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T. A. Cerny · G. Caetano-Anollés · R. N. Trigiano
T. W. Starman

Molecular phylogeny and DNA amplification fingerprinting of *Petunia* taxa

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Abstract The relationship of five species of *Petunia* and ten cultivars of the cultivated petunia, *Petunia* × *hybrida*, were investigated using DNA-amplification fingerprinting (DAF). Reproducible banding profiles were obtained from *P. parodii* and *P. axillaris* DNA from different seed sources. In contrast, other petunias such as *P. inflata*, *P. violacea* and *P. integrifolia* produced variable fingerprints when different plants were examined. However, representative profiles of the variable *Petunia* taxa were obtained by bulking the leaf tissue from ten different individual plants. Each of ten octamer primers revealed polymorphic loci between taxa. Among a total of 201 bands produced, 146 (73%) loci were polymorphic and distinguished all species and cultivars. Phenetic and cluster analysis using DAF markers separated *P. axillaris* from *P. parodii* and distinguished between the violet-flowered species, *P. inflata*, *P. violacea*, and *P. integrifolia*. *P. parodii* grouped together with the monophyletic set of the ten cultivars of *P.* × *hybrida* examined, indicating that it had made a major contribution to the development of these cultivars. Cultivars were distributed within the dendograms by flower color. The results demonstrated the utility of DAF in establishing relationships among closely related species and cultivars of *Petunia*.

Key words *Petunia* spp · Arbitrary primers · DAF · DNA fingerprinting · DNA bulking · Phylogenetic relationships

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T. A. Cerny · R. N. Trigiano · T. W. Starman (✉)
Department of Ornamental Horticulture and Landscape Design, Institute of Agriculture, The University of Tennessee, Knoxville, TN 37901-1071, USA

G. Caetano-Anollés
Plant Molecular Genetics, Institute of Agriculture and Center for Legume Research, The University of Tennessee, Knoxville, TN 37901-1071, USA

Introduction

The genus *Petunia* consists of over 30 species (Sink 1984), but the classification within the genus has been inconsistent. Earlier literature suggested that the hybrid *Petunia* (*Petunia* × *hybrida*) was a progeny resulting from a cross between *P. axillaris* and *P. violacea* (Bailey 1901; Ferguson and Ottley 1932), but the conflicting classification of other closely related species, *P. inflata*, *P. integrifolia* and *P. parodii*, made the validity of this proposed ancestry questionable (Sink 1984).

In the past 20 years, various chemotaxonomic studies have been used to investigate the parentage of *P.* × *hybrida*. A study using thin-layer chromatography (TLC) to investigate phenolic extracts of *Petunia* species and cultivars confirmed further that *P. axillaris* and *P. violacea* were the most probable parents (Natarella and Sink 1974). In contrast, an analysis of total proteins and peroxidases of the *Petunia* species concluded that *P. inflata* and *P. axillaris* were the more likely ancestors (Natarella and Sink 1975).

The generation of deoxyribonucleic-acid (DNA) markers has provided a more reliable method with which to detect taxonomic differences. The use of restriction fragment length polymorphisms (RFLPs) identified more informational traits between individuals than previous chemotaxonomic methods, but the time, expense, and requirement of radiolabeling, made this method less desirable. The isolation of repeated DNA sequences has already been used for phylogenetic and genomic studies of *Petunia* (Shepherd et al. 1990). Probes clearly distinguished the white-flowered species (*P. axillaris* and *P. parodii*) from the violet-flowered species (*P. inflata* and *P. violacea*), but the probe and restriction-enzyme combination that could distinguish the two white-flowered species from each other or the two violet-flowered species from each other was not found. Consequently, the parental species of *Petunia* were not identified. Koes et al. (1987) investigated the relationship between *Petunia* species and cultivars of *P.* ×

hybrida using chalcone synthase gene probes. A high degree of variability in gene organization and copy number was observed, and therefore the parental species were not determined.

New fingerprinting methods based on the amplification of DNA with arbitrary oligodeoxynucleotide primers (Welsh and McClelland 1990; Williams et al. 1990; Caetano-Anollés et al. 1991) generally use single synthetic primers to initiate DNA polymerase-directed in vitro replication of selected regions of a DNA template. These regions are copied many times through a temperature-cycling process, much as in the polymerase chain reaction (PCR). Arbitrarily primed-PCR (AP-PCR) (Welsh and McClelland 1990), randomly amplified polymorphic DNA (RAPD) (Williams et al. 1990), and DNA amplification fingerprinting (DAF) (Caetano-Anollés et al. 1991) are techniques that differ in the length of primer used, the primer-to-template DNA ratios, the temperatures employed during the amplification process, and the methods of separation and staining of DNA fragments.

