

Phylogenetic Analysis of *Petunia sensu* Jussieu (Solanaceae) using Chloroplast DNA RFLP

TOSHIO ANDO¹*, HISASHI KOKUBUN¹, HITOSHI WATANABE¹, NORIO TANAKA²,
TOMOHIKA YUKAWA², GORO HASHIMOTO³, EDUARDO MARCHESI⁴,
ENRIQUE SUÁREZ⁵ and ISABEL L. BASUALDO⁶

¹Faculty of Horticulture, Chiba University, 648 Matsudo, Matsudo, Chiba 271-8510, Japan, ²Tsukuba Botanical Garden, National Science Museum, 4-1-1 Amakubo, Tsukuba, Ibaraki 305, Japan, ³Centro de Pesquisas de História Natural, Rua Jaime Ribeiro Wright 618, Itaquera, 08201-970, São Paulo, Brazil, ⁴Facultad de Agronomía, Universidad de la República, Garzon 780, Montevideo, Uruguay, ⁵Instituto de Recursos Biológicos, INTA, Las Cabañas y Los Reseros, 1712 Castelar, Buenos Aires, Argentina and ⁶Sección Botánica, Facultad de Ciencias Químicas, Universidad Nacional de Asunción, San Lorenzo, Paraguay

Received: 29 December 2004 Returned for revision: 17 March 2005 Accepted: 26 April 2005 Published electronically: 8 June 2005

- **Background and Aims** The phylogenetic relationships of *Petunia sensu* Jussieu (*Petunia sensu* Wijsman plus *Calibrachoa*) are unclear. This study aimed to resolve this uncertainty using molecular evidence.
- **Methods** Phylogenetic trees of 52 taxa of *Petunia sensu* Jussieu were constructed using restriction fragment length polymorphism (RFLP) of chloroplast DNA digested with 19 restriction enzymes and hybridized with 12 cloned *Nicotiana* chloroplast DNA fragments as probes.
- **Key Results** In all, 89 phylogenetically informative RFLPs were detected and one 50 % majority consensus tree was obtained, using the maximum parsimony method, and one distance matrix tree, using the neighbour joining method. *Petunia sensu* Wijsman and *Calibrachoa* were monophyletic sister clades in both trees. *Calibrachoa parviflora* and *C. pygmaea*, previously thought to differ from the other species in terms of their cross-compatibility, seed morphology, and nuclear DNA content, formed a basal clade that was sister to the remainder of *Calibrachoa*. Several clades found in the phylogenetic trees corresponded to their distribution ranges, suggesting that recent speciation in the genus *Petunia sensu* Jussieu occurred independently in several different regions.
- **Conclusions** The separation of *Petunia sensu* Wijsman and *Calibrachoa* was supported by chloroplast DNA analysis. Two groups in the *Calibrachoa* were also recognized with a high degree of confidence.

Key words: *Calibrachoa*, cpDNA, distribution, *Petunia*, phylogeny, RFLP, Solanaceae.

INTRODUCTION

In 1803, Jussieu established the genus *Petunia* (Solanaceae), with two species as types: *P. parviflora* and *P. nictaginiflora* [= *P. axillaris* (Lam.) Britton, Sterns & Poggenb.]. Fries (1911) wrote the most recent formal generic treatment on *Petunia*. More recently, Sink (1984) provided a comprehensive list of taxonomic works by that time. Hereafter, the genus *Petunia* circumscribed by Jussieu (1803) is referred to as *Petunia sensu* Jussieu. Wijsman and de Jong (1985) and Wijsman (1990) divided *Petunia sensu* Jussieu into two genera, *Petunia* and *Calibrachoa* La Llave & Lex., and transferred 15 species of *Petunia sensu* Jussieu to *Calibrachoa*. Stehmann and Semir (1997) transferred nine additional species to *Calibrachoa* in a work that described a species of *Calibrachoa* using the circumscription of Wijsman (1990). In this paper, *Petunia* as circumscribed by Wijsman (1990) is referred to solely as *Petunia*.

Several attempts have been made to understand parts of the phylogeny of *Petunia sensu* Jussieu using molecular data, such as restriction fragment length polymorphism (RFLP) analysis of ribosomal DNA (Kabbaj *et al.*, 1995),

DNA amplification fingerprinting (Cerny *et al.*, 1996) and polymorphism in the chalcone synthase intron sequence (Griesbach *et al.*, 2000); however, no attempts have been made to analyse the phylogeny of the entire genus. The objective of the present study was to explore the phylogeny of *Petunia sensu* Jussieu using all available taxa of wild origin to elucidate the existence of clades equivalent to *Petunia*, *Calibrachoa* and their subgroups, and to understand their evolutionary history. Recent studies indicate that *Calibrachoa* can be divided into two subgroups based on cross-compatibility (Watanabe *et al.*, 1997), seed surface morphology (Watanabe *et al.*, 1999) and nuclear DNA content (Mishiba *et al.*, 2000). In all the analyses, *C. parviflora* (Juss.) Wijsman and *C. pygmaea* (R. E. Fr.) Wijsman formed one group, and the remaining *Calibrachoa* formed the other. Since *C. parviflora* is the type species of the genus *Calibrachoa*, it was attempted to resolve the relationships of these species to the other members of *Petunia sensu* Jussieu for nomenclatural reasons.

In this study, chloroplast DNA (cpDNA) RFLP was chosen to construct the phylogenetic trees. Contiguous clones are readily available for the entire cpDNA of *Nicotiana tabacum* L. (Sugiura *et al.*, 1986), which is in the same family as *Petunia sensu* Jussieu.

