Evaluating Genetic Relationships of Geranium Using Arbitrary Signatures from Amplification Profiles

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Abstract. Our objective was to distinguish between eight cultivars of two geranium species, *Pelargonium xhortorum* L.H. Bailey (cutting and seed geranium) and *Pelargonium peltatum* (L.) L'Hér. ex Ait. (ivy geranium), and evaluate their genetic relationships using the nucleic acid scanning techniques of DNA amplification fingerprinting (DAF) and/or arbitrary signatures from amplification profiles (ASAP). Cultivars used in the study represented three commercial types: cutting, seed, and ivy geranium. Two seed geranium cultivars from each of the Dynamo and Orbit series were included. Cutting geranium cultivars were 'Designer Lilac Chiffon' and 'Starburst Red' and the ivy geraniums were 'Bernardo Guiber' and 'Vincio Guivin'. The ASAP amplification protocol used one of two arbitrary octamer primers, followed by reamplification with one of four different mini-hairpin primers. ASAP profiles were complex, with 66% of bands being polymorphic and useful in distinguishing between cultivars. Genetic relationships were evaluated by principal coordinate analysis and cluster analysis based on the Jaccard distance estimator. This analysis grouped cultivars by species according to commercial type, i.e., seed geraniums were in one large group, the cutting geraniums were grouped together, and the ivy geraniums were a separate branch.

Geraniums are a major floriculture crop with a current wholesale value of \$201.5 million (U.S. Dept. of Agriculture, 1995), based on sales of cutting and seed geraniums grown as potted plants and bedding flats and ivy geraniums grown as hanging baskets. The commercial cutting geranium originated from crosses among species native to South Africa (Laughner, 1993). Today's cutting geraniums are mostly tetraploid and are believed to have originated from a mutation followed by years of selection toward tetraploidy. The diploid geranium was revived in the 1960s with the introduction of the first seed-produced inbred, followed by production of F_1 hybrids (Laughner, 1993). Two tetraploids propagated from seed have been released recently. The ivy geranium, first discovered in the Cape Province of South Africa, is thought to be a hybrid of several species; most plants are propagated asexually (Holcomb and O'Donovan, 1993).

Patents have contributed to the geranium industry's growth during the past decade because the resultant royalty income has helped to support research and development programs (Craig, 1993). Since 1940, 316 asexually produced geranium cultivars have been patented

in the United States. F_1 hybrids cannot be patented, but the parental inbred lines are protected as trade secrets (Craig, 1993). The most significant standard for the patentability of a plant under the U.S. Plant Patent Act is the requirement of distinctness (Peet, 1995). The inventor of a new, asexually propagated plant is required to describe the plant to the best of his or her ability when applying for a patent. This includes a complete botanical description, one or more photographs, and a signed declaration that the claimed plant is new and was produced in cultivation. DNA fingerprints could accompany visual descriptions to document the unique nature of a new geranium cultivar.

Restriction fragment length polymorphisms (RFLP) and randomly amplified polymorphic DNA (RAPD) have been used for cultivar identification of a limited number of floriculture crops. Cultivars of rose have been characterized using RFLP (Hubbard et al., 1992) and RAPD (Torres et al., 1993). Lee et al. (1993) used distinct polymorphic markers to distin-

guish *Lilium* species using RAPD. And RAPD markers were used to assess the relationship among species, cultivars, and hybrids of lilacs (Marsolais et al., 1993).

DNA amplification fingerprinting (DAF) (Caetano-Anollés et al., 1991) distinguished between three violet-flowered species and two white-flowered species of *Petunia* that the RFLP method failed to separate (Cerny et al., 1996). In addition, DAF characterized ten cultivars of *Petunia xhybrida* (Cerny et al., 1996). DAF was used to assess the genetic distance of closely related cultivars of chrysanthemum (Scott et al., 1996). Polymorphic loci were found that uniquely identified closely related cultivars within a series (Scott et al., 1996). In contrast, the RAPD technique could not distinguish chrysanthemum cultivars which were somatic mutants of a previous variety from the parent or other cultivars within the series (Wolff and Peters-Van Rijn, 1993).

The ASAP method (Caetano-Anollés and Gresshoff, 1996), is a new, dual-step procedure whereby amplification with an octamer primer is followed by reamplification with a mini-hairpin primer to produce a complex fingerprint, thus increasing the probability of detecting polymorphisms. Our objective was to use DAF and/or ASAP to distinguish between cultivars of geraniums that represent the most important commercial types and evaluate the relationship between species, cultivars within species, and closely related cultivars within series. The utility of DNA fingerprinting for plant patent protection to prevent infringement of inventors' rights was to be explored.