DNA amplification-based assays have been successfully used for the identification of closely related species and cultivars, as well as in phylogenetic analysis. For example, the parentage of maize (*Zea mays*) hybrids has been determined using AP-PCR (Welsh et al. 1991). Marsolais et al. (1993) used 13 decamer primers to produce RAPD markers to distinguish between nine different lilac (*Syringa spp.*) species and cultivars. Octamer primers were used to investigate the genetic relationships between centipedegrass (*Eremochloa ophiuroides*) cultivars (Weaver et al. 1995) and bermudagrass (*Cynodon*) cultivars (Caetano-Anollés et al. 1995) using DAF. Finally, the utility of amplified fragment length polymorphisms (AFLPs) as heritable genetic markers was demonstrated in a cross between the soybean species *Glycine max* and *Glycine soja* (Prabhu and Gresshoff 1994).

Our objective in the present study was to determine the genetic relationships between cultivars of *P.* × *hybrida* and five species of *Petunia*, thought to be involved in its development, using DAF. We also investigated the genetic variation within the genus *Petunia* for future breeding and genetic studies.

Materials and methods

Plant material

The *Petunia* species used were *P. axillaris*, *P. inflata*, *P. integrifolia*, *P. parodii*, and *P. violacea*; these are thought to be the most probable progenitors of the cultivated *P.* × *hybrida*. The ten cultivars of *P.* × *hybrida* chosen for the study differed as to introductory dates, flower color, and flower type. The cultivars 'Celebrity Chiffon', 'Coral Flash', 'Prime Time Rose', 'Prime Time White', 'Plum Crazy Madness', 'Purple Wave', 'Ultra Pink', and 'Ultra White', were newly developed commercial hybrids. 'Pale Face' was on the market before the prior eight and 'Mercury' was one of the oldest cultivars to be developed (C. Hope, personal communication). Various white-, violet-, rose- or pink-flowered cultivars were selected as well as white- and pink-flowered cultivars within the same series (i.e., 'Prime Time White' and 'Prime Time Rose', 'Ultra White' and 'Ultra Pink'). Cultivars of differing flowering type (i.e., grandiflora, multiflora, and floribunda) were also chosen. Cultivars represented the various morphological characteristics that are important for breeding and might influence the genetic relationships within the genus. 'Purple Wave' was of particular interest since it was from an entirely new breeding line of petunia (Rader 1993). Seed of the *Petunia* species and cultivars were obtained from several different sources from the United States and Europe (Table 1). Seeds of the *Petunia* species and cultivars of *P.* × *hybrida* were sown periodically over a 3-year period and grown according to standard floricultural practices.

DNA extraction

Extractions were performed initially using the method of Dellaporta et al. (1983). Due to its brevity, the method of Yoon et al. (1991) was also adopted. The extracted DNA was quantified using a TKO 100 mini-fluorimeter (Hoefer Scientific Instruments, San Francisco, Calif.) and the fluorescent intercalating agent Hoechst 33258.

Table 1 *Petunia* species and cultivars of *P.* × *hybrida*, flower color, and source

Species	Cultivar	Flower color	Source
P. axillaris		White	a
			b
			c
			d
			e
P. inflata		Violet	a
			b
			d
P. integrifolia		Violet	c
			e
P. parodii		White with dark veins	a
			b
			c
			d
			e
P. violacea		Violet	a
			b
			d
P. × *hybrida*	Celebrity Chiffon	Light pink	f
	Coral Flash	Coral	g
	Mercury	Blue-violet	b
	Pale Face	White	b
	Plum Crazy Madness	Violet with darker veins	h
	Prime Time Rose	Rose-pink	i
	Prime Time White	White	i
	Purple Wave	Violet	b
	Ultra Pink	Pink	i
	Ultra White	White	i

[a] Dr. John Power, Univ. of Nottingham, University Park, Nottingham NG7 2RD, United Kingdom
[b] PanAmerican Seed Company, P.O. Box 438, West Chicago, Ill 60119
[c] USDA Seed Storage Laboratory Fort Collins, Colo. 80523
[d] Dr. Ronald Koes, Dept. of Applied Genetics, Free University De Boelelaan 1087, HV Amsterdam, Netherlands
[e] Ms. Kim Krahl, Petunia Breeding Program, University of Georgia, Athens, Ga. 30602
[f] Bodger Seed Company, P.O. Box 5090, El Monte Calif 91734
[g] Sluis and Groot Seed Company, 5680 Greenwood Plaza, Englewood, Calif. 80111
[h] Ball Seed Company, P.O. Box 355, West Chicago, Ill. 60185
[i] Goldsmith Seed Company, P.O. Box 1349, Gilroy, Calif. 95021

