



# Biogeographical history and diversification of *Petunia* and *Calibrachoa* (Solanaceae) in the Neotropical Pampas grassland

JEFERSON N. FREGONEZI<sup>1†</sup>, CAROLINE TURCHETTO<sup>1†</sup>, SANDRO L. BONATTO<sup>2</sup> and LORETA B. FREITAS<sup>1\*</sup>

<sup>1</sup>*Molecular Evolution Laboratory, Genetics Department, Universidade Federal do Rio Grande do Sul, PO Box 15053, 91501-970 Porto Alegre, RS, Brazil*

<sup>2</sup>*Genomic and Molecular Biology Laboratory, Pontifícia Universidade Católica do Rio Grande do Sul, Ipiranga 6681, 90610-001 Porto Alegre, RS, Brazil*

Received 10 February 2012; revised 14 June 2012; accepted for publication 22 June 2012

The Pampas in the southern Neotropics is a vast region with vegetation composed mainly of grasses, and it may be the least-studied ecosystem in southern South America. Contrary to what was thought until recently, this region is heterogeneous and harbours rich biodiversity and many endemic species; however, little is known about the current geographical distribution and evolution of its plants. Here, we present results from phylogeographical studies on two genera typical of open environments (*Petunia* and *Calibrachoa*) that occur in both the Pampas and the high-altitude grasslands of southern Brazil. The rapid radiations of *Petunia* and *Calibrachoa* are examples of how strong selective pressures for different pollinators, coupled with adaptation to edaphic and climatic differences, may drive the diversification of plants in the Pampas. We also discuss factors that could have affected and driven the diversification and speciation of plants in this environment. Further studies, including some focusing on other taxa, are required to characterize the diversification of plant species in this region more accurately. © 2012 The Linnean Society of London, *Botanical Journal of the Linnean Society*, 2013, **171**, 140–153.

**ADDITIONAL KEYWORDS:** floral diversification – low-altitude grassland – Neotropics – plant speciation – pollinators – South America.

## INTRODUCTION

Biodiversity patterns in a biome are the product of a long and complex history of evolutionary trends involving ecological processes and external environmental forces (Rull, 2011). The current patterns of geographical distribution and plant evolution are the result of geological processes and historical events (e.g. Quaternary climatic episodes) that occurred in the species range (Hewitt, 2000; Vargas, 2003; Pielou, 2008).

Several plant phylogeographical studies have examined the biogeographical effects of Quaternary

climatic changes on speciation and intraspecific differentiation. These have been mostly in temperate regions (Aoki *et al.*, 2006; Bauert *et al.*, 2007; Chen *et al.*, 2007, 2008; Fujii, 2007; Li *et al.*, 2008; Ortiz *et al.*, 2008; Latch *et al.*, 2009; Espíndola *et al.*, 2010), and only a limited number of studies have addressed patterns of plant diversification in the Neotropics (Lorenz-Lemke *et al.*, 2005; Speranza *et al.*, 2007; Miller *et al.*, 2008; Antonelli *et al.*, 2009; Collevatti, Rabelo & Vieira, 2009; Palma-Silva *et al.*, 2009; Ramos, De Lemos & Lovato, 2009; Arana *et al.*, 2010; Hoorn *et al.*, 2010; Ireland *et al.*, 2010; Lage-Novaes *et al.*, 2010; Antonelli & Sanmartín, 2011). The biotic consequences of climate change in the Southern Hemisphere have been discussed in relation to the contraction and expansion of the Andean flora (Simpson & Todzia, 1990; Premoli, Kitzberger &

\*Corresponding author. E-mail: loreta.freitas@ufrgs.br

†These authors contributed equally to the preparation of the manuscript.

Veblen, 2000; Pastorino & Gallo, 2002; Pastorino, Gallo & Hattemer, 2004; Muellner *et al.*, 2005; Serrano-Serrano *et al.*, 2010), the formation of the Atlantic forest refugia (Carnaval & Moritz, 2008; Carnaval *et al.*, 2009), the contraction of the Amazonian forest to islands or refugia (Ledru, 1993; Colinvaux *et al.*, 1996; Prance, 1996; Miles, Grainger & Phillips, 2004), the expansion of tropical and subtropical open formations (Behling, 1995, 1997, 2002; Behling & Pillar, 2007; Behling *et al.*, 2007) and the expansion of seasonally dry forests (Pennington, Prado & Pendry, 2000; Pennington *et al.*, 2004); however, there are few studies on the biogeography and diversification patterns of plants in this context in the tropical and temperate plains of southern South American grasslands (Speranza *et al.*, 2007; Solis-Neffa, 2009).

In the highland fields of southern South America (Behling & Pillar, 2007; Lorenz-Lemke *et al.*, 2010), it has been proposed that the main driver of the speciation of grassland species was isolation by distance (allopatric speciation) in the glacial and interglacial periods. When the forest expanded to the south during the warmer periods (interglacials), it isolated grassland populations, thus disrupting gene flow and promoting diversification and speciation. Less is known about the speciation of lowland species in southern South America, specifically in the Pampas. Unfortunately, few studies have examined speciation in the Pampas.

As the vegetation in the Pampas was probably not affected directly by forest expansion and still contains high plant species diversity, the hypothesis to be tested in this study is whether the biodiversity of the Pampas is also a product of allopatry and geographical isolation, or whether other factors, such as different ecological interactions, can explain the diversity observed in lowland grasslands on the southern edge of the Neotropics. To this end, we show results based on molecular data from two genera typical of open environments (*Petunia* Juss. and *Calibrachoa* Cerv., Solanaceae) that occur in both the Pampas and the high-altitude grasslands of southern Brazil.

## STUDY AREA

### LOCATION

The Pampas are entirely located in the Neotropics, bounded by the Paranense province to the north and the Espinal province to the west and south (Cabrera & Willink, 1980). This region is one of the largest warm grassland areas in the world, covering approximately 500 000 km<sup>2</sup> between latitudes 29°S and 39°S. It includes the plains of east-central Argentina, the Uruguayan territory and the southern half of Rio Grande do Sul (RS) Brazilian state (Fig. 1; Berretta,

2001; Pallarés, Berretta & Maraschin, 2005). This study is confined to the northern portion, the Uruguayan province (Cabrera & Willink, 1980), which includes the southern half of RS state, the whole of Uruguay and the southern regions of the Santa Fé and Entre Rios provinces in Argentina.

### GEOMORPHOLOGY

The Pampas is a geomorphologically complex region. In the west, it is mostly of Quaternary sedimentary origin, whereas, east of the Uruguay River, sediments range from Devonian to Holocene and were deposited in and over the Brazilian Shield. The Uruguayan and Brazilian Pampas have clear geological continuity and rocky outcrops that date from the Proterozoic to the Quaternary eras. These regions contain a great variety of soil types (derived from basalt, granite, sandstone, silt, etc.) and landforms. The western region of Uruguay presents a major geological discontinuity, with changes in soil and physiography (Grela, 2004).

### CLIMATE

During the Quaternary, the glacial cycles resulted in cold, dry conditions interrupted by warmer, wet periods. Consequently, there were several pulses of expansion and retraction of grasslands and, concomitantly, advances and retreats of the northern tropical forests. A dry period occurred at the end of the Pleistocene, during the Last Glacial Maximum (LGM) around 18 000 years ago, with a briefer and less severe dry period in the upper Holocene (Iriondo & García, 1993). During the drier periods, pronounced aeolian activity deflated and redeposited large masses of silt and fine sand over most of the lowlands (Iriondo & García, 1993; Panario & Gutierrez, 1999). Since the LGM, the vegetation of the region has oscillated between xerophytic and tropical and subtropical species. Xerophytic species advanced repeatedly to the north-east during the dry and colder periods and retreated to the south-west during the humid and warmer periods (Iriondo & García, 1993; Iriondo, 1999).

At present, the environment of Uruguay is humid. The average temperatures in the warmest and coldest months are approximately 22 °C and 8 °C, respectively. Rainfall exhibits ample seasonal and annual variation and is more abundant in the north (1500 mm on average) than in the south (1000 mm; Berretta, 2001; Roesch *et al.*, 2009).

### VEGETATION

In a broad sense, the Pampas is pure grassland, but the region contains several different physiographic



**Figure 1.** Map representing the extension of the Pampas according to Cabrera & Willink (1980).

formations (Bredenkamp, Spada & Kazmierczak, 2002). In the north, the grassland is invaded by seasonal forests, especially in stream and river valleys. Psammophytic and halophytic steppes occupy the coastal areas, the continental dunes and areas with sandy soils. Shrubby woodlands generally occur on soils with calcareous crusts and consist of shrubland and thornscrub communities, with scarce trees (Quattrocchio *et al.*, 2008). Overbeck *et al.* (2007) suggested that the southern Brazilian grasslands should be referred to simply as ‘campos’, which is the most frequently used vernacular term in Uruguay and southern Brazil to refer to natural grazing grasslands covered by grasses, other herbaceous plants, bushes and shrubs, with few trees (Berretta & Nascimento, 1991).