Materials and Methods

We first determined the efficiency of the DAF protocol for distinguishing the geranium cultivars. When a lack of polymorphic content in the DAF banding patterns caused difficulty in distinguishing cultivars, the ASAP method was applied. The reproducibility of ASAP amplifications was first verified, followed by screening of primers for the final statistical analysis.

Plant material. Four cultivars of seed geranium from two series, and two cultivars each of cutting and ivy geranium were selected to represent diversity of geranium types (Table 1). Seed geranium cultivars were 'Dynamo White', 'Dynamo Deep Scarlet', 'Hot Pink Orbit', and 'Light Salmon Orbit'. Cutting geranium cultivars were 'Designer Lilac Chif-

Table 1. Geranium types, series, cultivars, sources and U.S. Plant Patent numbers.

Geranium type	Series	Cultivar	Source ²	Patent no.
Seed	Dynamo	Deep Scarlet	Sluis & Groot Seed Co.	
		White		
Cutting	Orbit	Hot Pink	Goldsmith	
		Light Salmon		
	Designer	Lilac Chiffon	Ball Seed Co.	PP 9, 552
		Starburst Red	Oglevee Ltd.	PP 9, 229
Ivy		Bernardo Guiber	Paul Ecke Ranch	PP 9, 587
		Vincio Guivin		PP 9, 620

²Sluis and Groot Seed Co., 5680 Greenwood Plaza, Englewood, CO 80111; Goldsmith Seed Co., P.O. Box 1349, Gilroy, CA 95021; Ball Seed Co., P.O. Box 355, West Chicago, IL 60185; Oglevee Ltd., 152 Oglevee Lane, Connellsville, PA 15425-3888; Paul Ecke Ranch, P.O. Box 230488, Encinitas, CA 92023-0488.

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fon' and 'Starburst Red'; 'Bernardo Guiber' and 'Vincio Guivin' were the ivy geranium cultivars.

Plants were grown according to standard floricultural practices. Young, not fully expanded (≈ 1 –2 cm in length) leaves were collected for DNA extraction. Leaf tissue was collected in early morning, frozen in liquid nitrogen, and transported immediately to storage at -80°C until needed for extraction. Leaf tissue of ten individual plants from each cultivar of seed geranium was bulked for DNA extraction, while the cutting and ivy geranium cultivars were bulks of five individual plants of each cultivar. DNA was bulked from different individual plants as a strategy to avoid plant-to-plant variation.

DNA extraction. Extractions of the bulked leaf tissue were performed using the Puregene Kit (Gentra Systems, Research Triangle Park, N.C.). The extracted DNA was quantified using a TKO 100 mini-fluorimeter (Hofer Scientific Instruments, San Francisco) and the fluorescent intercalating agent Hoechst 33258.

DNA amplification. Amplification was performed according to the DAF protocol (Caetano-Anollés et al., 1991). The reaction cocktail, contained in a final volume of 20 μL , included 9.2 μL deionized H_2O , 2 μL deoxyribonucleosides (200 μM of each dNTP), 1.2 μL MgCl_2 (25 mM stock), 2 μL primer (30 μM stock), 2 μL reaction buffer [10 mM Tris, 10 mM KCl (pH 8.3)], 3 μL DNA template (diluted to 1.0 ng- μL^{-1} with deionized sterile H_2O), and 0.6 μL DNA polymerase (10 units per μL) Ampli-Taq Stoffel Fragment (Perkin-Elmer/Cetus, Norwalk, Conn.). Ten octamer primers were screened. The DNA was amplified in a twin-block thermocycler (Ericomp, San Diego, Calif.) using a 35-cycle program, with cycles of 30 s at 96°C , 30 s at 30°C , and an extension phase of 30 s at 72°C .

Fig. 1. DAF profiles of cultivars of geranium using the primer 8.6H (GAAACGCC). Lane 1 = 'Dynamo White', Lane 2 = 'Dynamo Deep Scarlet', Lane 3 = 'Hot Pink Orbit', Lane 4 = 'Light Salmon Orbit', Lane 5 = 'Vincio Guivin', Lane 6 = 'Bernardo Guiber', Lane 7 = 'Starburst Red', Lane 8 = 'Designer Lilac Chiffon'. Molecular weights are given in bp.