DNA amplification

Amplification was done according to the DAF protocol (Caetano-Anollés et al. 1991). The amplification reaction was optimized by varying the concentrations of reaction components. The reaction cocktail contained in a final volume of 10 µl: 5 µl deionized H_2O, 1 µl deoxyribonucleosides (200 µM of each dNTP), 0.6 µl $MgCl_2$ (25 mM stock), 1 µl primer (30 µM stock), 1 µl reaction buffer [100 mM Tris, 100 mM KCl (pH 8.3)], 1 µl DNA template (diluted to 1.0 ng/µl with deionized sterile H_2O), 0.4 µl DNA polymerase (10 units/µl) Ampli-*Taq* Stoffel Fragment (Perkin-Elmer/Cetus, Norwalk, Conn.).

The DNA was amplified in an Ericomp (San Diego, Calif.) thermocycler using a 35-cycle program, with cycles of 10 s at 96 °C and 10 s at 30 °C. An extension phase at 72 °C was omitted. DNA amplification fragments were separated using polyacrylamide-gel electrophoresis (PAGE) (Caetano-Anollés and Gresshoff 1994) and silver staining (Bassam et al. 1991). Twenty five octamer and heptamer primers varying in GC content were screened. The ten most effective primers for petunia chosen for use in the phylogenetic analysis were GAGCCTGT (8.6A), GTAACGCC (8.6D), GATCGCAG (8.6F), GAAACGCC (8.6H), GTTACGCC (8.6I), GTATCGCC (8.6J), GTAACGGC (8.6M), AATGCAGC (8.7A), CCGAGCTG (8.7D), and CGCGGCCA (8.9A).

Evaluation of genetic variability and bulking

Plants of *P. axillaris*, *P. inflata*, *P. parodii*, *P. violacea*, *P. integrifolia* and *P.* × *hybrida* 'Mercury' both from the same seed source and different sources were checked for any variation between DNA banding patterns using three different primers (8.6H, 8.6J, and 8.6M). When individuals from the same or different sources produced varying fingerprints, DNA was bulked from different plants as a strategy to avoid plant to plant variation.

To validate the bulking strategy, extraction bulks, amplification bulks, and leaf-tissue bulks were compared. *P. inflata* showed the greatest degree of variation between individuals, so it was used as the representative species in this study. The 8.6J primer was employed because it identified the most variability between and within sources.

Extraction bulks

DNA was extracted from 20 different seedlings of *P. inflata* and each tube of template diluted to 10 ng/µl. Equal quantities of the diluted DNA from five or ten different seedlings were mixed together. The bulks were amplified using 1 ng/µl of template and the DNA fragments were separated using PAGE.

Amplification bulks

Diluted DNA (1 ng/µl) was first amplified from each of the 20 different seedlings and then equal quantities of the amplified products were bulked from five or ten individuals. The amplified products were thoroughly mixed and 3.0 µl from each bulk was loaded in the gel and separated.

Leaf tissue bulks

When the bulked extractions and amplifications showed identical banding profiles, equal quantities of leaf tissue from each of five or ten individuals was collected and pooled together. The bulked tissue was extracted, amplified, and the DNA fragments separated by PAGE.

Equal quantities of leaf tissue from the determined number of individuals of the other 14 *Petunia* taxa were bulked in the greenhouse and then extracted for phylogenetic analysis. Extractions of bulked leaf tissue, followed by amplification and separation, were done in replicate for each of these remaining species.

Phylogenetic analysis

The genetic relationships between species and cultivars of *Petunia* were explored by phylogenetic analysis using parsimony (PAUP) version 3.1 (Swofford 1993), by the neighbor-joining (NJ) method with the NTSYS-pc program (Numerical taxonomy and multivariate analysis system; version 1.7, Exeter Software, Setauket, N.Y.) and the distance estimator of Dice, and by principal coordinate analysis (PCO) using the Jaccard coefficient. In PCO, the distance matrix was transformed using the double-centering option, eigenvectors calculated, and the multidimensional hyperellipsoid viewed as a three-dimensional model using the NTSYS-pc program.

Banding patterns of the bulked leaf tissue from ten plants of *Petunia* species and cultivars were evaluated using ten octamer primers that were previously screened. Bands generated by each primer ($\leq$ 400 bp in length) were visually scored as present (1) or absent (0) and entered into the PAUP analysis as unordered. non-directed, and unweighted Wagner binary characters. Ambiguous bands were scored as missing data. Because exhaustive searches of data exceeded memory and time capabilities, we used the heuristic algorithm to identify minimal trees. The tree-bisection-reconnection (TBR) branch-swapping with MULPARS was used in a simple addition sequence.