* For correspondence. E-mail: andot@faculty.chiba-u.jp

TABLE 1. Names and voucher specimens of *Petunia sensu Jussieu* taxa used in the cpDNA RFLP analysis

Taxa	Botanical name, collection locality, voucher specimen number and herbaria code*
<i>Petunia sensu</i> Wijsman (1990), $2n = 2x = 14$	
1	<i>P. altiplana</i> T.Ando & Hashim. BRAZIL: Rio Grande do Sul, Ando et al. B319 (S, US)
2	<i>P. axillaris</i> (Lam.) Britton, Sterns & Poggenb. subsp. <i>axillaris</i> . URUGUAY: Montevideo, Ando & Kokubun U1 (MVFA, Ando)
3	<i>P. axillaris</i> subsp. <i>parodii</i> (Steere) Cabrera. URUGUAY: Artigas, Ando & Kokubun U27 (MVFA, Ando)
4	<i>P. axillaris</i> subsp. <i>subandina</i> T.Ando. ARGENTINA: Salta, Ando & Iida A100 (S, SI, Ando)
5	<i>P. bajeensis</i> T.Ando & Hashim. BRAZIL: Rio Grande do Sul, Hashimoto et al. B662 (BM, MBM, MVFA, R, S, SI, SP, U, US, GHSP, Ando)
6	<i>P. bonjardinensis</i> T.Ando & Hashim. BRAZIL: Rio Grande do Sul, Ando et al. B174 (S, GHSP, Ando)
7	<i>P. exserta</i> Stehmann BRAZIL: Rio Grande do Sul, Hashimoto et al. B931 (GHSP)
8	<i>P. guarapuavensis</i> T.Ando & Hashim. BRAZIL: Paraná, Hashimoto et al. B65 (MBM, BM, HBR, S, US, GHSP, Ando)
9	<i>P. inflata</i> R.E.Fr. ARGENTINA: Misiones, Ando & Buto A7 (SI, GHSP, Ando)
10	<i>P. integrifolia</i> (Hook.) Schinz & Thell. subsp. <i>integrifolia</i> . URUGUAY: Río Negro, Ando & Watanabe U106 (MVFA, S, SI, GHSP, Ando)
11	<i>P. integrifolia</i> subsp. <i>depauperata</i> R.E.Fr. BRAZIL: Rio Grande do Sul, Hashimoto et al. B59 (MVFA, SI, GHSP, Ando)
12	<i>P. interior</i> T.Ando & Hashim. BRAZIL: Santa Catarina, Hashimoto et al. B569 (MBM, BM, R, S, US, GHSP, Ando)
13	<i>P. littoralis</i> L.B.Sm. & Downs. BRAZIL: Santa Catarina, Hashimoto et al. B29 (MVFA, GHSP, Ando)
14	<i>P. mantiqueirensis</i> T.Ando & Hashim. BRAZIL: Minas Gerais, Hashimoto et al. B357 (S, BM, K, SP, U, US, GHSP, Ando)
15	<i>P. occidentalis</i> R.E.Fr. ARGENTINA: Jujuy, Ando & Iida A109 (MVFA, S, SI, GHSP, Ando)
16	<i>P. reitzii</i> L.B.Sm. & Downs. BRAZIL: Santa Catarina, Hashimoto et al. B28 (GHSP, Ando)
17	<i>P. riograndensis</i> T.Ando & Hashim. BRAZIL: Rio Grande do Sul, Hashimoto et al. B860 (MBM, S, GHSP, Ando)
18	<i>P. saxicola</i> L.B.Sm. & Downs. BRAZIL: Santa Catarina, Hashimoto et al. B113 (GHSP, Ando)
19	<i>P. scheideana</i> L.B.Sm. & Downs. BRAZIL: Santa Catarina, Hashimoto et al. B109 (BM, MBM, MVFA, S, SI, US, GHSP, Ando)
20	Unnamed taxon P1. BRAZIL: Rio Grande do Sul, Hashimoto et al. B990 (GHSP, Ando)
<i>Petunia sensu</i> Jussieu (1803) with $2n = 2x = 18$ chromosomes sharing similar morphological characters with <i>Calibrachoa</i>	
21	<i>P. alpicola</i> L.B.Sm. & Downs. BRAZIL: Santa Catarina, Hashimoto et al. B130 (BM, MBM, MVFA, S, SI, US, GHSP, Ando)
22	<i>P. helianthemoides</i> Sendtn. ARGENTINA: Misiones, Ando & Buto A4 (SI, Ando)
23	<i>P. kleinii</i> L.B.Sm. & Downs. BRAZIL: Santa Catarina, Hashimoto et al. B105 (MBM, GHSP, Ando)
24	<i>P. pubescens</i> (Spreng.) R.E.Fr. URUGUAY: Durazno, Ando & Kokubun U23 (S, MVFA, Ando)
25	<i>P. variabilis</i> R.E.Fr. URUGUAY: Tacuarembó, Ando & Kokubun U6 (MVFA, Ando)
<i>Calibrachoa</i> La Llave & Lexarza (1825), $2n = 2x = 18$	
26	<i>C. calycina</i> (Sendtn.) Wijsman. ARGENTINA: Corrientes, Ando & Buto A15 (MBM, GHSP, Ando)
27	<i>C. dusenii</i> (R.E.Fr.) Stehmann & Semir. BRAZIL: Paraná, Hashimoto et al. B484 (MBM, S, GHSP, Ando)
28	<i>C. eglandulata</i> Stehmann & Semir. BRAZIL: Santa Catarina, Hashimoto et al. B917 (GHSP, Ando)
29	<i>C. elegans</i> (Miers) Stehmann & Semir. BRAZIL: Minas Gerais, Hashimoto et al. B489 (GHSP, Ando)
30	<i>C. ericaefolia</i> (R.E.Fr.) Wijsman. BRAZIL: Paraná, Hashimoto et al. B63 (MBM, GHSP, Ando)
31	<i>C. heterophylla</i> (Sendtn.) Wijsman. BRAZIL: Rio Grande do Sul, Hashimoto et al. B60 (BM, MBM, GHSP, Ando)
32	<i>C. linearis</i> (Hook.) Wijsman. URUGUAY: Río Negro, Ando & Buto U165 (MVFA, Ando)
33	<i>C. linoides</i> (Sendtn.) Wijsman. BRAZIL: Rio Grande do Sul, Hashimoto et al. B282 (GHSP, Ando)
34	<i>C. macrodactylon</i> (L.B.Sm. & Downs) Wijsman. BRAZIL: Santa Catarina, Hashimoto et al. B138 (Ando)
35	<i>C. micrantha</i> (R.E.Fr.) Stehmann & Semir. BRAZIL: Paraná, Hashimoto et al. B486 (BM, MBM, S, US, GHSP, Ando)
36	<i>C. parviflora</i> (Juss.) Wijsman. URUGUAY: Tacuarembó, Ando & Kokubun U42 (MVFA, Ando)
37	<i>C. pygmaea</i> (R.E.Fr.) Wijsman. URUGUAY: Artigas, Ando & Kokubun U39 (BM, MVFA, S, US, Ando)
38	<i>C. rupestris</i> (Dusén) Wijsman. BRAZIL: Paraná, Hashimoto et al. B479 (BM, MBM, GHSP, Ando)
39	<i>C. selloviana</i> (Sendtn.) Wijsman. BRAZIL: Rio Grande do Sul, Hashimoto et al. B892 (MBM, S, US, GHSP, Ando)
40	<i>C. sendmeriana</i> (R.E.Fr.) Stehmann & Semir. BRAZIL: Santa Catarina, Hashimoto et al. B447 (BM, US, GHSP, Ando)
41	<i>C. serrulata</i> (L.B.Sm. & Downs) Stehmann & Semir. BRAZIL: Santa Catarina, Hashimoto et al. B1031 (BM, S, US, U, GHSP, Ando)
42	<i>C. spathulata</i> (L.B.Sm. & Downs) Stehmann & Semir. BRAZIL: Santa Catarina, Hashimoto et al. B90 (GHSP, Ando)
43	<i>C. thymifolia</i> (A.St.-Hil.) Stehmann & Semir. BRAZIL: Santa Catarina, Hashimoto et al. B36 (BM, MBM, MVFA, S, SI, US, GHSP, Ando)
44	unnamed taxon C1. BRAZIL: Rio Grande do Sul, Hashimoto et al. B7 (GHSP, Ando)
45	unnamed taxon C2. BRAZIL: Rio Grande do Sul, Hashimoto et al. B201 (GHSP, Ando)
46	unnamed taxon C3. BRAZIL: Santa Catarina, Hashimoto et al. B215 (GHSP, Ando)
47	unnamed taxon C4. BRAZIL: Rio Grande do Sul, Hashimoto et al. B755 (GHSP, Ando)
48	unnamed taxon C5. BRAZIL: Rio Grande do Sul, Hashimoto et al. B789 (GHSP, Ando)
49	unnamed taxon C6. BRAZIL: Santa Catarina, Hashimoto et al. B1126 (GHSP, Ando)
50	unnamed taxon C7. BRAZIL: Rio Grande do Sul, Hashimoto et al. B1176 (GHSP, Ando)
51	unnamed taxon C8. BRAZIL: Santa Catarina, Hashimoto et al. B1248 (GHSP, Ando)
52	unnamed taxon C9. URUGUAY: Salto, Ando & Iida U229 (GHSP, Ando)
53	<i>Nicotiana</i> L. (1735) $2n = 18, 20, 24, 32, 36, 38, 40, 42, 44, 46, 48$
55	<i>Nicotiana langsdorffii</i> Weinm. ($2n = 2x = 18$). BRAZIL: Rio Grande do Sul, Hashimoto et al. HB622 (Ando)