The ASAP strategy (Caetano-Anollés and Gresshoff, 1996) was the second phase of a dual step amplification procedure whereby amplification with octamer primers [GAAACGCC (8.6H) and GTAACGGC (8.6M)] was followed by reamplification with one of four different mini-hairpin primers, HpA12 (GCGAAAGC-CGA), HpB52 (GCGACAGC-TCA), HpC19 (GCGAGAGC-TCG), and HpD4 (GCGATAGC-CCA). Mini-hairpin primers consist of a stem loop structure with a three nucleotide arbitrary core sequence that serves as the annealing site. The ASAP amplification reaction contained in a final volume of 20 μL included 6 μL deionized H_2O , 2 μL deoxyribonucleosides (200 μM of each dNTP), 1.2 μL MgSO_4 (67-mM stock), 6 μL primer (30- μM stock), 2 μL reaction buffer (10X TTNK), 2 μL DNA template (1:50 dilution of the DAF amplification), and 0.8 μL DNA polymerase (10 units per μL) Ampli-Taq Stoffel Fragment.

Separation and staining. DNA amplification fragments were separated using polyacrylamide gel electrophoresis (PAGE) (Caetano-Anollés and Gresshoff, 1994) with modification (Bassam and Bentley, 1995). The fragments were then silver stained (Bassam et al., 1991) for visualization.

Data analysis. A total of eight primer combinations (two octamers $\times$ four mini-hairpins) were used in the analysis of the eight geranium cultivars. Bands generated by each primer combination (≤ 1000 bp in length) were visually scored on a light box as present (1) or absent (0). Ambiguous bands were scored as missing data. To estimate genetic distance

Fig. 2. Reproducibility of ASAP reactions. Lanes 1–4 are separate amplifications of 'Designer Lilac Chiffon' DNA with primer 8.6H (GAAACGCC) followed by reamplification with HpA12 (GCGAAAGC-CGA), and lanes 5–8 are separate amplifications of 'Designer Lilac Chiffon' DNA with primer 8.6M (GAAACGCC) followed by reamplification with HpB52 (GCGACAGC-TCA). Lanes 1, 2, 5, and 6 were amplified in one block of the thermocycler and lanes 3, 4, 7, and 8 were amplified at the same time in a separate block.

among taxa, Jaccard similarity coefficients were computed using the simple matching method of the NTSYS-pc program (numerical taxonomy and multivariate analysis system, version 1.8; Exeter Software, Setauket, N.Y.). The resultant matrix was then used in the SAHN clustering procedure to analyze relationships among cultivars. Both an unweighted pair group cluster analysis (UPGMA) and principal coordinate analysis (PCO) were used.

Results and Discussion

DAF using ten different octamer primers resulted in very few polymorphisms. Similar banding profiles were seen between individual cultivars of cutting and seed geranium, although ivy geranium cultivars were somewhat distinguishable from the others (Fig. 1). Because distinguishing different cultivars within a species was difficult, and sometimes impossible, the ASAP method was employed. Mini-hairpin primers used for ASAP have a stem-loop structure with a three nucleotide annealing site that produces complex fingerprints, thus increasing the probability of detecting polymorphisms by increasing the number of annealing sites (Caetano-Anollés and Gresshoff, 1994). In other studies, DAF profiles have provided exquisite detail of differences in plant cultivars and have been used to distinguish closely related species and cultivars, as well as for phylogenetic analysis (Cerny, et al., 1996; Scott, 1996). In addition, DAF identified closely related chrysanthemum cultivars within a series (Scott et al., 1996), whereas RAPD could not distinguish cultivars within series (Wolff and Peters-Van Rijn, 1993).