Distance matrices showing the number of markers which were different (non-shared) between each pair of taxa and homoplasy values were calculated. Minimum trees were rooted by the midpoint rooting mode. The data set examined by the NJ method was used to construct a dendogram.

Results

Morphological differences were observed between the two white-flowered species, *P. parodii* and *P. axillaris*. *P. parodii* had a longer corolla tube with smaller petals than *P. axillaris*. The throat of the flowers had violet-purple veining that was less distinct in the flowers of *P. axillaris*. The violet-flowered species, *P. inflata*, *P. violaea* and *P. integrifolia*, had small flowers with short, slightly inflated corolla tubes and darker violet coloring in the center of the flower and were difficult to distinguish. *P. parodii* and *P. axillaris* could be distinguished from the *P. inflata*-*P. violacea*-*P. integrifolia* group by flower color and shape as well as by growth habit. The *P.* × *hybrida* group displayed intermediate characteristics of the former species but produced much larger flowers.

Our DAF studies showed distinguishable banding profiles between each of the species and cultivars. The optimized amplification reaction conditions generated reproducible results between amplifications and extractions (Fig. 1A).

DAF profiles of *P. parodii* did not show variation within or among seed sources. Fingerprints of *P. axillaris* were also consistent within and among different seed sources using the primers 8.6M and 8.6J; the primer 8.6H produced some variation between sources. Polymorphic profiles were generated between seedlings of *P. inflata* (Fig. 1 B), *P. violacea*, and *P. integrifolia* both within and among seed sources using primers 8.6M, 8.6J and 8.6H. The *P.* × *hybrida* cultivar 'Mercury' showed variation within its population using the primers 8.6M and 8.6J.

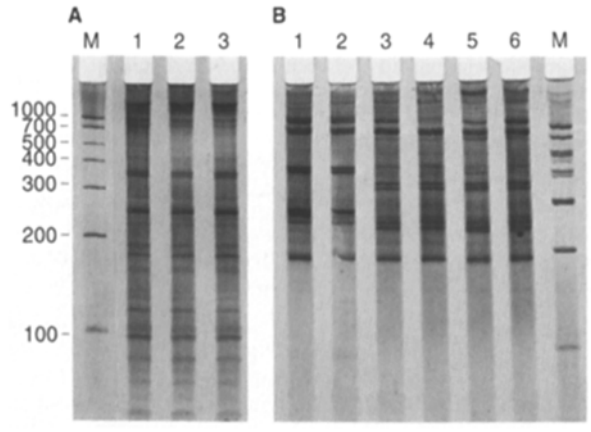

Fig. 1 Reproducibility of DAF profiles between three different extractions from the same plant of *P. inflata* using the primer 8.6 M (GTAACGGC) (**A**). Polymorphic DAF profiles of *P. inflata* seedlings from the University of Nottingham (*lanes 1 and 2*), PanAmerican Seed Co. (*lanes 3 and 4*), and the Free University, Amsterdam (*lanes 5 and 6*), using the primer 8.6J (GTATCGCC). Adjacent lanes are different amplifications from the same extracted plant tissue (**B**). Molecular weights (*M*) are given in bp

DNA polymorphisms were seen between fingerprints generated from extraction bulks made of equal quantities of DNA isolated from five plants of *P. inflata* (Fig. 2A). In contrast, DAF profiles of two ten-plant extraction bulks were identical and contained a combination of bands present in the four five-plant extraction bulks (Fig. 2A). The same variation was produced in the banding patterns among four five-plant amplification bulks (Fig. 2B). Again, identical banding patterns were found between two ten-plant amplification bulks. These results confirmed the monomorphic patterns produced between the ten bulked individual extractions, suggesting that competition among DNA annealing sites during the amplification process plays a minor role in determining fingerprint pattern. The banding patterns in the five- and ten-leaf extraction bulks were identical to those in the corresponding amplification bulks. A combination of all the bands present in each of the ten individual fingerprints of *P. inflata* seemed to be present in the ten-plant amplification and extraction bulks. Variation also occurred among the profiles of the four five-plant leaf-tissue bulks. The bulked leaf-tissue samples containing ten individuals produced identical fingerprints, except for a minor single-band difference (Fig. 2C).

Since our results indicate that bulking of ten individuals can generate an average fingerprint pattern representing the genetic variation present within the population, we extended our findings to other species. Two groups of ten-leaf tissue bulks from each of the other four *Petunia* species were compared and identical banding patterns were obtained (Fig. 3). Leaf-tissue bulks from ten different plants also produced identical patterns in the ten cultivars used in this study.