Poaceae are the most species-rich family in the Pampas, with approximately 200 species, including both warm season (C4) and winter (C3) species that are highly characteristic of these grasslands (Berretta, 2001). The most frequently found tribes are Paniceae (including genera with a large number of species, such as *Paspalum* L., *Panicum* L., *Axonopus* P.Beauv., *Setaria* P.Beauv. and *Digitaria* Haller),

Andropogoneae (with *Andropogon* L., *Bothriochloa* Kuntze and *Schizachyrium* Nees), Eragrostaceae (with *Eragrostis* Wolf and *Distichlis* Raf.), Chlorideae (with *Chloris* Sw., *Eleusine* Gaertn. and *Bouteloua* Lag.), Poaceae (with *Bromus* L., *Poa* L., *Melica* L., *Briza* L., *Lolium* L., *Dactylis* L. and *Festuca* L.), Stipeae (with *Stipa* L. and *Piptochaetium* J.Presl) and Agrostideae (with *Calamagrostis* Adans and *Agrostis* L.). Other families that occur in the region include Asteraceae, Fabaceae, Cyperaceae, Apiaceae, Rubiaceae, Plantaginaceae, Oxalidaceae and Solanaceae (Boldrini, 1997; Berretta, 2001).

Anthropogenic land use started approximately 9000 BP (Behling, Pillar & Bauermann, 2005) and, more recently, has had profound impacts on the natural structure of the communities through the introduction of exotic grass species, the conversion of native areas to agricultural lands and grazing areas, and the establishment of *Pinus* L. and *Eucalyptus* L’Her. forests. For example, it is estimated that 50% of the Brazilian Pampas is now composed of vegetation under human management (Roesch *et al.*, 2009). Current human activity in southern Brazil, including extensive areas used as pasture, sometimes makes it

difficult to ascertain whether some areas are open because of natural or anthropogenic causes.

#### PETUNIA AND CALIBRACHOA

*Petunia* and *Calibrachoa* are included in tribe Petunieae of Solanaceae, members of which are broadly distributed throughout South America. Considering the phylogenetic structure of the genera of Petunieae and their geographical distribution, it has been suggested that *Petunia* and *Calibrachoa* have ancestors of Andean origin (Olmstead *et al.*, 2008).

The species of *Petunia* and *Calibrachoa* were generally considered to be part of the same genus until 1985. Wijsman (1982, 1983) investigated the origin of the garden petunia by crossing different species and, based on these successful crosses, Wijsman & Jong (1985) concluded that the species should be classified into two different groups according to the chromosome numbers and certain morphological characters, such as leaf margins, aestivation, flower symmetry, calyx, anther colour and seed coats, and their classification is supported by cytotaxonomic, reproductive, anatomical and chemical studies (Ellinger *et al.*, 1992; Stehmann *et al.*, 1996; Watanabe *et al.*, 1996a, b; Reis, Sajo & Stehmann, 2002).

The evolutionary history of *Petunia* and *Calibrachoa* has been investigated recently using various molecular tools, and these analyses have revealed short genetic distances among species in each genus, with resultant poorly resolved phylogenetic trees indicating recent diversification (Ando *et al.*, 2005; Kulcheski *et al.*, 2006; Chen *et al.*, 2007). The monophyly of the genera was confirmed, and there is a large genetic distance between the clade *Petunia* plus *Calibrachoa* and other genera in the tribe.

*Petunia* and *Calibrachoa* spp. serve as excellent model taxa in which to investigate diversification and speciation in the Pampas. The 14 *Petunia* spp. are exclusively South American and most are found in southern and south-eastern Brazil (Stehmann *et al.*, 2009) in grasslands, including the Pampas. Known as the garden petunia, this genus has a long history of artificial crosses, including hybrids between *P. axillaris* (Lam.) Britton, Sterns & Poggenb and *P. integrifolia* (Hook.) Schinz & Thell, which are disseminated worldwide as ornamental plants (*Petunia* × *hybrida*, Hort. ex Vilm.).

Recent plastid phylogenetic analyses divided *Petunia* into two clades (Kulcheski *et al.*, 2006; Lorenz-Lemke *et al.*, 2010): a highland clade (> 500 m above sea level) with a time to the most recent common ancestor ( $T_{\text{MRCA}}$ ) estimated at ~0.9 Mya (0.6–1.3 Mya) and a lowland clade (species that grow in the Pampas region) with a  $T_{\text{MRCA}}$  estimated at ~1.1 Mya (0.8–1.5 Mya). These estimates were obtained from *trnH*-

*psbA* and *trnS-trnG* plastid spacer sequences and derived by a Bayesian method using a biogeographical calibration point. For both clades, species diversification may have been affected by the climate changes during the glacial and interglacial periods during the Pleistocene (Lorenz-Lemke *et al.*, 2010).

*Calibrachoa* encompasses 27 species that are found in open areas of southern South America, with an Atlantic subtropical distribution lying between 18°S and 37°S and occurring most densely in the east, along the coast. From the southern boundary, the genus is widely distributed in the Pampas, but is more restricted in the north-west, where rocky and shallow soils support grasslands that extend to the highlands of Santa Catarina and Paraná states. In south-eastern Brazil (the northern limit of the genus distribution in South America), it is represented by a few populations of *C. linoides* (Sendtn.) Wijsman, a relatively abundant species in the south, and *C. elegans* (Miers) Stehmann & Semir, an isolated micro-endemic species found at altitudes > 1000 m in Minas Gerais state. *Calibrachoa parviflora* (Juss.) D'Arcy is an exception, as it also occurs in North America and Europe. In South America, 15 *Calibrachoa* spp. inhabit the Paranense biogeographical province and 12 the Pampean and Espinal provinces (according to Cabrera & Willink, 1980).

Most *Calibrachoa* spp. are self-incompatible and have bee-pollinated flowers (melittophilous). Two exceptions are *C. sendtneriana* (R.E.Fr.) Stehmann & Semir and *C. serrulata* (L.B.Sm. & Downs) Stehmann & Semir, which are endemic to the high-altitude grasslands in Santa Catarina and have flowers adapted to hummingbird pollination (Stehmann & Semir, 2001). *Calibrachoa parviflora* and *C. pygmaea* (R.E.Fr.) Wijsman differ substantially from the other species in their reproductive biology and habit. The former is the only self-compatible species in the genus (Tsukamoto *et al.*, 2002), although it has floral traits of the melittophilous syndrome, with extremely small flowers compared with those of other bee-pollinated species. The latter has a unique white hypocrateriform corolla, which is most probably adapted to pollination by hawkmoths. *Calibrachoa parviflora* and *C. pygmaea* are also typically herbaceous species with an annual life cycle, whereas other *Calibrachoa* spp. are usually perennial shrubs (Fregonezi *et al.*, 2012).

The melittophilous species have magenta or purple or (infrequently) white or pink corollas. Some taxa have contrasting colours, such as *C. excellens* (R.E.Fr.) Wijsman *atropurpurea* Stehmann & Semir and *C. heterophylla* (Sendtn.) Wijsman, which have a dark purplish ring surrounding the opening of the corolla. In white or pale pink flowers, a striking vein can be observed, as in *C. humilis* (R.E.Fr.) Stehmann & Semir and *C. pubescens* (Spreng.) Stehmann.

## MATERIAL AND METHODS

## PETUNIA DATA

The sequences of the plastid gene spacers *trnH-psbA* and *trnS-trnG* were obtained by Lorenz-Lemke *et al.* (2006) and Lorenz-Lemke *et al.* (2010) from lowland species and highland species, respectively. Unpublished sequences from the *Petunia integrifolia* group (*P. integrifolia*, *P. inflata* R.E.Fr., *P. interior* T.Ando & Hashim. and *P. bajeensis* T.Ando & Hashim.) of the same genetic markers were included (A.M.C. Ramos-Fregonezi, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, pers. comm.). These sequences were obtained using the same methods as described in the two publications cited above.