To determine reproducibility of the ASAP amplification reaction for geranium, four sepa-

Fig. 3. Polymorphic ASAP profiles of geranium cultivars using primer 8.6H (GAAACGCC) followed by reamplification with primer HpA12 (GCGAAAGC-CGA). Lane 1 = 'Dynamo White', Lane 2 = 'Dynamo Deep Scarlet', Lane 3 = 'Hot Pink Orbit', Lane 4 = 'Light Salmon Orbit', Lane 5 = 'Vincio Guivin', Lane 6 = 'Bernardo Guiber', Lane 7 = 'Starburst Red', Lane 8 = 'Designer Lilac Chiffon'. Molecular weights are given in bp.

rate amplifications with two primer combinations [8.6H (GAAACGCC) with HpA12 (GCGAAAGC-CGA) and 8.6H (GAAACGCC) with HpB52 (GCGACAGC-TCA)] and 'Designer Lilac Chiffon' DNA template from a single extraction were repeated twice in each block of the twin-block thermocycler and amplified at the same time. Polymorphic DNA between amplifications was not detected (Fig. 2). This demonstrated that, although some variation in staining intensity may be evident, overall profile patterns were consistent for independent DNA amplifications. Reproducibility has been verified repeatedly between extractions and amplifications of floriculture crops using the DAF technique (Cerny et al., 1996; Scott et al., 1996). ASAP amplifications were also determined to be reproducible with soybean (Caetano-Anollés and Gresshoff, 1996).

In contrast with the results with DAF, the ASAP method generated complex polymorphic banding profiles facilitating separation of all eight cultivars (Fig. 3). Using the ASAP method with eight primer combinations, 330 bands were generated and used for analysis of the cultivars of geranium. The average number of bands (≤ 1000) per primer was ≈ 41 , ranging from 27 to 51 (Table 2). About 66% of the ASAP fragments scored were polymorphic and distinguished between all cultivars. Of the eight total profiles produced by each primer, the percentage of unique fingerprints was usually 100%, depending on primer.

The similarity matrix was used to generate a dendrogram (Table 3, Fig. 4). As expected, relatively low similarity values were observed between the cultivars of the two species, differentiating the *P. xhortorum* and *P. peltatum* representatives into separate clusters. Cluster analysis of the coefficients of similarity for taxa further revealed that seed geraniums clustered in one group, cutting geraniums were clustered together, and the ivy geranium cultivars were separate from the other species. Cutting and seed geraniums of the same species were separated into two groups. This was expected because tetraploid and diploid geraniums have evolved separately for over one hundred years (Laughner, 1993). Cultivars were clearly distinguished within the two series of seed geranium.

To prevent infringement of the inventors' rights, geraniums have been protected by plant patents, general patents, or trade secrets. During the past several years, morphological traits such as flower color and patterning and leaf color have been used to establish uniqueness

Table 2. DAF and ASAP primers, number of bands produced, polymorphic bands, and unique profiles among eight cultivars of geranium.

DAF primer	ASAP primer	Bands (no.)	Polymorphisms (no.)	Unique profiles ² (no.)
8.6m	HpA12	45	25	8
	HpB52	46	26	8
	HpC19	45	29	8
	HpD4	27	12	6
8.6h	HpA12	51	37	8
	HpB52	28	16	8
	HpC19	40	32	8
	HpD4	48	39	8

²Eight profiles total.

Fig. 4. Relationships among geranium cultivars using the SAHN cluster analysis based on the Jaccard distance estimator, 3-dimensional graph (top), and principal coordinate analysis dendrogram (bottom). Numbers 1-8 refer to geranium cultivars. 1 = 'Dynamo White', 2 = 'Dynamo Deep Scarlet', 3 = 'Hot Pink Orbit', 4 = 'Light Salmon Orbit', 5 = 'Vincio Guivin', 6 = 'Bernardo Guiber', 7 = 'Starburst Red', 8 = 'Designer Lilac Chiffon'.

Table 3. Similarity matrix based on Jaccard coefficients among eight geranium cultivars.

Cultivar	Cultivar							
	1	2	3	4	5	6	7	8
1. Dynamo White	1.0000000							
2. Dynamo Deep Scarlet	0.7782427	1.0000000						
3. Orbit Hot Pink	0.7887931	0.8165939	1.0000000					
4. Orbit Light Salmon	0.8193833	0.7692308	0.8280543	1.0000000				
5. Vincio Guivin	0.5703704	0.5342960	0.5485075	0.5763359	1.0000000			
6. Bernardo Guiber	0.5390335	0.5145985	0.5399240	0.5742188	0.8157895	1.0000000		
7. Starburst Red	0.7327935	0.6889764	0.7314050	0.7521008	0.5340502	0.5144928	1.0000000	
8. Designer Lilac Chiffon	0.7007874	0.6653846	0.7056452	0.7254098	0.5211268	0.5125448	0.8319328	1.0000000

of new geranium cultivars. In the future, such traits as host-plant resistance, interspecific hybrids, postproduction characteristics, and stress-related tolerances will be important traits distinguishing similar geranium cultivars (Craig, 1993). In other studies involving soybean breeding, increased numbers of cultivars and a finite number of conventional characters have revealed that such morphological traits will not suffice to establish uniqueness (Rongwen et al., 1995). In an infringement case concerning misappropriation of maize inbreds held as trade secrets, the patent owner was successful in proving genetic relatedness of the misappropriated vs. patented plant varieties with genetic fingerprint data (Peet, 1995).