Ten selected primers of varying GC content generated 201 bands that were used for phylogenetic analysis of the five *Petunia* species and ten cultivars of *P.* × *hybrida*. Primer 8.7D was one of the primers used in the analysis (Fig. 4). In this study, the average number of bands (≤ 400 bp in length) per primer was 20. The number of bands for each primer ranged from 14 bands (for primers 8.6F and 8.7A) to 26 bands (for the primer 8.6D). One hundred and forty six (73%) of the 201 fragments scored were polymorphic and distinguished between each of the species and cultivars. The frequency of polymorphic DNA ranged from 61 to 88% per primer. The number of shared amplified fragments per primer ranged from 3 to 9.

Dendograms generated by PAUP and neighbor-joining (NJ) analysis using the data obtained with the ten octamer primers are shown in Fig. 5A and B. Phenetic and cluster analysis produced comparable results. *P. parodii* was grouped with the ten cultivars of *P.* × *hybrida*. The three violet-flowered species, *P. inflata*, *P. violacea* and *P. integrifolia*, were grouped together, while *Petunia* cultivars distributed generally according to their flower color. Pairwise marker differences generated with PAUP are given in Table 2. The average marker distance was 62 between species and 44 between cultivars of *P.* × *hybrida*. The morphologically similar violet-flowered species had an average marker distance of 40. Principal coordinate analysis (PCO) illustrates the three-dimensional relationships between

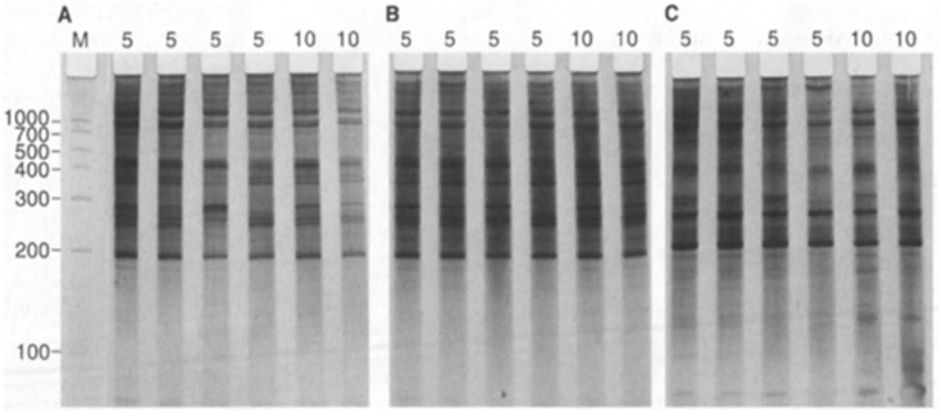

Fig. 2 DAF profiles from *P. inflata* DNA of four five-plant bulks (*lanes 5*) and two ten-plant bulks (*lanes 10*) made of equal quantities of extracted DNA (**A**), amplified DNA (**B**), and leaf tissue (**C**) using the primer 8.6J (GTATCGCC). Molecular weights (*M*) are given in bp

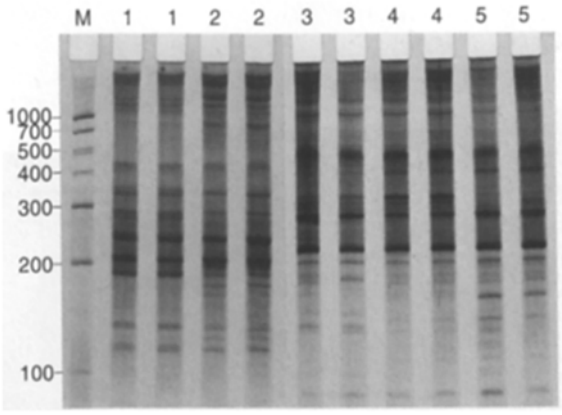

Fig. 3 DAF profiles of two ten-plant tissue bulks of *P. axillaris* (*lanes 1*), *P. parodii* (*lanes 2*), *P. inflata* (*lanes 3*), *P. integrifolia* (*lanes 4*), and *P. violacea* (*lanes 5*) using the primer 8.6J (GTATCGCC). Molecular weights (*M*) are given in bp

the different taxa (Fig. 5C) and supports the PAUP and NJ dendograms. Clustering and ordination techniques were used here to analyze the data and confirm the relationships inferred by parsimony analysis of our hybrid plant material. Please note that phylogenetic analysis implies a natural bifurcating evolutionary tree without hybridizations between its elements.

Discussion

Morphological characteristics are subject to environmental influences and, therefore, thorough and extensive observation of mature plants is often required for taxonomic classification. DNA fingerprinting, however, offers a faster and more precise way of determining relationships between individuals without such a constraint.