*Abbreviations of herbaria are after Holmgren et al. (1990) except GHSP (Centro de Pesquisas História Natural, São Paulo, Brazil) and Ando (temporary collection of Toshio Ando).

MATERIALS AND METHODS

Plant materials

Table 1 shows the taxa used in this study. These 52 taxa cover almost the entire genus *Petunia sensu* Jussieu. Ando

and Hashimoto (e.g. 1993, 1998) described most of the new taxa of *Petunia*, except unnamed taxon P1, while many *Calibrachoa* remain undescribed (unnamed taxa C1 to C9). *Nicotiana langsdorffii* Weinm. was used as the out-group. The seeds were collected randomly from respective

native populations and were raised in a greenhouse using standard cultivation techniques for garden petunias.

Petunia alpicola L. B. Sm. & Downs, *P. helianthemoides* Sendtn., *P. kleinii* L. B. Sm. & Downs, *P. pubescens* (Spreng.) R. E. Fr. and *P. variabilis* R. E. Fr. are five species of *Petunia sensu* Jussieu that share the same morphology and chromosome count ($2n = 2x = 18$) with *Calibrachoa* (Watanabe *et al.*, 1996, 1997). Here, they are tentatively treated as *Calibrachoa* (*C. alpicola*, *C. helianthemoides*, *C. kleinii*, *C. pubescens* and *C. variabilis*), except in Table 1.

Southern hybridization

Total *Petunia* DNA was extracted from approx. 5 g of fresh leaves using a modified CTAB method (Doyle and Doyle, 1987). However, an unidentified substance, possibly a polysaccharide, prevented the use of this method with many of the *Calibrachoa* species. For these samples, the modified CTAB method described by Lassner *et al.* (1989) was used.

Approximately 1 µg each of extracted DNA was digested using 19 endonucleases that recognize four to six base pairs (*AluI*, *AvaI*, *AvaII*, *BamHI*, *BclI*, *BglIII*, *Clal*, *DraI*, *EcoO109I*, *EcoRI*, *EcoRV*, *EcoT22I*, *HaeIII*, *HhaI*, *HincII*, *HindIII*, *StyI*, *VspI*, *XbaI*) according to the manufacturers' instructions. The recognition sequence of four base pair recognition enzymes was not included in those of six base pair recognition enzymes. DNA fragments were separated by electrophoresis in 1.5 % agarose gels and transferred onto Hybond N+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, UK) by capillary blotting with 20× SSC.

Twelve contiguous clones of *Nicotiana tabacum* cpDNA (pTBa1, pTBa2, pTB7, pTB8, pTB10, pTB15, pTB19, pTB20, pTB22, pTB25, pTB28, pTB29; Sugiura *et al.*, 1986), which cover 99.4 % of the total sequence, were used as probes to detect RFLP. These probes were labelled with the ECLTM direct nucleic acid labelling and detection system (Amersham), according to the manufacturer's instructions, and hybridized with given cpDNA fragments for 12 h at 42°C. The hybridized blot was washed in 0.5× SSC with 0.4 % SDS for 2 × 20 min at 42°C, then in 5× SSC for 2 × 5 min at room temperature before detection.

Phylogenetic analysis

The data set was constructed by scoring the presence and absence of a restriction site as 1 and 0, respectively. When the site mutation was not unambiguously determined due to the detection limit of approx. 300 base pairs (bp), length mutation of a particular fragment was scored 1 and 0, according to the length. Other fragments that cannot be reasonably explained were recorded as presence/absence of the fragments. The most-parsimonious trees were sought using PAUP* version 4.0b10 (Swofford, 1998), using a heuristic search with the MULPARS and TBR branch-swapping options, following the strategy of Maddison *et al.* (1992), as adopted by Cantino *et al.* (1998), to search as many islands as practical. In this analysis, the 50 %

majority consensus tree was calculated from the set of equally most-parsimonious trees obtained. To test the support for each clade in the parsimony tree, bootstrap analysis (Felsenstein, 1985) was performed using 1000 replicates with PAUP*. A decay analysis (Bremer, 1988) was also performed using TreeRot version 2 (Sorenson, 1999) in conjunction with PAUP*.