The phylogenetic tree for the plastid haplotypes of *Petunia* spp. was constructed using a Bayesian approach with Beast version 1.6.1 (Drummond & Rambaut, 2007). Two independent runs of  $5 \times 10^7$  chains were performed, each sampling every 5000 generations. The parameters used were as follows: HKY substitution model with four gamma categories, a Yule tree prior and a relaxed clock model. The software Tracer version 1.5 (available at <http://tree.bio.ed.ac.uk/software/tracer/>) was used to check for convergence and adequate effective sample sizes (> 200) after the first 10% of generations had been discounted as burn-in. The maximum-clade-credibility trees were estimated using the program TreeAnnotator, which is part of the Beast package. Statistical support for the clades was determined by assessing the Bayesian posterior probability. The haplotype network obtained by Kulcheski *et al.* (2006) was used to compare those results with this phylogenetic tree and with flower morphology.

## CALIBRACHOA DATA

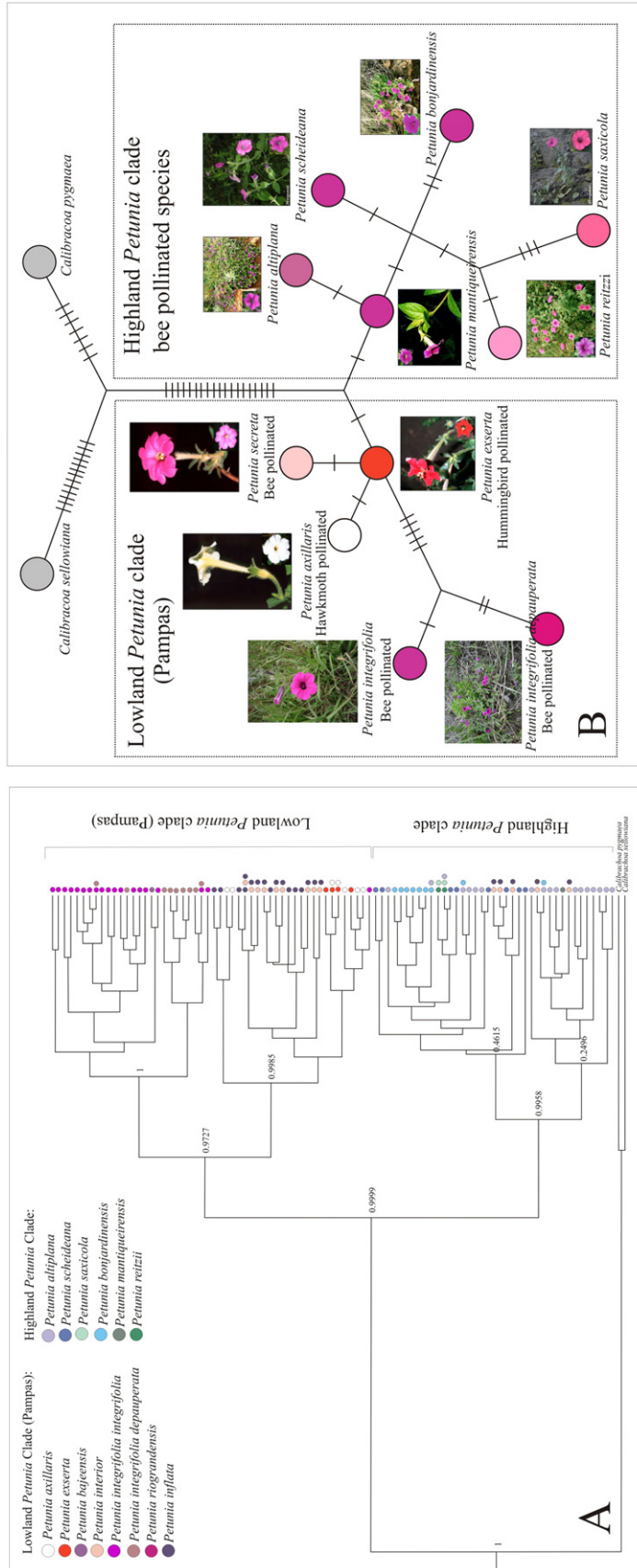
The phylogenetic tree for *Calibrachoa* spp. was based on five noncoding plastid DNA regions: the intergenic spacers *psbB-psbH* and *trnS-trnG* (described by Hamilton, 1999), the intergenic spacer *trnL-trnF* and the *trnL* intron (Taberlet *et al.*, 1991) and the intergenic spacer *trnH-psbA* (Sang, Crawford & Stuessy, 1997). The sequences of the first four regions above were described in a study by Fregonezi *et al.* (2012), and the *trnH-psbA* intergenic spacer was added to the dataset to increase the resolution. All samples used were collected in the field, and detailed information about the *trnH-psbA* samples used is given in Supporting Information Appendix S1. Samples of *P. axillaris* (Lam.) Britton, Sterns & Poggenb. and *P. integrifolia* were used as the outgroup. The maximum likelihood (ML) phylogenetic analysis was estimated using PAUP\* version 4.0b10 (Swofford, 2002) as described in Fregonezi *et al.* (2012) and in the legend of Figure 3.

## RESULTS AND DISCUSSION

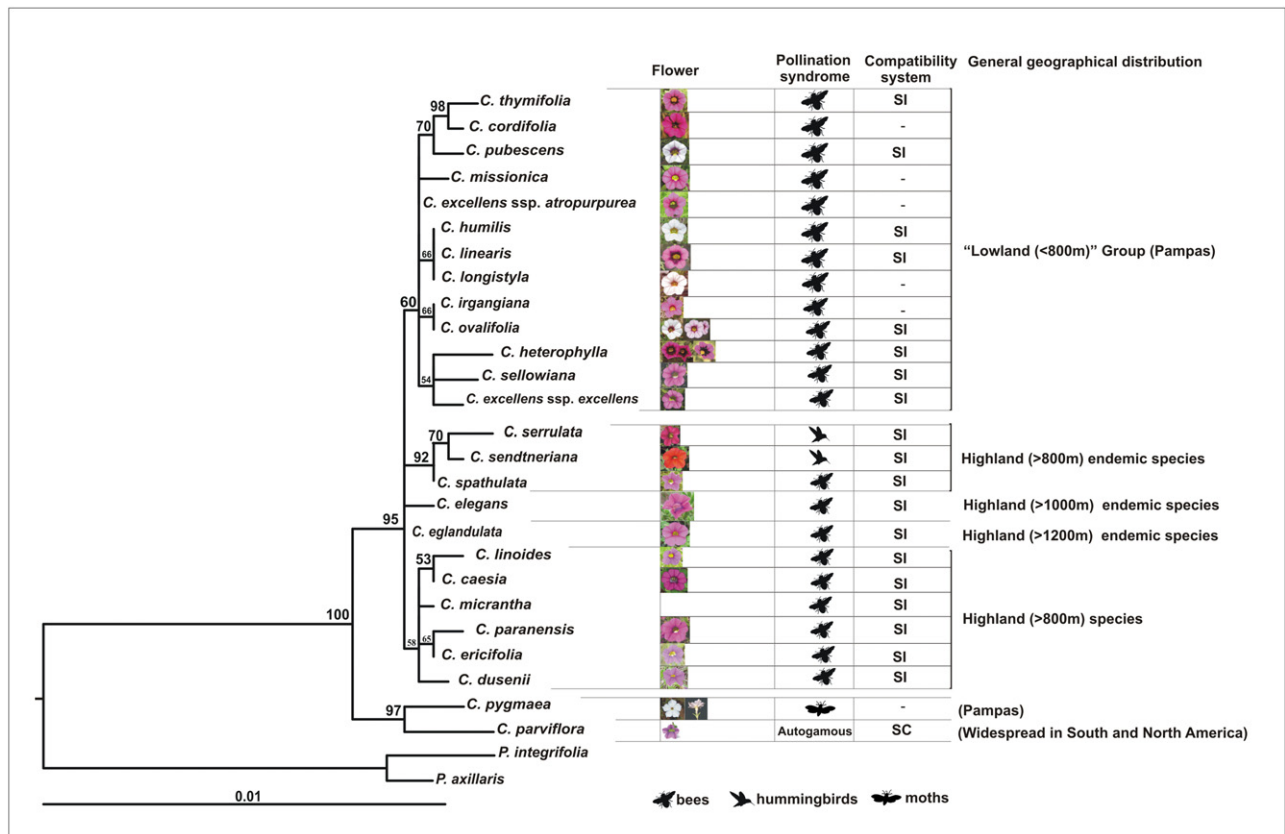
As shown in Figure 2, the lowland (Pampas) *Petunia* clade comprises species with flowers of different shapes and colours. *Petunia axillaris*, *P. exserta* Stehmann and *P. secreta* Stehmann & Semir have white, red and purple hypocrateriform corollas, respectively, with filaments adnate to the middle of the tube and yellow pollen; *P. integrifolia* has a purple funnel-form corolla, with filaments adnate to the base of the tube and bluish pollen (Kulcheski *et al.*, 2006; Stehmann *et al.*, 2009). In the Pampas region, these species are often sympatric, with some populations growing together in specific locations (Lorenz-Lemke *et al.*, 2006; personal observations in the field). The plants are self-compatible (except for some lineages of *P. axillaris*) and the flowers are pollinated by moths, birds and bees, respectively (Ando *et al.*, 1995, 2001; Stehmann *et al.*, 2009; Venail, Dell'Olivo & Kuhlemeier, 2010; Klahre *et al.*, 2011). The highland species have funnel-form or campanulate pink or purple corollas, filaments adnate to the base of the tube and bluish pollen. All highland species have allopatric distributions, are self-incompatible and probably are pollinated by bees (Stehmann *et al.*, 2009; Lorenz-Lemke *et al.*, 2010).