We have applied the DAF technique to the analysis of species and cultivars of *Petunia*. A careful optimization of amplification parameters resulted in the generation of reproducible banding profiles. Optimum conditions were similar to those found in the amplification of centipedegrass (Weaver et al. 1995) and bermudagrass (Caetano-Anollés et al. 1995).

Because certain taxa exhibited reproducible variation of DNA fingerprints between seedlings, we used the strategy of template and amplification bulking for the analysis of the genus *Petunia*. Consistencies were found among DAF profiles of each of the *Petunia* species by bulking extractions, amplifications, or leaf tissue from ten different individuals. Only five individual plants were necessary for consistent RAPD banding patterns for bulks of celery cultivars (Yang and Quiros 1993) but the bulking of ten plants was necessary in our study. The consistencies found between profiles of the bulked samples demonstrates that bulk assays are valuable for studying populations and for avoiding sampling of several different individuals within the population. Bulk sampling of different subpopulations could be desirable for locating unique markers in the profiles, and monitoring the introduction of new or lost variation into breeding programs.

DAF analysis indicated that the gametophytically self-incompatible *P. inflata, P. violacea, P. integrifolia*, and *P.* × *hybrida* (Ascher 1984) showed more genetic variation among and within seed sources than the self-compatible species *P. parodii* (Sink 1975). These results agreed with other studies associating increased variability with outcrossing species (Miller and Tanksley 1990). *P. axillaris* showed low levels of variability within its DAF profiles.

The overall genetic variability within species and cultivars of *Petunia* was considerable but comparable to other plants. The percentage of polymorphic loci in *Petunia* (73%) compares with the frequency of RAPD bands (92%) produced by 30 clones from different species of *Populus* (Castiglione et al. 1993). In contrast, the narrow genetic base of celery cultivars resulted in only 6.1% polymorphic RAPD bands (Yang and Quiros 1993). Similarly, of the 221 DAF bands scored for centipedegrass, only 30 were polymorphic (Weaver et al. 1995). Conversely, DAF analysis of soybeans showed a low frequency of DNA polymorphisms between *Glycine soja* and *G. max* ranging from 0 to 17% per primer (Prabhu and Gresshoff 1994).

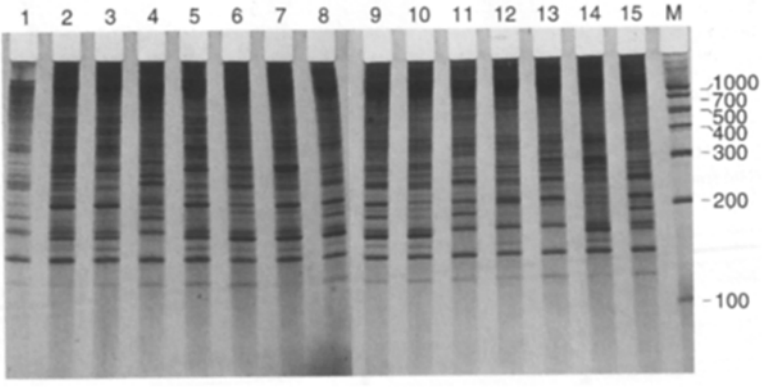

Fig. 4 DAF profiles using the primer 8.7D (CCGAGCTG) of five species (*lane 1- P. axillaris, lane 2- P. inflata, lane 3- P. integrifolia, lane 4- P. parodii, lane 5- P. violacea*) and ten cultivars of *P.* × *hybrida* (*lane 6-* 'Celebrity Chiffon', *lane 7-* 'Coral Flash', *lane 8-* 'Mercury' *lane 9-* 'Pale Face', *lane 10-*'Prime Time Rose', *lane 11-* 'Prime Time White', *lane 12-* 'Purple Wave', *lane 13-* 'Plum Crazy Madness', *lane 14-* 'Ultra Pink', *lane 15-* 'Ultra White') scored for phylogenetic analysis. Molecular weights (*M*) are given in bp