Another phylogenetic tree was also reconstructed for comparison using a neighbour joining (NJ) distance matrix method, which will be referred to as the distance tree. The distance matrix consisting of the number of nucleotide substitutions per site was calculated from only the unambiguously identified site mutations. Calculation of the matrix was done with the RESTDATA program (Ota, 1994) that employs the estimation method of Nei and Tajima (1983). The matrix was reformatted as NEXUS file, then the distance tree was calculated using the NJ option of PAUP*. The purpose of this analysis was to assess overall sequence similarity while avoiding the effect of homoplasy.

RESULTS

RFLPs

A total of 212 RFLPs was detected with all 19 restriction enzymes distributed in all 12 *Nicotiana* cpDNA clone groups for all 20 taxa of *Petunia* and 32 taxa of *Calibrachoa*. *Nicotiana langsdorffii* produced sufficiently similar RFLPs to *Petunia sensu* Jussieu for site mutations to be inferred without exact restriction site maps. Of the 212 mutations, 89 were shared by more than one taxon and were phylogenetically informative. Tree search for the parsimony tree was carried out using these 89 site mutations.

Of the 212 RFLPs, 85 (41 %) were unambiguously identified as gain or loss of a particular restriction site with this method. These site mutation data were used to calculate a distance matrix. Due to the length of the fragments produced and the detection limit of approx. 300 bp, it was not possible to unambiguously identify such restriction sites for some enzymes. There were 83 such cases (39 %), and the mutation was recorded as length mutation of a fragment when applicable. When this failed, a presence of unexplained fragment was recorded as 1.

Most of the taxa in *Petunia sensu* Jussieu that were studied had at least one unique site mutation that distinguished that taxon from all others. However, two pairs of taxa shared exactly the same site mutations. The first pair was *Calibrachoa macrodactylon* (L. B. Sm. & Downs) Wijsman and *C. selloviana* (Sendtn.) Wijsman, and the second pair was *C. thymifolia* (A. St.-Hil.) Stehmann & Semir and unnamed taxon C3. Both pairs of species had overlapping or similar distribution ranges [high altitude plateau of Santa Catarina (SC) and Rio Grande do Sul (RS) for the former pair and a localized area on the Atlantic coast in SC for the latter].

Deletion, insertion and reversion of the cpDNA

Two cases of deletion of a DNA fragment were detected in two taxa, *Petunia altiplana* T. Ando & Hashim. and

unnamed taxon C1, both in the cpDNA overlap region of pTB7 and pTB20. The estimated length of the deletion was approx. 0.2 kilobase pairs (kbp) for *P. altiplana* and approx. 0.4 kbp for taxon C1. No insertion or reversion of a DNA fragment was unambiguously identified in any taxon studied.

Phylogenetic trees

The heuristic search with PAUP* found 3420 equally parsimonious trees. From these trees, the 50% majority consensus tree was calculated (Fig. 1). Due to the paucity of synapomorphic site mutations, many clades contained polytomies, so that the relationships of the terminal taxa in some clades were not resolved completely. However, both *Petunia* and *Calibrachoa* were monophyletic, and *Calibrachoa* was further subdivided into two clades: one consisted of *C. parviflora* and *C. pygmaea* (clade B in Fig. 1) and the other comprised the remaining *Calibrachoa* (clade A in Fig. 1). All of these clades had 100% bootstrap support (Fig. 1). The five species of *Petunia sensu* Jussieu that have $2n = 2x = 18$ chromosomes and morphological characters of *Calibrachoa* (Table 1) were included in the clade of *Calibrachoa*. In the distance tree (Fig. 2), the two genera, *Petunia* and *Calibrachoa*, and the two subgroups within *Calibrachoa* were again clearly identified.

The most significant difference between the 50% majority consensus tree and the distance tree was the position of *P. altiplana*. This was the taxon in which a deletion of part of the cpDNA was detected. The other taxon with the deletion (unnamed taxon C1) was found at the same place in both trees.

DISCUSSION

Phylogeny of *Petunia sensu* Jussieu

The phylogenetic trees obtained in this study (Figs 1 and 2) clearly identify two major clades in *Petunia sensu* Jussieu: the clades *Petunia* and *Calibrachoa*. Bootstrap support for both the *Petunia* and *Calibrachoa* clades in the 50% majority consensus tree was 100%, with 12 and 11 decay values, respectively (Fig. 1). This further validates the treatment dividing *Petunia* and *Calibrachoa* (Wijsman, 1990). Watanabe *et al.* (1996, 1997, 2001) and Ando *et al.* (2001) have confirmed that species of *Petunia* and *Calibrachoa* are genetically isolated from each other.

The *Calibrachoa* clade was further divided into two clades: the first with two species (*C. parviflora* and *C. pygmaea*, Figs 1 and 2; clade B), and the second with the remaining *Calibrachoa* (clade A). Each clade had a bootstrap probability of 100% with decay values of seven or nine, respectively (Fig. 1).

Calibrachoa parviflora and *C. pygmaea* are unique in *Petunia sensu* Jussieu in terms of seed morphology (Watanabe *et al.*, 1999) and nuclear DNA content (Mishiba *et al.*, 2000). Moreover, these two species cross only with each other, and are cross-incompatible with all other *Calibrachoa*. In contrast, all *Calibrachoa* species except these two are cross-compatible with *C. pubescens*

(Watanabe *et al.*, 1997). In other words, the *Calibrachoa* species in clades A and B (Figs 1 and 2) are genetically isolated from each other. The great difference in the site mutations among the three subgroups of *Petunia sensu* Jussieu described above may reflect prolonged genetic isolation from one another.

