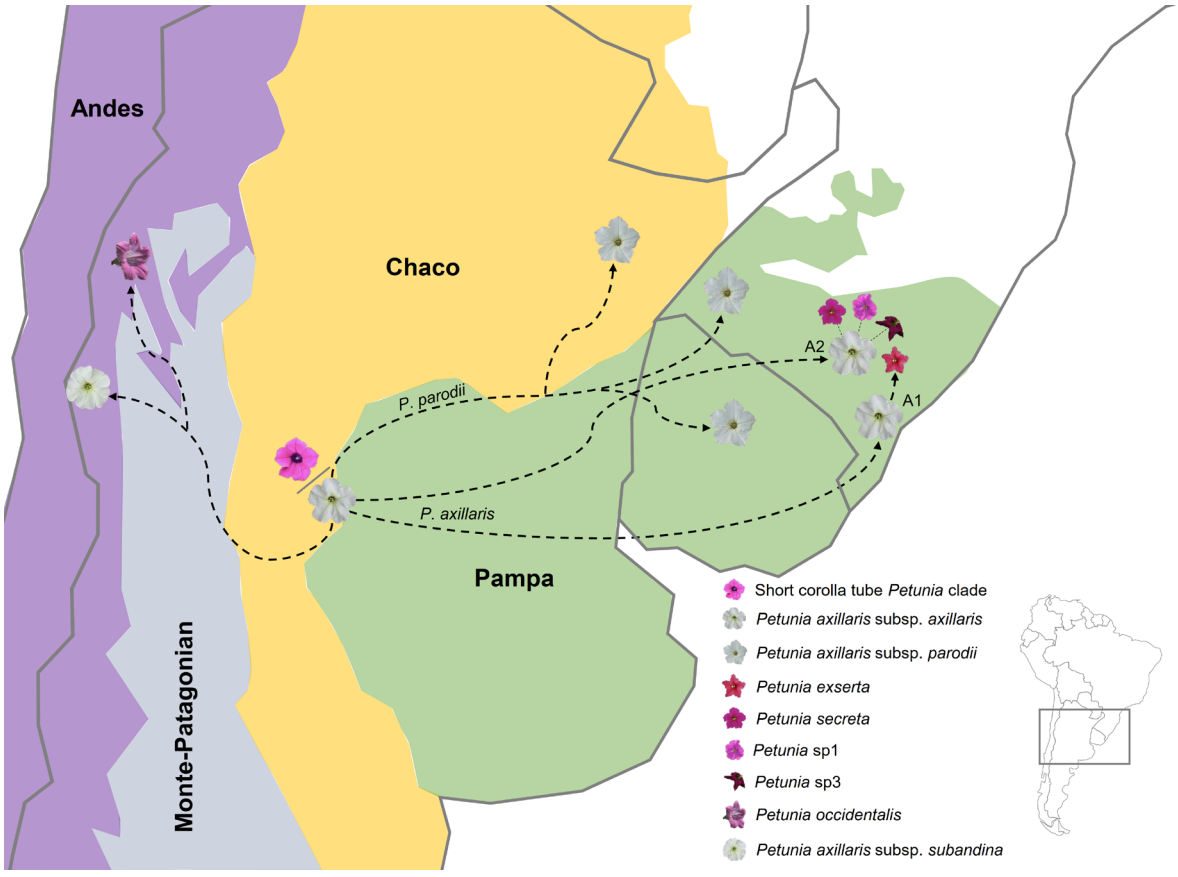

Figure 3 – Putative migratory routes and diversification for *Petunia* corolla tube clade species.

These last two lineages, *P. parodii* and *P. axillaris* A2, could have given rise to the colored lineages in the clade as they advanced colonizing new environments. The albino *P. parodii* and *P. axillaris* A1 and the red-flowered *P. exserta* share several polymorphisms (e.g., Segatto *et al.*, 2014; Li *et al.*, 2023), despite currently not being found close. Mainly regarding *P. exserta*, this species inhabits a very particular microenvironment, inside small caves where plants grow protected from direct sunlight and rain (Stehmann *et al.*, 2009; Segatto *et al.*, 2014), an inhospitable environment for other *Petunia* species. The two *P. exserta* lineages (E1 and E2) differ mainly in flower color hue (Figure 1) and distribution as each inhabits a different rock formation. *Petunia exserta* E2 is sympatric to some *P. axillaris* A2 populations, whereas *P. exserta* E1 occurs in the same formation as *P. secreta*. The red color of *P. exserta* petals is reached through a complex gene interaction that begins with a moderate upregulation and shifts in tissue specificity of the *Deep Purple* gene that restores anthocyanin biosynthesis (Berardi *et al.*, 2021). *P. exserta* retains the same nonfunctional *AN2* copy present in *P. axillaris.*

The pink-flowered *P. secreta* and *P.* sp1 differ from *P. axillaris* only based on the flower color (Stehmann and Semir, 2005), and this difference is due to the regain in *AN2* gene function (Esfeld *et al.*, 2018). *Petunia secreta* and *P.* sp1 occur in the same geographic area as *P. axillaris* A2. Still, whereas *P.* sp1 occupies a similar environment closely distributed to *P. axillaris*, *P. secreta* is found ca. 40 Km away from the closest *P. axillaris* A2 population and in an entirely diverse microenvironment (Turchetto *et al.*, 2016; Rodrigues *et al.*, 2019). *Petunia* sp3 is the *P. secreta* sister lineage, despite being morphologically similar to *P. exserta,* mainly regarding the exserted styles and anthers (Figure 1). Indeed, *P.* sp3, *P. secreta*, and *P. exserta* E1 are endemic to the same rock formation. Still, whereas *P. exserta* E1 occupies shaded locations, *P. secreta* and *P.* sp3 individuals grow in sunny places. Our results did not discard a hybrid status for *P.* sp3.

In conclusion, we described the evolutionary relationships among the *Petunia* long corolla tube clade due to ancestral geographic expansion with local adaptation and pollinator interaction as the vital diversification drivers. Structuring in LT lineages depends on isolation by distance, and high polymorphism-sharing is due to a common ancestor and rapid adaptive radiation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

Author Contributions

LBF led the project; CT, ALAS, and GM obtained original data; AB, GM, and SLB perfomed the analyses. All authors have commented on and approved the final manuscript.

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Internet Resources

FigTree v. 1.4.1, http://tree.bio.ed.ac.uk/software/figtree/ (accessed 10 September 2023).

Supplementary material

The following online material is available for this article:
Table S1 – Sampling information for *Petunia* long corolla tube clade and outgroups.

Table S2 – Genetic markers that were used to obtain the phylogenetic tree for the *Petunia* long corolla tube clade.

Table S3 – GenBank numbers for phylogenetic markers.

Table S4 – Nuclear microsatellite markers used to genotype *Petunia* long corolla tube clade.

Table S5 – Number of individuals per lineage per DAPC group (best K = 3).

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