Lorenz-Lemke *et al.* (2010) suggested that diversification of the highland species occurred as a result of expansion of the ancestral species during glacial periods, followed by fragmentation when forests of *Araucaria* Juss. spread and surrounded the grassland areas, which subsequently became isolated islands in the areas of highest altitude. This process most probably led to population fragmentation and local differentiation. Which factors might have been important to the speciation of Pampas *Petunia* (the lowland clade)? Considerable evidence indicates that ecological factors, such as climate and geomorphology, are important to diversification in plant species in the Pampas (see previous studies mentioned above), and may also be important factors in the diversification of lowland *Petunia* spp. Another question about these species, given the observed variation in reproductive characters (Ando *et al.*, 2005; Lorenz-Lemke *et al.*, 2006), is the role of pollinators; the differences observed among the species could be selected by pollinators and/or by different environmental conditions.

Although the *Petunia* spp. in the Pampas occur in sympatry and may artificially cross-fertilize (Wijsman, 1982; Watanabe *et al.*, 1996a, b; Ando *et al.*, 2001), hybrid forms are rare in nature (Ando *et al.*, 2001; Lorenz-Lemke *et al.*, 2006). Reproductive isolation in the species is thus probably effective. *Petunia hybrida* has been widely used as a model system for molecular genetics, providing a range of genetic and molecular tools (Gerats & Vandenbussche, 2005). These tools



**Figure 2.** A, Bayesian phylogenetic tree based on two plastid sequences (*trnG-trnS* and *trnH-psbA*) for *Petunia* spp. sampled across the geographical range, except for *P. occidentalis* (Lorenz-Lemke *et al.*, 2006, 2010; A.M.C. Ramos-Fregonezi, Universidade Federal do Rio Grande do Sul, Brazil, unpubl. data). The tips represent one sequence per haplotype. B, Haplotype network representing evolutionary relationships among *Petunia* spp. modified from Kulcheski *et al.* (2006). Note the morphological diversification and pollination syndromes found in *Petunia* spp. from the Pampas (Lowland *Petunia* clade). *Calibrachoa* spp. were used as the outgroup.



**Figure 3.** Majority rule consensus tree for the genus *Calibrachoa* of the maximum likelihood analysis from 1000 bootstrap replicates using the Transversal model of nucleotide substitution (TVM) model, based on the combined dataset for the plastid *psbB-psbH*, *trnH-psbA*, *trnL-trnF* and *trnS-trnG* intergenic spacers and the *trnL* intron. The numbers on the branches indicate bootstrap values > 50%. The flowers, floral syndromes and compatibility systems (compiled from Tsukamoto *et al.*, 2002) for each species are indicated, if available.

offer the prospect of an integrated ecological and molecular approach to the analysis of isolation mechanisms in this genus. Previous studies have identified major quantitative trait loci (QTLs) for several traits relevant to pollinator preferences (Stuurman *et al.*, 2004; Galliot *et al.*, 2006; Venail *et al.*, 2010). Dell'Olivo *et al.* (2011) suggested that the emergence of divergent pollination syndromes alone is unlikely to lead to complete genetic isolation in *P. axillaris* and *P. integrifolia* in Uruguay. In addition, gametic isolation is also high in sympatric populations. The floral characters of these species suggest that each conforms to a different pollination syndrome (Gübitz *et al.*, 2009). *Petunia integrifolia* has small, purple, almost scentless flowers that produce hexose-rich nectar (Stuurman *et al.*, 2004; Galliot *et al.*, 2006; Hoballah *et al.*, 2007) and are pollinated by bees (Ando *et al.*, 2001). In contrast, *P. axillaris* flowers are white, have a long corolla tube and produce a strong scent at night; the nectar is sucrose-rich (Stuurman *et al.*, 2004; Oyama-Okubo *et al.*, 2005; Galliot *et al.*, 2006; Hoballah *et al.*, 2007)

and the flowers are pollinated by nocturnal hawk-moths (Ando *et al.*, 2001; Hoballah *et al.*, 2007), although other animals may visit (Ando *et al.*, 2001; our field observations). Although both species occur in sympatry in the wild, natural hybrids have never been found (Ando *et al.*, 2001), suggesting that, in addition to spatial isolation, other strong isolation barriers exist.

*Petunia axillaris*, *P. exserta* and *P. secreta* constitute another example of speciation in the Pampas. They are closely related and share several morphological characteristics, including a long and salverform (hypocrateriform) corolla tube, an erect growth habit and yellow pollen (Fig. 2). The last two species are endemic to the Serra do Sudeste region, RS state, one of the centres of diversity of the genus. *Petunia axillaris* has a large range throughout the Pampas, and even grows in sympatry with *P. exserta* in the Serra do Sudeste region (Fig. 1). The major differences among the three species may be associated with different floral syndromes. *Petunia exserta* has red, nonfragrant flowers

with exerted styles and anthers (hummingbird pollination syndrome), whereas *P. axillaris* has white flowers that are strongly fragrant after dusk (hawkmoth pollination syndrome). *Petunia secreta* has magenta flowers and melittophily typical of species with very long and narrow tubes; the nectar is virtually inaccessible to bees with short tongues and only pollen is available to visitors. These morphological characteristics suggest that the latter species may have secondarily adjusted melittophily and probably originated from ancestral long-tongued pollinators able to reach the nectar chamber, as in *P. axillaris* and *P. exserta*. Observations in a population of *P. secreta* in RS state showed that this species is visited by bees of the genus *Pseudagapostemon* (Stehmann, 1999). *Petunia axillaris* grows in open and sunny habitats, from the Andean slopes in Argentina to Uruguay and southern Brazil, whereas *P. exserta* can be found only in shady cracks (shelters) in sandstone towers, where it is protected from direct rain and sunlight. Such ecological requirements make *P. exserta* endemic to specific rock formations in the Serra do Sudeste region of RS state, where it is sympatric with *P. axillaris*, although the latter is found only in open habitats. Lorenz-Lemke *et al.* (2006) described the occurrence of natural hybrids between *P. exserta* and *P. axillaris*; these hybrids are characterized morphologically by pinkish corolla lobes and weakly exerted stamens and styles, and were found only inside shelters, but this phenomenon is not yet well understood.

A. L. A. Segatto (Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, unpubl. data) proposed a scenario in which recent and sympatric speciation occurred between *P. axillaris* and *P. exserta*, suggesting that *P. exserta* may have diverged from a regional population of the widely distributed *P. axillaris* during the LGM and that hybridization has occurred subsequently in some seasons and populations. These species could be an example of sympatric speciation in the Pampas that occurred by adaptive radiation of the floral syndrome and adaptation to different microhabitats.