When *Petunia* and *Calibrachoa* were compared in the distance tree (Fig. 2), larger genetic distances were found within the genus *Calibrachoa* than within *Petunia*. The average genetic distances within *Calibrachoa* and *Petunia* calculated from the distance matrix were 0.0144 and 0.0112, respectively. This suggests that divergence is further advanced in the genus *Calibrachoa* than in the genus *Petunia*. If the genetic distance is proportional to time, this may indicate that the genus *Calibrachoa* is older than the genus *Petunia*. However, the differences between genetic distances are only very small, hence more molecular phylogenetic data would be needed to be able to either accept or reject this hypothesis.

Clades associated with geographic distribution

The native habitats of *Petunia sensu* Jussieu in South America have been studied for 17 seasons since 1988. A start is being made to understand the distributions and geographical proximities of the members of *Petunia sensu* Jussieu, and overall distribution maps of *Petunia* (Tsukamoto *et al.*, 1998) and *Calibrachoa* (Tsukamoto *et al.*, 2002) have been published. In the 50% majority consensus tree (Fig. 1) and the distance tree (Fig. 2), the three clades marked a, b and c are considered to be associated with geographic distribution enclosing species endemic to their respective regions. Based on the present field observation data, a distribution map illustrating the distributions of these clades was made (Fig. 3).

Clade a (Figs 1 and 2) includes *Calibrachoa calycina* (Sendtn.) Wijsman, *C. helianthemoides*, *C. linealis* (Hook.) Wijsman, *C. linoides* (Sendtn.) Wijsman, *C. pubescens*, *C. variabilis* and unnamed taxa C4, C5, C7 and C9. This clade was monophyletic in both trees (Figs 1 and 2) with 91% bootstrap support in the 50% majority consensus tree (Fig. 1) and two unique site mutations (Table 2). Except for *Calibrachoa linoides*, which has a wide distribution in southern Brazil and adjacent regions, these *Calibrachoa* taxa exclusively occupy the most south-western territory of the *Calibrachoa*, from southern Uruguay (*C. pubescens*) to southern Paraguay (*C. calycina*; region a in Fig. 3). Because these hilly lowlands are a continuation of the Pampa, the vast grassland extending widely in temperate Argentina, this region can be summarized as the Eastern Pampa.

Clade b in both trees (Figs 1 and 2) included *C. ericaefolia* (R. E. Fr.) Wijsman, *C. kleinii*, *C. micrantha* (R. E. Fr.) Stehmann & Semir, *C. rupestris* (Dusén) Wijsman and unnamed taxon C8. Although the bootstrap value for the clade was relatively low (57%), and characterized with only one unambiguous, unique site mutation (Fig. 1 and Table 2), these taxa are exclusively found in eastern Paraná (PR) in Brazil (region b in Fig. 3), except *C. kleinii* and unnamed

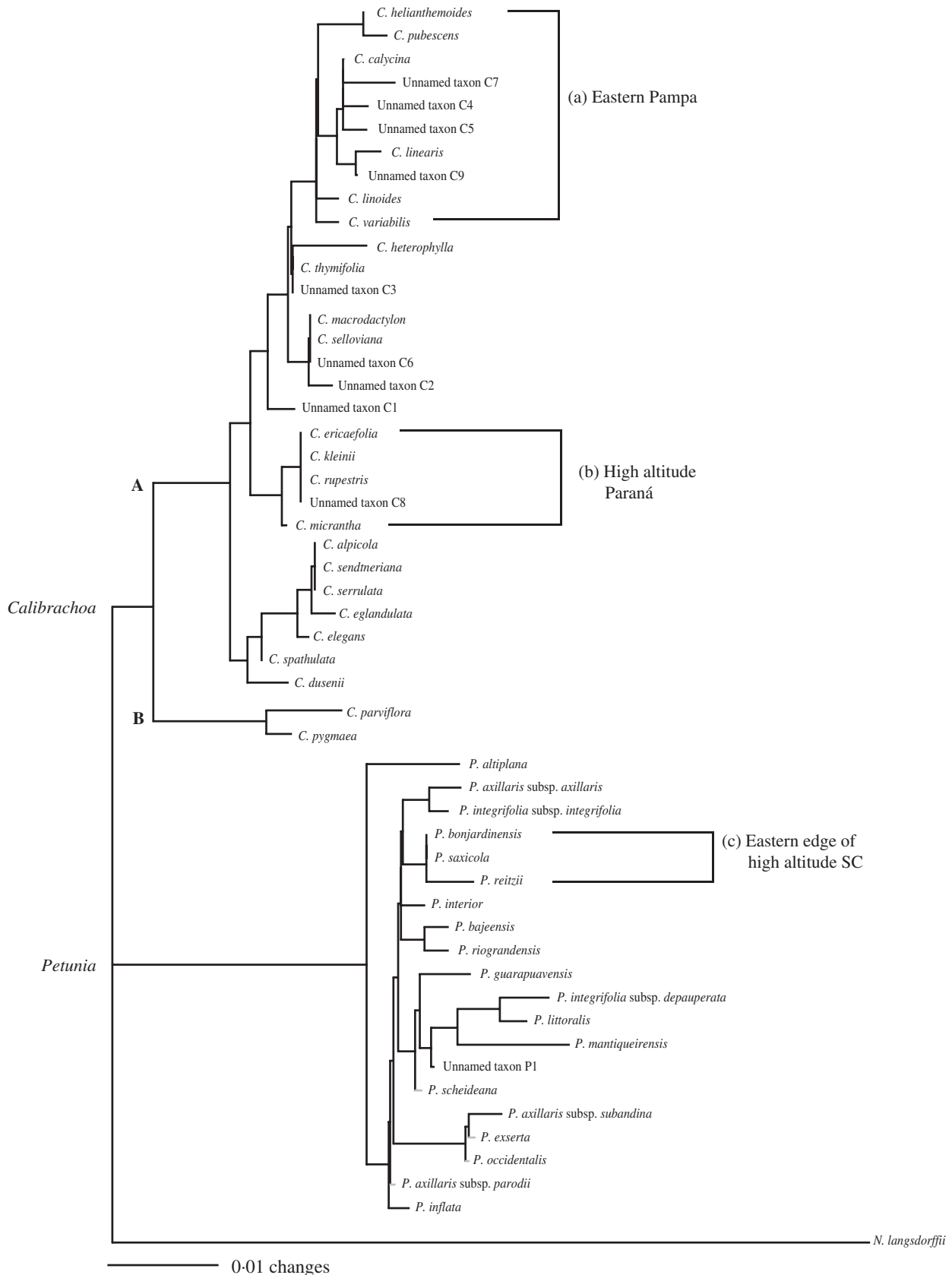


FIG. 2. Distance matrix tree calculated using the NJ method. The distance matrix was calculated from 85 unambiguous site mutation data with the method of Nei and Tajima (1983) as implemented in RESTDATA program (Ota, 1994).