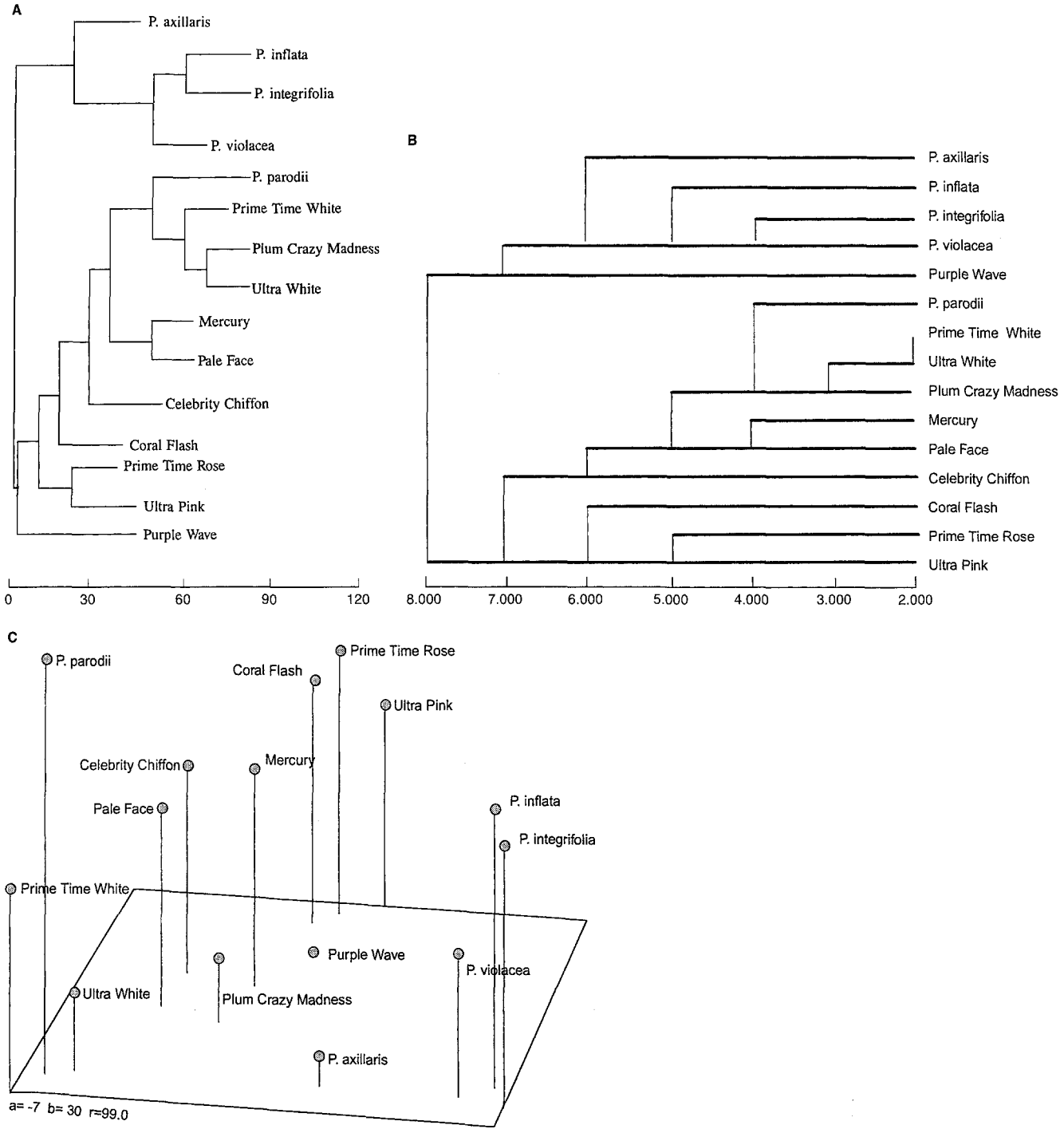

Fig. 5 Relationships among *Petunia* taxa based on PAUP (**A**), neighbor joining (**B**), and principal coordinate analysis using the distance estimator of Dice (**C**)

Phenetic, cluster and principal coordinate analysis of the DAF data separated *P. axillaris* from *P. parodii* and distingished between the violet-flowered species, *P. inflata, P. violacea,* and *P. integrifolia.* Other taxonomic studies using RFLP markers were unable to distinguish the white-flowered species or the violet-flowered species from each other (Koes et al. 1987; Shepherd et al. 1990). Classification of *P. axillaris* and *P. parodii* as separate species supported the conclusions of earlier morphological and chemotaxonomic studies (Natarella and Sink 1974, 1975: Sink 1984; Bahadur et al. 1989).