A similar process may have occurred in the *P. axillaris* Pampas complex. This species has three recognized subspecies (Ando, 1996): *P. axillaris* ssp. *axillaris* (exclusively Pampas), *P. axillaris* ssp. *parodii* (Steere) Cabrera (Pampas and Chaco) and *P. axillaris* ssp. *subandina* Ando (Pampas and sub-Andean Sierras). These subspecies are geographically delimited on the basis of flower morphology (Ando, 1996; Kokubun *et al.*, 2006). These authors proposed that the Pampas subspecies, *P. axillaris axillaris* and *P. axillaris parodii*, are allopatric in Uruguay and that the limit of the distribution of the two subspecies corresponds to the Rio Negro, which could act as a gene flow barrier. In addition, C. Turchetto (Univer-

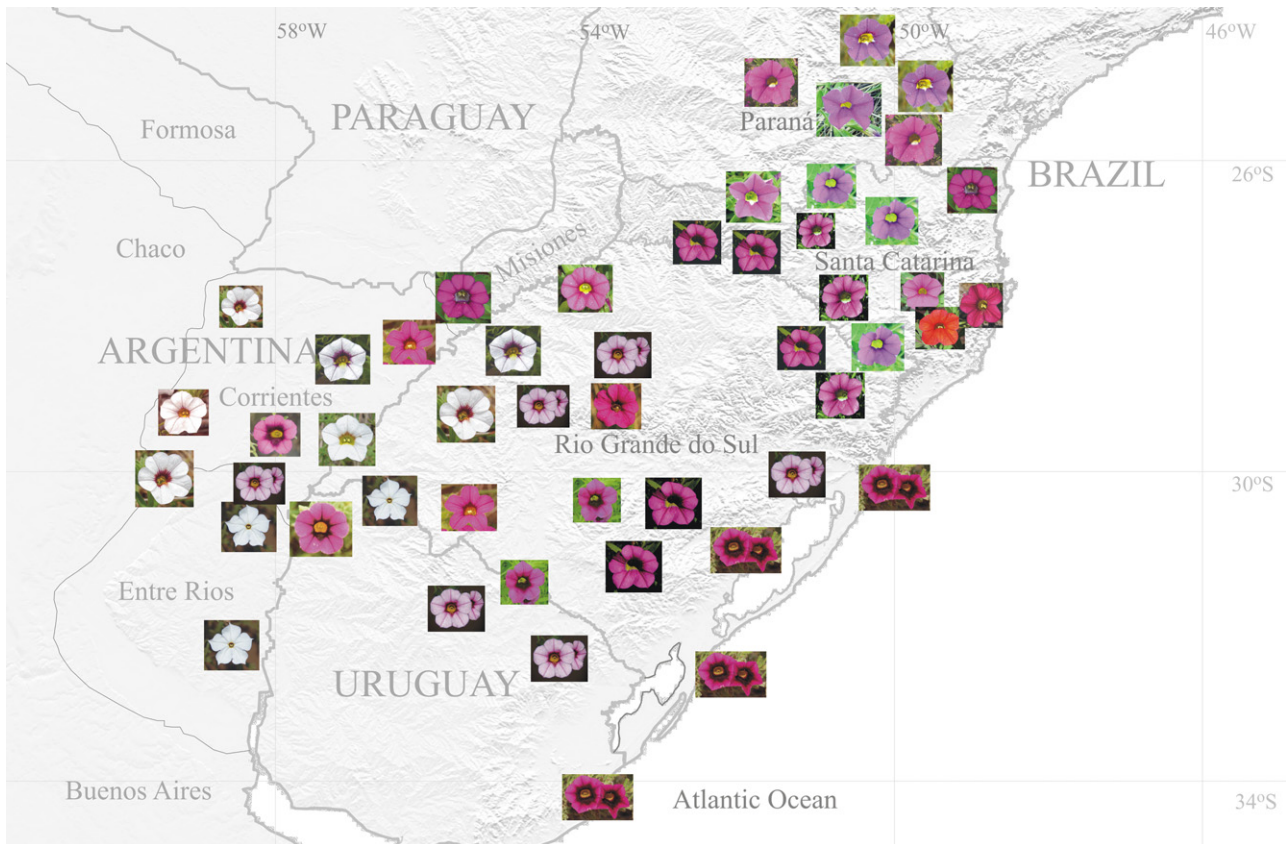
sidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, unpubl. data) identified a major phylogeographical break in the distribution of the subspecies corresponding to climatic discontinuities. Soils could also act as a limiting factor and barrier, as the range of *P. axillaris* ssp. *parodii* includes areas on basaltic soil (see description on geology above), suggesting that the environment could affect morphology through selection. Moreover, Venail *et al.* (2010) studied *P. axillaris* ssp. *axillaris* and ssp. *parodii*, which are similar in all relevant traits for pollinator behaviour, except for floral limb size and tube length. Both of these traits segregate in an interspecific F2 population. Genetically, these traits appear to be complex; three and four medium-effect QTLs were found for limb area and tube length. F2 individuals that differed exclusively in either tube length or limb area were used for pollinator choice experiments. The hawkmoth, *Manduca sexta*, was more strongly attracted to flowers with larger limb sizes and to flowers with shorter tube lengths. In this sense, the relative importance of pollinator selection could be attributed to the morphology of *P. axillaris*, and our results for the Pampas subspecies of *P. axillaris* might suggest incipient speciation driven by adaptation to different environmental conditions and pollinator-mediated selection (C. Turchetto, unpubl. data).

In summary, studies with *Petunia* spp. are consistent with the hypothesis that historical events, such as climate change in glacial periods, affected the distribution of these species, and that ecological factors, with selective pressure of pollinators on morphological characters, may have been important drivers of speciation.

In relation to *Calibrachoa*, the phylogenetic tree presented in Figure 3 shows a clear division of the genus into two clades: one containing the Pampas species *C. parviflora* and *C. pygmaea* and another comprising the remaining species. In the second clade, subclades appear to be associated with the geographical distribution of species. The 'lowland' region refers to the species that are distributed in the Pampas, with specimens collected in the lowlands of RS state, Uruguay and Argentina. In these areas, *C. cordifolia* Stehmann & L.Aguiar, *C. excellens atropurpurea*, *C. excellens* Wijsman *excellens*, *C. heterophylla* (Sendtn.) Wijsman, *C. humilis*, *C. irgangiana* Stehmann, *C. linearis* (Hook.) Wijsman, *C. longistyla* Stehmann & Greppi, *C. missionica* Stehmann & Semir, *C. ovalifolia* (Miers) Stehmann & Semir, *C. pubescens*, *C. thymifolia* (A.St.-Hil.) Stehmann & Semir, *C. parviflora* and *C. pygmaea* can be found.

This phylogenetic tree has short branches and low support values, even using markers with high levels of variation in other studies, including population analysis (Hamilton, Braverman & Soria-Hernanz,





**Figure 4.** Overall geographical distribution of flower morphological types in *Calibrachoa* spp. The arrangement of figures on the map reflects the general area of occurrence for the representative species of each morphology.

2003; Oh, Kajita & Murata, 2003; Lorenz-Lemke *et al.*, 2006; Bettin *et al.*, 2007). These data suggest rapid morphological divergence in the genus, given the low variation found in the markers used.

The major floral and reproductive divergences probably occurred in the Pampas region. As most species of the genus have melittophilous pollination syndromes, and related genera also have species pollinated by bees, we suggest that the ancestor of the whole group was melittophilous. The Pampas species (also including *C. parviflora* and *C. pygmaea*) have different reproductive strategies, with autogamous species (*C. parviflora*) and self-incompatible species pollinated either by bees or by moths (*C. pygmaea*).

The geographical distribution of flower types in the genus also shows that the highest floral diversity is found in the Pampas (Fig. 4). In the subtropical highlands (the central and northern distribution of the genus), the majority of species have melittophilous syndromes. The exceptions are *C. sendtneriana* and *C. serrulata*, which inhabit the highest mountains in Santa Catarina state and have flowers pollinated by hummingbirds.

Thus, these two main regions (lowland and highland areas of species distribution) appear to have

undergone distinct mechanisms of differentiation and speciation in a short period of time. In the highlands, the grasslands were abundant during the colder periods, but were restricted to higher altitudes during the interglacial periods (Behling, 1995, 1997, 2002; Behling & Lichte, 1997). The consecutive cycles of expansion and contraction of grasslands and forests accordingly changed the geographical distribution of *Calibrachoa* populations. The main speciation mechanism therefore appears to be allopatric, without major changes in floral structures.

The Pampas were not strongly affected by the expansion of forest over the grasslands, which have persisted through glacial and interglacial times. The gallery forests found today appeared in the pollen record only approximately 5000 years ago (Behling *et al.*, 2005; Behling & Pillar, 2007), but the number of plant species is as high as that observed in subtropical highlands. In this case, the higher speciation rate may be attributed to the type of substrate and to a larger number of pollinators. In the phylogenetic tree (Fig. 3), three groups of closely related species should be highlighted: (1) *C. thymifolia*, *C. cordifolia* and *C. pubescens*, with purple flowers with dark rings, purple and white flowers, respectively; (2)

*C. humilis*, *C. linearis* and *C. longistyla* with white flowers, purple flowers with dark rings and white flowers, respectively; and (3) *C. irgangiana* and *C. ovalifolia*, with purple flowers and flowers ranging from pink to white, respectively. Species are found inhabiting mainly sandy soils (e.g. *C. heterophylla*, *C. linearis* and *C. thymifolia*), flooded or wet soils (e.g. *C. pygmaea*) or latosols (e.g. *C. excellens*), with the majority being found on rocky outcrops. Although most melittophilous species have magenta or purple flowers, in the Pampas region, bright flowers (white or pink) and purple flowers with a dark ring are common, suggesting that different pollinators exist in the region. These pollinators may be a mechanism of isolation among groups of sister species. Therefore, in the Pampas region, mechanisms of speciation other than allopatry may have driven the speciation of this group; these could include adaptive evolution to different pollinators. In melittophilous species, the putative pollinators can differ slightly in the way in which they detect flower colour.