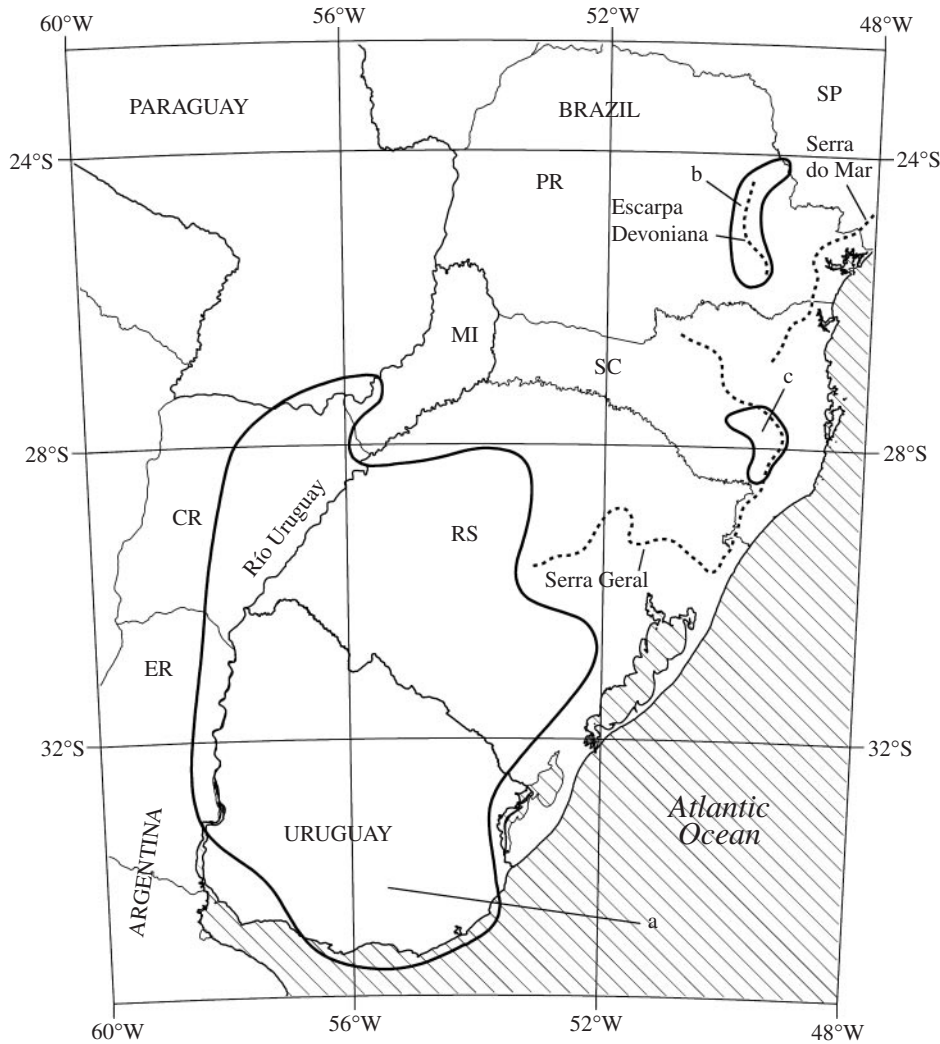


FIG. 3. Distribution of selected clades (a, b and c, corresponding to the previous figures) in *Petunia sensu* Jussieu found in this study. Abbreviation of states and provinces: Argentina, CR = Corrientes, ER = Entre Ríos, MI = Misiones; Brazil, PR = Paraná, RS = Rio Grande do Sul, SC = Santa Catarina, SP = São Paulo.

taxon C8. *Calibrachoa kleinii* is distributed more widely along the border of PR and SC in Brazil, the province of Misiones in Argentina, and Paraguay, and taxon C8 was from SC. Region b is highland near an escarpment called Escarpa Devoniana (the broken line in region b in Fig. 3), which is approx. 1000 m a.s.l., and is the eastern edge of a second high plateau (Segundo Planalto) that declines gradually to the west (Maack, 1968). This region, one may call it high-altitude Paraná, represents the most northern territory of *Calibrachoa*, except *C. elegans* (Miers) Stehmann & Semir, which is isolated far north in Minas Gerais in Brazil (Fries, 1911; Tsukamoto *et al.*, 2002). Although *Calibrachoa kleinii* has a wider range, it is apparently a northern element. *Calibrachoa* species are abundant in region b, while no species of *Petunia* is found there.

Clades c in both trees (Figs 1 and 2) with weak bootstrap support of 53% included three *Petunia* species: *P. bonjardinensis* T. Ando & Hashim., *P. reitzii* L. B. Sm. & Downs and *P. saxicola* L. B. Sm. & Downs. These are rather rare species endemic to region c (Fig. 3) along the

Serra Geral in south-eastern SC, where the altitude exceeds 1000 m. Serra Geral (dotted line in eastern SC and RS in Fig. 3) is the edge of a lava plateau that covers much of eastern SC and north-eastern RS. The highest regions on the plateau are located near Serra Geral, where the altitude drops abruptly toward the eastern lowlands. The western side of the plateau declines gradually toward the River Uruguay (Fig. 3). This region can suitably be called the eastern edge of high-altitude SC. This region is also the endemic distribution range of some *Calibrachoa* species [*C. alpicola*, *C. eglandulata* Stehmann & Semir, *C. sendtneriana* (R.E. Fr.) Stehmann & Semir and *C. serrulata* (L.B.Sm. & Downs) Stehmann & Semir].