We have used ASAP to develop unique DNA profiles for eight cultivars of geranium. DNA fingerprints could be used in conjunction with visual descriptions to enhance the portrayal of uniqueness of a new geranium cultivar when applying for a plant patent. Our results demonstrate the ease and effectiveness of ASAP in establishing cultivar identification, as well as its potential use for evaluating relationships among closely related species and cultivars of geranium.

Literature Cited

- Bassam, B.J. and S. Bentley. 1995. Electrophoresis of polyester-backed polyacrylamide gels. *BioTechniques* 19:568–572.
- Bassam, B.J., G. Caetano-Anollés, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. *Anal. Biochem.* 196:80–83.
- Caetano-Anollés, G., B.J. Bassam, and P.M. Gresshoff. 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide primers. *Bio/Technology* 9:553–557.
- Caetano-Anollés, G. and P.M. Gresshoff. 1996. Generation of sequence signatures from DNA amplification fingerprints with mini-hairpin and microsatellite primers. *BioTechniques* 20:1044–1056.
- Caetano-Anollés, G. and P.M. Gresshoff. 1994. DNA amplification fingerprinting using arbitrary mini-hairpin oligonucleotide primers. *Bio/Technology* 12:619–623.
- Cerny, T.A., G. Caetano-Anollés, R.N. Trigiano, and T.W. Starman. 1996. Molecular phylogeny and DNA amplification fingerprinting of *Petunia*. *Theor. Appl. Genet.* 92:1009–1016.
- Craig, R. 1993. Intellectual property protection, p. 389–404. In: J.W. White (ed.). *Geraniums IV: The grower's manual*. Ball Publishing, Geneva, Ill.
- Holcomb, E.J. and E.J. O'Donovan. 1993. Ivy geraniums, p. 191–199. In: J.W. White (ed.). *Geraniums IV: The grower's manual*. Ball Publishing, Geneva, Ill.
- Hubbard, M., J. Kelly, S. Rajapakse, A. Abbott, and R. Ballard. 1992. Restriction fragment length polymorphisms in rose and their use for cultivar identification. *HortScience* 27:172–173.
- Laughner, L.J. 1993. History, p. 363–371. In: J.W. White (ed.). *Geraniums IV: The grower's manual*. Ball Publishing, Geneva, Ill.
- Lee, W.B., S.Y. Choi, and Y.S. Kim. 1993. An application of random amplified polymorphic DNA (RAPD) to systematics of some species of *Lilium* in Korea. *Kor. J. Plant Tax.* 23(2):35–42.
- Marsolais, J.V., J.S. Pringle, and B.N. White. 1993. Assessment of random amplified polymorphic DNA (RAPD) as genetic markers for determining the origin of interspecific lilac hybrids. *Taxon.* 42:531–537.
- Peet, J.D. 1995. Protection of plant-related inventions in the United States. *Foley & Lardner Biotechnology and Pharmaceutical Practice Group, Foley & Lardner* (internet communication).
- Rongwen, J., M.S. Akkaya, A.A. Bhagwat, and U. Lavi. 1995. The use of microsatellite DNA markers for soybean genotype identification. *Theor. Appl. Genet.* 90:43–48.
- Scott, M.C., R.N. Trigiano, and G. Caetano-Anollés. 1996. DNA amplification fingerprinting identifies closely related *Chrysanthemum* cultivars. *J. Amer. Soc. Hort. Sci.* 121:1043–1048.
- Torres, A.M., T. Millán, and J.I. Gubero. 1993. Identifying rose cultivars using random amplified polymorphic DNA markers. *HortScience* 28:333–334.
- U.S. Department of Agriculture. 1995. *Agriculture Statistics Board. Floriculture crops* (internet communication).
- Wolff, K. and J. Peters-Van Rijn. 1993. Rapid detection of genetic variability in chrysanthemum (*Dendranthema grandiflora* Tzvelev) using random primers. *Heredity* 71:335–341.