#### CONCLUSIONS AND FUTURE PROSPECTS

Speciation during grassland formation does not seem to have been affected directly by the expansion and contraction of forests or by ice-sheet advances in relation to *Petunia* and *Calibrachoa* lowland clades. In the Pampas region, a climatic gradient and significant soil differences are apparent, and ecological factors were important in the speciation process of these plants. As discussed here, these appear to affect the patterns of diversification and distribution of these species in the Pampas. Studies on other taxa are crucial to the development of a better understanding and, in the future, to the production of a general hypothesis of diversification in this region. Studies of the pollination biology and mating patterns in *Petunia* and *Calibrachoa* spp. and other taxa are essential to the elucidation of the mechanisms of speciation in the Pampas, as pollinators seem to be essential in the evolution of floral traits and in subsequent ecological speciation. In addition to these high-level studies, there is an urgent need for more basic taxonomic studies. Unfortunately, time is running out fast for this area, given the increasing anthropogenic degradation of the biome, with the introduction of exotic plant species as pasture cover and use by the timber industry.

#### ACKNOWLEDGEMENTS

The authors thank A. P. Lorenz-Lemke, A. M. C. Ramos-Fregonezi, G. Mäder and J. R. Stehmann (Brazil), V. Sollis-Neffa and G. Seijo (Argentina) and P. Speranza (Uruguay) for assistance in field collec-

tion, and two anonymous reviewers and the Associate Editor for criticisms and suggestions that improved the manuscript. This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS).

#### REFERENCES

- Ando T.** 1996. Distribution of *Petunia axillaris* (Solanaceae) and its new subspecies in Argentina and Bolivia. *Acta Fitotaxonomica et Geobotanica* **47**: 19–30.
- Ando T, Iida S, Kokubun H, Ueda Y, Marchesi E.** 1995. Distribution of *Petunia axillaris sensu lato* in Uruguay as revealed by discriminant analysis of the live plants. *Journal of the Japanese Society for Horticultural Science* **64**: 381–391.
- Ando T, Kokubun H, Watanabe H, Tanaka N, Yukawa T, Hashimoto G, Marchesi E, Suárez E, Basualdo IL.** 2005. Phylogenetic analysis of *Petunia sensu* Jussieu (Solanaceae) using chloroplast DNA RFLP. *Annals of Botany* **96**: 289–297.
- Ando T, Nomura M, Tsukahara J, Watanabe H, Kokubun H, Tsukamoto T, Hashimoto G, Marchesi E, Kitching IJ.** 2001. Reproductive isolation in a native population of *Petunia sensu* Jussieu (Solanaceae). *Annals of Botany* **88**: 403–413.
- Antonelli A, Nylander JAA, Persson C, Sanmartin I.** 2009. Tracing the impact of the Andean uplift on Neotropical plant evolution. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 9749–9754.
- Antonelli A, Sanmartin I.** 2011. Why are there so many plant species in the Neotropics? *Taxon* **60**: 403–414.
- Aoki K, Matsumura T, Hattori T, Murakami N.** 2006. Chloroplast DNA phylogeography of *Photinia glabra* (Rosaceae) in Japan. *American Journal of Botany* **93**: 1852–1858.
- Arana MV, Gallo LA, Vendramin GG, Pastorino MJ, Sebastiani F, Marchelli P.** 2010. High genetic variation in marginal fragmented populations at extreme climatic conditions of the Patagonian cypress *Austrocedrus chilensis*. *Molecular Phylogenetics and Evolution* **54**: 941–949.
- Bauert MR, Kaelin M, Edwards PJ, Baltisberger M.** 2007. Genetic structure and phylogeography of alpine relict populations of *Ranunculus pygmaeus* and *Saxifraga cernua*. *Botanica Helvetica* **117**: 181–196.
- Behling H.** 1995. A high-resolution Holocene pollen record from Lago do Pires, SE Brazil – vegetation, climate and fire history. *Journal of Paleolimnology* **14**: 253–268.
- Behling H.** 1997. Late Quaternary vegetation, climate and fire history of the *Araucaria* forest and campos region from Serra Campos Gerais, Parana State (South Brazil). *Review of Palaeobotany and Palynology* **97**: 109–121.
- Behling H.** 2002. South and southeast Brazilian grasslands during late Quaternary times: a synthesis. *Palaeogeography, Palaeoclimatology, Palaeoecology* **177**: 19–27.

- Behling H, Lichte M. 1997.** Evidence of dry and cold climatic conditions at glacial times in tropical Southeastern Brazil. *Quaternary Research* **48**: 348–358.
- Behling H, Pillar VD. 2007.** Late Quaternary vegetation, biodiversity and fire dynamics on the Southern Brazilian highland and their implication for conservation and management of modern *Araucaria* forest and grassland ecosystems. *Philosophical Transactions of the Royal Society B: Biological Sciences* **362**: 243–251.
- Behling H, Pillar VD, Bauermann SG. 2005.** Late Quaternary grassland (Campos), gallery forest, fire and climate dynamics, studied by pollen, charcoal and multivariate analysis of the São Francisco de Assis core in western Rio Grande do Sul (Southern Brazil). *Reviews of Palaeobotany and Palynology* **133**: 235–248.
- Behling H, Pillar VD, Muller SC, Overbeck GE. 2007.** Late-Holocene fire history in a forest–grassland mosaic in southern Brazil: implications for conservation. *Applied Vegetation Science* **10**: 81–90.
- Berretta EJ. 2001.** Ecophysiology and management response of the subtropical grasslands of Southern America. In: Gomide JA, Mattos WRS, Silva SC, eds. *Proceedings of the XIX International Grassland Congress*. Piracicaba: Programa Cooperativo para el Desarrollo Tecnológico Agroalimentario y Agroindustrial del Cono Sur, 939–946.
- Berretta EJ, Nascimento D. 1991.** *Glosario estructurado de términos sobre pasturas y producción animal*. Montevideo: IICA – PROCISUR.
- Bettin O, Cornejo C, Edwards PJ, Holderegger R. 2007.** Phylogeography of the high alpine plant *Senecio halleri* (Asteraceae) in the European Alps: *in situ* glacial survival with postglacial stepwise dispersal into peripheral areas. *Molecular Ecology* **16**: 2517–2524.
- Boldrini II. 1997.** Campos do Rio Grande do Sul: caracterização fisionômica e problemática ocupacional. *Boletim do Instituto de Biociências* **53**: 1–39.
- Bredenkamp GJ, Spada F, Kazmierczak E. 2002.** On the origin of Northern and Southern Hemisphere grasslands. *Plant Ecology* **163**: 209–229.
- Cabrera AL, Willink A. 1980.** *Biogeografía de América Latina*. Washington DC: OEA.
- Carnaval AC, Hickerson MJ, Haddad CFB, Rodrigues MT, Moritz C. 2009.** Stability predicts genetic diversity in the Brazilian Atlantic Forest hotspot. *Science* **323**: 785–789.
- Carnaval AC, Moritz C. 2008.** Historical climate modeling predicts patterns of current biodiversity in the Brazilian Atlantic forest. *Journal of Biogeography* **35**: 1187–1201.
- Chen KM, Abbott RJ, Milne RI, Tian XM, Liu JQ. 2008.** Phylogeography of *Pinus tabulaeformis* Carr. (Pinaceae), a dominant species of coniferous forest in northern China. *Molecular Ecology* **17**: 4276–4288.
- Chen S, Matsubara K, Kokubun H, Kodama H, Watanabe H, Marchesi E, Ando T. 2007.** Reconstructing historical events that occurred in the petunia *Hf1* gene, which governs anthocyanin biosynthesis, and effects of artificial selection by breeding. *Breeding Science* **57**: 203–211.
- Colinvaux PA, DeOliveira PE, Moreno JE, Miller MC, Bush MB. 1996.** A long pollen record from lowland Amazonia: forest and cooling in glacial times. *Science* **274**: 85–88.
- Collevatti RG, Rabelo SG, Vieira RF. 2009.** Phylogeography and disjunct distribution in *Lychnophora ericoides* (Asteraceae), an endangered Cerrado shrub species. *Annals of Botany* **104**: 655–664.
- Dell’Olivo A, Hoballah ME, Guebitz T, Kuhlemeier C. 2011.** Isolation barriers between *Petunia axillaris* and *Petunia integrifolia* (Solanaceae). *Evolution* **65**: 1979–1991.
- Drummond AJ, Rambaut A. 2007.** BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7**: 214.
- Ellinger CA, Wong RY, Benson M, Gaffield W, Wais AC. 1992.** Diterpenes of *Calibrachoa parviflora*. *Journal of Natural Products (Lloydia)* **55**: 1477–1487.
- Espindola A, Buerki S, Bedalov M, Küpfer P, Alvarez N. 2010.** New insights into the phylogenetics and biogeography of *Arum* (Araceae): unravelling its evolutionary history. *Botanical Journal of the Linnean Society* **163**: 14–32.
- Fregonezi JN, Freitas LB, Bonatto S, Semir J, Stehmann JR. 2012.** Infrageneric classification of *Calibrachoa* (Solanaceae) based on morphological and molecular evidence. *Taxon* **61**: 120–130.
- Fujii N. 2007.** Chloroplast DNA phylogeography of *Pedicularis* ser. *Gloriosae* (Orobanchaceae) in Japan. *Journal of Plant Research* **120**: 491–500.
- Galliot C, Hoballah ME, Kuhlemeier C, Stuurman J. 2006.** Genetics of flower size and nectar volume in *Petunia* pollination syndromes. *Planta* **225**: 203–212.
- Gerats T, Vandenbussche M. 2005.** A model system comparative for research: *Petunia*. *Trends in Plant Science* **10**: 251–256.
- Grela IA. 2004.** *Geografía florística de las especies arbóreas de Uruguay: propuesta para la delimitación de dendrofloras*. Programa de Desarrollo de las Ciencias Básicas. Montevideo: Universidad de la República.
- Gübitz T, Hoballah ME, Dell’Olivo A, Kuhlemeier C. 2009.** *Petunia* as a model system for the genetics and evolution of pollination syndromes. In: Gerats T, Strommer J, eds. *Petunia: evolutionary, developmental and physiological genetics*. New York: Springer, 29–50.
- Hamilton MB. 1999.** Four primers pairs for the amplification of chloroplast intergenic regions with intraspecific variation. *Molecular Ecology* **8**: 513–525.
- Hamilton MB, Braverman JM, Soria-Hernanz DF. 2003.** Patterns and relative rates of nucleotide and insertion/deletion evolution at six chloroplast intergenic regions in new world species of the Lecythidaceae. *Molecular Biology and Evolution* **20**: 1710–1721.
- Hewitt GM. 2000.** The genetic legacy of the Quaternary ice ages. *Nature* **405**: 907–913.
- Hoballah ME, Guebitz T, Stuurman J, Broger L, Barone M, Mandel M, Dell’Olivo A, Arnold M, Kuhlemeier C. 2007.** Single gene-mediated shift in pollinator attraction in *Petunia*. *The Plant Cell* **19**: 779–790.
- Hoorn C, Wesselingh FP, Steege H, Bermudez MA, Mora A, Sevink J, Sanmartin I, Sanchez-Meseguer A, Anderson CL, Figueiredo JP, Jaramillo C, Riff D,**