Compared with the total ranges of *Petunia* (Tsukamoto *et al.*, 1998) and *Calibrachoa* (Tsukamoto *et al.*, 2002), the species enclosed in clades b and c seem to be concentrated at the eastern margin of the territories (Fig. 3). It is assumed that the large amount of precipitation delimiting the highland regions (Maack, 1968) is one of the reasons why many species of both *Petunia* and *Calibrachoa* are found in such

TABLE 2. Synapomorphic site mutations for selected clades in the 50% majority consensus tree

Clade	Number of taxa	Synapomorphic site mutations*
<i>Petunia</i> (<i>sensu</i> Wijsman)	20	<u>AluI+pTB19, AvaI+pTB19, AvaII+pTBa1, AvaII+pTBa2, AvaII+pTB25, BamHI+pTBa1, BamHI+pTB19, BcnI+pTB25, ClaI+pTB28, EcoT22I+pTB25, HhaI+pTB8, Hind III+pTBa1</u>
<i>Calibrachoa</i>	32	<u>AluI+pTB19, AvaII+pTBa2, AvaII+pTB8, AvaII+pTB19, BamHI+pTB29, BgIII+pTB25, ClaI+pTBa1, DraI+pTBa2, EcoO109I+pTBa1, EcoO109I+pTB8, EcoO109I+pTB22, EcoRV+pTB25, HincII+pTB15, HincII+pTB25, HindIII+pTB8</u>
Clade A <i>Calibrachoa</i> other than <i>C. parviflora</i> and <i>C. pygmaea</i>	30	<u>AluI+pTBa2, AluI+pTB7, AluI+pTB25, BamHI+pTB20, BcnI+pTB10, BcnI+pTB29, EcoRI+pTBa2, HhaI+pTB7, HhaI+pTB19</u>
Clade B <i>C. parviflora</i> and <i>C. pygmaea</i>	2	<u>AluI+pTB20, AvaII+pTB20, BgIII+pTBa2, EcoRV+pTB7-20, EcoT22I+pTBa1, EcoT22I+pTB20, XbaI+pTB22</u>
Clades with geographic association		
Clade a Eastern Pampa— <i>Calibrachoa</i>	10	<u>AvaII+pTB19, AvaII+pTB25, EcoO109I+pTB15-28, HhaI+pTB19</u>
Clade b High altitude Paraná— <i>Calibrachoa</i>	5	<u>HhaI+pTBa1</u>
Clade c Eastern edge of high altitude Santa Catarina— <i>Petunia</i>	3	<u>ClaI+pTB15-28</u>

*The underline indicates that the mutation occurred exclusively in that clade.

small areas. In contrast, clade a is characterized by the members with wider distributions (Fig. 3). The hilly lowland of this region may have allowed rapid dispersal or active genetic exchange among the members.

Infrageneric relationships of *Petunia*

In the 50% majority consensus tree, *Petunia axillaris* together with the infraspecific taxa, *P. exserta* Stehmann and *P. occidentalis* R. E. Fr. formed the most basal branch in the *Petunia* clade (Fig. 1). They have a thick, consistently inflexed pedicel in contrast to the remaining members of *Petunia*, which have a thin, deflexed pedicel in the fruiting state. An exception is *P. inflata* R. E. Fr., whose thin pedicel is inflexed at the upper stem, but is often deflexed at the lower stem in younger plants.

The RFLP data did not appear particularly useful for resolving infraspecific relationships in *Petunia*, since there was some incongruity between the present results and the traditional infraspecific taxonomy. *Petunia axillaris* occurs widely in southern Bolivia, Paraguay, RS (Brazil), Uruguay, and the northern half of Argentina. Three allopatric subspecies are known today: subsp. *axillaris*, subsp. *parodii* (Steere) Cabrera and subsp. *subandina* T. Ando (Ando, 1996). In the phylogenetic trees produced in the present study, these three subspecies never formed a clade (Figs 1 and 2). The two most likely causes are convergence (three subspecies appear similar, but represent three distinctive lineages) and geographic isolation (they share a common ancestor, but have been separated in each region for enough time not to appear monophyletic when analysed).

Petunia inflata has been treated in various ways taxonomically. Fries (1911) described it as an independent species, while Smith and Downs (1966) treated it as synonymous to

P. integrifolia, and Wijsman (1982) regarded it an extreme form of *P. integrifolia*. In the present study, *P. inflata* was distinguished from *P. integrifolia* by eight site mutations (*AvaII* + pTB7, *BamHI* + pTB7-20, *BcnI* + pTB15-28, *BgIII* + pTBa1-15, *DraI* + pTB19, *EcoRI* + pTB25, *StyI* + pTB7, *XbaI* + pTB19), and the two taxa were situated in different locations in the phylogenetic trees (Figs 1 and 2). The present results seem to support the treatment of Fries (1911).

As shown here, the phylogenetic trees constructed from cpDNA RFLP did not necessarily correspond to the current infrageneric taxonomy of *Petunia*. Additional molecular data from nuclear DNA or a more rapidly changing region of cpDNA should be analysed to explain this discrepancy.

CONCLUSIONS

It is concluded that *Petunia sensu* Jussieu is a monophyletic clade when rooted using *Nicotiana langsdorffii*, and that it consists of two major monophyletic sister clades: *Petunia* and *Calibrachoa*. Two unusual *Calibrachoa* species, *C. parviflora* (the type species) and *C. pygmaea*, form a basal clade that is sister to the remaining *Calibrachoa*. Therefore, it is not necessary to consider a different nomenclature for the remaining *Calibrachoa* for now. Some of the infrageneric clades seemed to be tied with geographic distribution, although the support from the RFLP data was weak.

ACKNOWLEDGEMENTS

We thank Mr Tsuguyoshi Aoki (Buenos Aires, Argentina), Mr Sebastião T. Nagase, Mr Nobuyuki Hiranaka, Mr Tomio Koshizawa, Mr Hideo Ohkubo and Mr Roberto H. Ohkubo (São Paulo, Brazil) and Mr Masao Udagawa (Montevideo,

Uruguay) for help in surveying the natural habitat. We also thank the DNA Bank of the Center for Gene Research of Nagoya University for kindly providing the tobacco cpDNA clones. This work was partly supported by a Grant-in-Aid for Scientific Research (B; project number 08456016) from the Ministry of Education, Science, Sports and Culture of Japan.