The three violet-flowered species were the most similar morphologically and have resulted in considerable discrepancies in past classifications (Ferguson and Ottley 1932; Natarella and Sink 1974; Sink 1984; Bahadur et al. 1989). The banding similarities shown by the low average-marker difference indicates a close relationship between these species. Our phylogenetic analysis separ-

Table 2 Pairwise marker difference among five species of *Petunia* (1–5) and ten cultivars of *P.* × *hybrida* (6–15) generated using the PAUP analysis

Taxa	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1 *P. axillaris*	–													
2 *P. inflata*	62	–												
3 *P. integrifolia*	68	40	–											
4 *P. parodii*	68	84	80	–										
5 *P. violacea*	58	45	34	82	–									
6 *'Celebrity Chiffon'*	57	71	69	51	63	–								
7 'Coral Flash'	58	59	61	65	59	35	–							
8 'Mercury'	57	59	59	53	55	38	39	–						
9 'Pale Face'	48	56	61	47	55	31	40	26	–					
10 'Prime Time Rose'	60	55	57	56	59	44	37	39	30	–				
11 'Prime Time White'	64	74	75	52	71	42	53	53	36	56	–			
12 'Purple Wave'	61	76	70	75	66	51	58	56	49	49	71	–		
13 'Plum Crazy Madness'	54	71	65	58	61	48	49	49	41	46	42	57	–	
14 'Ultra Pink'	53	50	46	59	52	43	30	40	40	21	57	45	40	–
15 'Ultra White'	51	72	70	57	65	43	52	36	25	53	33	60	33	46

ated the three violet-flowered species and supported the views of Bahadur et al. (1989) based on seed micro-characters. The taxonomic key of Fries (Sink 1984) and studies done on phenolic extracts of *Petunia* (Natarella and Sink 1974) also separated *P. inflata* from *P. violacea*. The species *P. integrifolia* was not referred to in previous studies.

Our analysis placed all the cultivars of *P.* × *hybrida* in one large group. *P. parodii* grouped in a cluster that included the *P.* × *hybrida* cultivars 'Prime Time White', 'Plum Crazy Madness' and 'Ultra White'. This implies that these three cultivars have *P. parodii* as a parent and that there is a progression of cultivars from this species. Studies on the inheritance of independently assorted genes for morphological characters (Sink 1975) and an electrophoretic analysis of proteins and peroxidases (Natarella and Sink 1975) showed close relationships between *P. parodii* and *P.* × *hybrida*.

Our analysis also indicated a major contribution of the violet-flowered *P. violacea* and *P. integrifolia* to the development of *P.* × *hybrida*. *Petunia inflata* is not compatible with *P. parodii* and should therefore be considered an unlikely contributor. In past studies, *P. axillaris*, *P. inflata*, *P. violacea* and *P. parodii* all intercrossed with breeding lines and cultivars of *P.* × *hybrida* (Sink 1975). With the exception of the *P. inflata* hybridization with *P. parodii*, interspecific crosses were successful using standard pollination techniques (Sink et al. 1978).

The date of introduction of the cultivars seemed to have little influence on their position in the dendogram relative to the wild species. This may indicate that many of these cultivars have other hybrid petunias as ancestors. 'Mercury' was in the center of the tree but grouped together with the less-recently developed cultivar 'Pale Face'. Flower type (i.e., grandiflora, multiflora, and floribunda), which is controlled by a single pair of alleles, G and g (Ewart 1984), also showed little influence on the taxa's position in the dendogram.

Surprisingly, cultivars were distributed within the dendogram by flower color. The cultivars 'Prime Time White' and 'Prime Time Rose' as well as 'Ultra White' and 'Ultra Pink' were grouped according to color and not by cultivar series. The white-flowered cultivars, 'Prime Time White' and 'Ultra White' were clustered with *P. parodii* along with 'Plum Crazy Madness'. The light-violet petal of the latter cultivar had dark-violet veining, similar to the veining present in the throat of the flowers of *P. parodii*.

The rest of the *P.* × *hybrida* cultivars had flowers that exhibited shades of pink, rose and violet. 'Mercury', a cultivar with a light blue-violet colored flower was positioned between the white and rose-pink groups. In particular, the violet flowers of 'Purple Wave' are similar morphologically to those of *P. inflata*, *P. violacea* and *P. integrifolia*. Our DAF analysis also identified 'Purple Wave' as the *P.* × *hybrida* cultivar more closely related to these violet-flowered species.

Of the 100 genes identified in *Petunia*, 32 are known to influence flower color either by regulating the pH of the corolla tube, deciding its venation patterns, or synthesizing specific flavonoids (Cornu 1984). Arbitrary primers target genomic sequences unrestrictively (Caetano-Anollés 1994). Since the distribution of cultivars within groups based on DAF markers appears to follow that based on flower color, petal coloration should be regarded as a complex character determined by loci randomly distributed throughout the *Petunia* genome.

Although the five species used in this study are thought to be the most likely progenitors of *P.* × *hybrida*, a broader observation of the genus *Petunia* may serve to identify other closely related wild species. Unique markers found in the wild species could be of value in future genetic-improvement programs. Our results demonstrate the utility of DAF in establishing relationships among closely related species and cultivars of *Petunia* as well as its potential use in other floriculture crops.

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