- Negri FR, Hooghiemstra H, Lundberg J, Stadler T, Saerkinen T, Antonelli A. 2010. Amazonia through time: Andean uplift, climate change, landscape evolution, and biodiversity. *Science* **330**: 927–931.
- Ireland HE, Kite GC, Veitch NC, Chase MW, Schrire B, Lavin M, Linares J, Pennington RT. 2010. Biogeographical, ecological and morphological structure in a phylogenetic analysis of *Ateleia* (Swartzieae, Fabaceae) derived from combined molecular, morphological and chemical data. *Botanical Journal of the Linnean Society* **162**: 39–53.
- Iriondo MH. 1999. Climatic changes in the South American plains: records of a continent-scale oscillation. *Quaternary International* **57–58**: 93–112.
- Iriondo MH, García NO. 1993. Climatic variations in the Argentina plains during the last 18,000 years. *Palaeogeography, Palaeoclimatology, Palaeoecology* **101**: 209–220.
- Klahre U, Gurba A, Hermann K, Saxenhofer M, Bosso lini E, Guerin PM, Kuhlemeier C. 2011. Pollinator choice in *Petunia* depends on two major genetic loci for floral scent production. *Current Biology* **21**: 730–739.
- Kokubun H, Nakano M, Tsukamoto T, Watanabe H, Hashimoto G, Marchesi E, Bullrich L, Kao T, Ando T. 2006. Distribution of self-compatible and self-incompatible populations of *Petunia axillaris* (Solanaceae) outside Uruguay. *Journal of Plant Research* **119**: 419–430.
- Kulcheski FR, Muschner VC, Lorenz-Lemke AP, Stehmann JR, Bonatto SL, Salzano FM, Freitas LB. 2006. Molecular phylogenetic analysis of *Petunia* Juss. (Solanaceae). *Genetica* **126**: 3–14.
- Lage-Novaes RM, De Lemos Filho JP, Ribeiro RA, Lovato MB. 2010. Phylogeography of *Plathymenia reticulata* (Leguminosae) reveals patterns of recent range expansion towards northeastern Brazil and southern cerrados in eastern tropical South America. *Molecular Ecology* **19**: 985–998.
- Latch EK, Heffelfinger JR, Fike JA, Rhodes OE. 2009. Species-wide phylogeography of North American mule deer (*Odocoileus hemionus*): cryptic glacial refugia and postglacial recolonization. *Molecular Ecology* **18**: 1730–1745.
- Ledru MP. 1993. Late Quaternary environment and climatic changes in central Brazil. *Quaternary Research* **39**: 90–98.
- Li EX, Yi S, Qiu YX, Guo JT, Comes HP, Fu CX. 2008. Phylogeography of two East Asian species in *Croomia* (Stemonaceae) inferred from chloroplast DNA and ISSR fingerprinting variation. *Molecular Phylogenetics and Evolution* **49**: 702–714.
- Lorenz-Lemke AP, Mäder G, Muschner VC, Stehmann JR, Bonatto SL, Salzano FM, Freitas LB. 2006. Diversity and natural hybridization in a highly endemic species of *Petunia* (Solanaceae): a molecular and ecological analysis. *Molecular Ecology* **15**: 4487–4497.
- Lorenz-Lemke AP, Muschner VC, Bonatto SL, Cervi AC, Salzano FM, Freitas LB. 2005. Phylogeographic inferences concerning evolution of Brazilian *Passiflora actinia* and *P. elegans* (Passifloraceae) based on ITS (nrDNA) variation. *Annals of Botany* **95**: 799–806.
- Lorenz-Lemke AP, Togni PD, Mäder G, Kriedt RA, Stehmann JR, Salzano FM, Bonatto SL, Freitas LB. 2010. Diversification of plant species in a subtropical region of eastern South American highlands: a phylogeographic perspective on native *Petunia* (Solanaceae). *Molecular Ecology* **19**: 5240–5251.
- Miles L, Grainger A, Phillips O. 2004. The impact of global climate change on tropical forest biodiversity in Amazonia. *Global Ecology and Biogeography* **13**: 553–565.
- Miller MJ, Bermingham E, Klicka J, Escalante P, Do Amaral FSR, Weir JT, Winker K. 2008. Out of Amazonia again and again: episodic crossing of the Andes promotes diversification in a lowland forest flycatcher. *Proceedings of the Royal Society B: Biological Sciences* **275**: 1133–1142.
- Muellner AN, Tremetsberger K, Stuessy T, Baeza CM. 2005. Pleistocene refugia and recolonization routes in the Southern Andes: insights from *Hypochaeris palustris* (Asteraceae, Lactuceae). *Molecular Ecology* **14**: 203–212.
- Oh T, Kajita T, Murata AJ. 2003. Distinct geographic structure as evidenced by chloroplast DNA haplotypes and ploidy level in Japanese *Aucuba* (Aucubaceae). *American Journal of Botany* **90**: 1645–1652.
- Olmstead RG, Bohs L, Migid HA, Santiago-Valentin E, Garcia VF, Collier SM. 2008. A molecular phylogeny of the Solanaceae. *Taxon* **57**: 1159–1181.
- Ortiz MA, Tremetsberger K, Terrab A, Stuessy TF, Garcia-Castano JL, Urtubey E, Baeza CM, Ruas CF, Gibbs PE, Talavera S. 2008. Phylogeography of the invasive weed *Hypochaeris radicata* (Asteraceae): from Moroccan origin to worldwide introduced populations. *Molecular Ecology* **17**: 3654–3667.
- Overbeck GE, Muller SC, Fidelis A, Pfdenhauer J, Pillar VD, Blanco CC, Boldrini II, Both R, Forneck ED. 2007. Brazil's neglected biome: the South Brazilian campos. *Perspectives in Plant Ecology, Evolution and Systematics* **9**: 101–116.
- Oyama-Okubo N, Ando T, Watanabe N, Marchesi E, Uchida K, Nakayama M. 2005. Emission mechanism of floral scent in *Petunia axillaris*. *Bioscience, Biotechnology and Biochemistry* **69**: 773–777.
- Pallarés OR, Berretta EJ, Maraschin GE. 2005. The South American Campos ecosystem. In: Suttie J, Reynolds SG, Batello C, eds. *Grasslands of the world*. Rome: Food and Agriculture Organization of the United Nations, 171–219.
- Palma-Silva C, Lexer C, Paggi GM, Bárbara T, Bered F, Bodanese-Zanettini MH. 2009. Range-wide patterns of nuclear and chloroplast DNA diversity in *Vriesea gigantea* (Bromeliaceae), a Neotropical forest species. *Heredity* **103**: 503–512.
- Panario D, Gutierrez O. 1999. The continental Uruguayan Cenozoic: an overview. *Quaternary International* **62**: 75–84.
- Pastorino MJ, Gallo LA. 2002. Quaternary evolutionary history of *Austrocedrus chilensis*, a cypress native to the Andean–Patagonian forest. *Journal of Biogeography* **29**: 1167–1178.
- Pastorino MJ, Gallo LA, Hattemer HH. 2004. Genetic variation in natural populations of *Austrocedrus chilensis*, a cypress of the Andean–Patagonian Forest. *Biochemical Systematics and Ecology* **32**: 993–1008.
- Pennington RT, Lavin M, Prado DE, Pendry CA, Pell SK, Butterworth CA. 2004. Historical climate change and