LITERATURE CITED

- Ando T. 1996.** Distribution of *Petunia axillaris* (Solanaceae) and its new subspecies in Argentina and Bolivia. *Acta Phytotaxonomica et Geobotanica* **44**: 19–30.
- Ando T, Hashimoto G. 1993.** Two new species of *Petunia* (Solanaceae) from southern Brazil. *Botanical Journal of the Linnean Society* **111**: 265–280.
- Ando T, Hashimoto G. 1998.** Two new species of *Petunia* (Solanaceae) from southern Rio Grande do Sul, Brazil. *Brittonia* **50**: 483–492.
- 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.
- Bremer K. 1988.** The limits of amino acid sequence data in angiosperm phylogenetic reconstruction. *Evolution* **42**: 795–803.
- Cantino PD, Wagstaff SJ, Olmstead RG. 1998.** *Caryopteris* (Lamiaceae) and the conflict between phylogenetic and pragmatic considerations in botanical nomenclature. *Systematic Botany* **23**: 369–386.
- Cerny TA, Caetano-Anollés G, Trigiano RN, Starman TW. 1996.** Molecular phylogeny and DNA amplification fingerprinting of *Petunia* taxa. *Theoretical and Applied Genetics* **92**: 1009–1016.
- Doyle JJ, Doyle JL. 1987.** A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin* **19**: 11–15.
- Felsenstein J. 1985.** Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783–791.
- Fries RE. 1911.** Die Arten der Gattung *Petunia*. *Kungliga Svenska Vetenskapsakademiens Handlingar* **46**: 1–72.
- Griesbach RJ, Beck RM, Stehmann JR. 2000.** Molecular heterogeneity of the chalcone synthase intron in *Petunia*. *HortScience* **35**: 1347–1349.
- Jussieu AL. 1803.** Sur le *Pétunia*, genre nouveau de la famille des plantes solanées. *Annales du Muséum National d'Histoire Naturelle* **2**: 214–216.
- Holmgren PK, Holmgren NH, Barnett LC. 1990.** *Index Herbariorum Part I: the herbaria of the world*. Bronx, NY: New York Botanical Garden.
- Kabbaj A, Zeboudj F, Peltier D, Tagmount A, Tersac M, Dulieu H, Berbillé A. 1995.** Variation and phylogeny of the ribosomal DNA unit types and 5 S DNA in *Petunia* Jussieu. *Genetic Resources and Crop Evolution* **42**: 311–325.
- Lassner MW, Peterson P, Yoder JI. 1989.** Simultaneous amplification of multiple DNA fragments by polymerase chain reaction in the analysis of transgenic plants and their progeny. *Plant Molecular Biology Reporter* **7**: 116–128.
- Maack R. 1968.** As zonas das paisagens naturais. In: Maack R. *Geografia física do estado do Paraná*. Curitiba: Banco de Desenvolvimento do Paraná, 85–88.
- Maddison DR, Ruvolo M, Swofford DL. 1992.** Geographic origins of human mitochondrial DNA: phylogenetic evidence from control region sequences. *Systematic Biology* **41**: 111–124.
- Mishiba K, Ando T, Mii M, Watanabe H, Kokubun H, Hashimoto G, Marchesi E. 2000.** Nuclear DNA content as an index character discriminating taxa in the genus *Petunia sensu Jussieu* (Solanaceae). *Annals of Botany* **85**: 665–673.
- Nei M, Tajima F. 1983.** Maximum likelihood estimation of the number of nucleotide substitutions for restriction sites data. *Genetics* **105**: 207–216.
- Ota T. 1994.** RESTDATA: restriction data and phylogenetic analysis (<http://mep.bio.psu.edu>).
- Sink KC. 1984.** Taxonomy. In: Sink KC. ed. *Petunia*. Berlin: Springer, 3–9.
- Smith LB, Downs RJ. 1966.** *Petunia*. In: Reitz PR. ed. *Flora Illustrada Catarinense. Solanáceas*. Itajai: Herbario Barbosa Rodrigues, 261–291.
- Sorenson MD. 1999.** TreeRot, version 2. Boston: Boston University.
- Stehmann JR, Semir J. 1997.** A new species and new combinations in *Calibrachoa* (Solanaceae). *Novon* **7**: 417–419.
- Sugiura M, Shinozaki K, Zaita N, Kusuda M, Kumano M. 1986.** Clone bank of the tobacco (*Nicotiana tabacum*) chloroplast genome as a set of overlapping restriction endonuclease fragments: mapping of eleven ribosomal protein genes. *Plant Science* **44**: 211–216.
- Swofford DL. 1998.** PAUP*. *Phylogenetic analysis using parsimony (*and other methods)*. Version 4. Sunderland, MA: Sinauer Associates.
- Tsukamoto T, Ando T, Kokubun H, Watanabe H, Tanaka R, Hashimoto G, Marchesi E, Kao T. 1998.** Differentiation in the status of self-incompatibility among all natural taxa of *Petunia* (Solanaceae). *Acta Phytotaxonomica et Geobotanica* **49**: 115–133.
- Tsukamoto T, Ando T, Watanabe H, Kokubun H, Hashimoto G, Sakazaki U, Suárez E, Marchesi E, Oyama K, Kao T-H. 2002.** Differentiation in the status of self-incompatibility among *Calibrachoa* species (Solanaceae). *Journal of Plant Research* **115**: 185–193.
- Watanabe H, Ando T, Iida S, Suzuki A, Buto K, Tsukamoto T. 1996.** 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 S, Buto K, Tsukamoto T, Kokubun H, Hashimoto G, Marchesi E. 1997.** Cross-compatibility of *Petunia pubescens* and *P. pygmaea* with native taxa of *Petunia*. *Journal of the Japanese Society for Horticultural Science* **66**: 607–612.
- Watanabe H, Ando T, Nishino E, Kokubun H, Tsukamoto T, Hashimoto G, Marchesi E. 1999.** Three groups of species in *Petunia sensu Jussieu* (Solanaceae) inferred from the seed morphology. *American Journal of Botany* **86**: 302–305.
- Watanabe H, Ando T, Tsukamoto T, Hashimoto G, Marchesi E. 2001.** Cross-compatibility of *Petunia exserta* with other *Petunia* taxa. *Journal of the Japanese Society for Horticultural Science* **70**: 33–40.
- 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. 1990.** On the inter-relationships of certain species of *Petunia*. VI. New names for the species of *Calibrachoa* formerly included into *Petunia* (Solanaceae). *Acta Botanica Neerlandica* **39**: 101–102.
- Wijsman HJW, de Jong JH. 1985.** On the interrelationships of certain species of *Petunia*. IV. Hybridization between *P. linearis* and *P. calycina* and nomenclatural consequences in the *Petunia* group. *Acta Botanica Neerlandica* **34**: 337–349.