- speciation: neotropical seasonally dry forest plants show patterns of both Tertiary and Quaternary diversification. *Philosophical Transactions of the Royal Society B: Biological Sciences* **359**: 515–537.
- Pennington RT, Prado DE, Pendry CA. 2000.** Neotropical seasonally dry forests and Quaternary vegetation changes. *Journal of Biogeography* **27**: 261–273.
- Pielou EC. 2008.** Plankton, from the last ice age to the year 3007. *Ices Journal of Marine Science* **65**: 296–301.
- Prance GT. 1996.** Islands in Amazonia. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences* **351**: 823–833.
- Premoli AC, Kitzberger T, Veblen TT. 2000.** Isozyme variation and recent biogeographical history of the long-lived conifer *Fitzroya cupressoides*. *Journal of Biogeography* **27**: 251–260.
- Quattrocchio ME, Borrromei AM, Deschamps CM, Grill SC, Zavala CA. 2008.** Landscape evolution and climate changes in the Late Pleistocene–Holocene, southern Pampa (Argentina): evidence from palynology, mammals and sedimentology. *Quaternary International* **181**: 123–138.
- Ramos ACS, De Lemos JP, Lovato MB. 2009.** Phylogeographical structure of the Neotropical forest tree *Hymenaea courbaril* (Leguminosae: Caesalpinioideae) and its relationship with the vicariant *Hymenaea stigonocarpa* from Cerrado. *Journal of Heredity* **100**: 206–216.
- Reis C, Sajo MG, Stehmann JR. 2002.** Leaf structure and taxonomy of *Petunia* and *Calibrachoa*. *Brazilian Archives in Biology and Technology* **45**: 59–66.
- Roesch LFW, Vieira FCB, Pereira VA, Schünemann AL, Teixeira IF, Senna AJT, Stefenon VM. 2009.** The Brazilian Pampa: a fragile biome. *Diversity* **1**: 182–198.
- Rull V. 2011.** Neotropical biodiversity: timing and potential drivers. *Trends in Ecology and Evolution* **26**: 508–513.
- Sang T, Crawford DJ, Stuessy TF. 1997.** Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paonia* (Paeoniaceae). *American Journal of Botany* **84**: 1120–1136.
- Serrano-Serrano ML, Hernandez-Torres J, Castillo-Villamizar G, Debouck DG, Sanchez MIC. 2010.** Gene pools in wild lima bean (*Phaseolus lunatus* L.) from the Americas: evidences for an Andean origin and past migrations. *Molecular Phylogenetics and Evolution* **54**: 76–87.
- Simpson BB, Todzia CA. 1990.** Patterns and processes in the development of the high Andean flora. *American Journal of Botany* **77**: 1419–1432.
- Solis-Neffa VG. 2009.** Geographic patterns of morphological variation in *Turnera sidoides* subsp. *pinnatifida* (Turneraceae). *Plant Systematics and Evolution* **284**: 231–253.
- Speranza PR, Seijo JG, Grela IA, Solis-Neffa VG. 2007.** Chloroplast DNA variation in the *Turnera sidoides* L. complex (Turneraceae): biogeographical implications. *Journal of Biogeography* **34**: 427–436.
- Stehmann JR. 1999.** Estudos taxonômicos da tribo Nicotianeae G. Don (Solanaceae): revisão de *Petunia* Jussieu, das espécies brasileiras de *Calibrachoa* La Llave & Lexarza e o estabelecimento do novo gênero *Petuniopsis*. PhD Thesis, Universidade Estadual de Campinas, Campinas, SP.
- Stehmann JR, Lorenz-Lemke AP, Freitas LB, Semir J. 2009.** The genus *Petunia*. In: Gerats T, Strommer J, eds. *Petunia: evolutionary, developmental and physiological genetics*. New York: Springer, 1–28.
- Stehmann JR, Semir J. 2001.** Biologia reprodutiva de *Calibrachoa elegans* (Miers) Stehmann & Semir (Solanaceae). *Revista Brasileira de Botânica* **24**: 43–49.
- Stehmann JR, Semir J, Dutilh JHA, Forni-Martins ER. 1996.** Solanaceae. IOPB chromosome data. *Newsletter of the International Organization of Plant Biosystematists* **26–27**: 24.
- Stuurman J, Hoballah ME, Broger L, Moore J, Basten C, Kuhlemeier C. 2004.** Dissection of floral pollination syndromes in *Petunia*. *Genetics* **168**: 1585–1599.
- Swofford DL. 2002.** *PAUP\*: phylogenetic analysis using parsimony (\*and other methods)*, version 4.0b10. Sunderland, MA: Sinauer Associates.
- Taberlet P, Gielly L, Pautou G, Bouvet J. 1991.** Universal primers for amplification of three non-coding regions of chloroplast DNA. *Plant Molecular Biology* **17**: 1105–1109.
- Tsukamoto T, Ando T, Watanabe H, Kokubun H, Hashimoto G, Sakazaki U, Suárez E, Marchesi E, Oyama K, Kao T. 2002.** Differentiation in the status of self-compatibility among *Calibrachoa* species (Solanaceae). *Journal of Plant Research* **115**: 185–193.
- Vargas P. 2003.** Molecular evidence for multiple diversification patterns of alpine plants in Mediterranean Europe. *Taxon* **52**: 463–476.
- Venail J, Dell’Olivo A, Kuhlemeier C. 2010.** Speciation genes in the genus *Petunia*. *Philosophical Transactions of the Royal Society B: Biological Sciences* **365**: 461–468.
- Watanabe H, Ando T, Iida S, Suzuki A, Buto K, Tsukamoto T, Hashimoto G, Marchesi E. 1996a.** Cross compatibility of *Petunia* cultivars and *P. axillaris* with native taxa of *Petunia* in relation to their chromosome number. *Journal of the Japanese Society for Horticultural Science* **65**: 625–634.
- Watanabe H, Ando T, Iida SI, Suzuki A, Buto KI, Tsukamoto T, Kokubun H, Hashimoto G, Marchesi E. 1996b.** Cross compatibility of *Petunia pubescens* and *P. pygmaea* with native taxa of *Petunia*. *Journal of the Japanese Society for Horticulture and Science* **66**: 607–612.
- Wijsman HJW. 1982.** On the interrelationships of certain species of *Petunia*. I. Taxonomic notes on the parental species of *Petunia hybrida*. *Acta Botanica Neerlandica* **31**: 477–490.
- Wijsman HJW. 1983.** On the interrelationships of certain species of *Petunia*. II. Experimental data: crosses between different taxa. *Acta Botanica Neerlandica* **32**: 97–107.
- Wijsman HJW, Jong JH. 1985.** On the interrelationships of certain species of *Petunia*. IV. Hybridization and nomenclatural consequences in the *Petunia* group. *Acta Botanica Neerlandica* **34**: 337–349.

## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

**Appendix S1. Supplementary Material.** GenBank accession numbers for *trnH-psbA* sequences used in this study. The information about the collection sites, voucher information and GenBank accession numbers for the other sequences are available in the study of Fregonezi *et al.* (2012).