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Target Region Amplification Polymorphism (TRAP) as a Tool for Detecting Genetic Variation in the Genus *Pelargonium*

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***Abstract*. *Pelargonium* was a priority genera collected by the Ornamental Plant Germplasm Center (OPGC) until a recent reorganization. To preserve genetic diversity for future breeders, OPGC collects heirloom cultivars, breeding lines, and wild species. The current *Pelargonium* collection at OPGC consists primarily of cultivars originating from *P.* ×*hortorum* and *P.* ×*domesticum*. Target region amplification polymorphism (TRAP) has the advantage of producing a large number of markers through use of sequence information that is already available. Our first goal was to determine the feasibility of TRAP for the analysis of this large collection, so that in the future the most diverse genotypes may be retained. To achieve this goal, we first modified existing DNA extraction techniques to account for the high levels of phenolic compounds present in some *Pelargonium* species by combining several washes to remove the phenolics with the addition of high levels of antiphenolic compounds. Second, we evaluated the TRAP procedure using the DNA isolated from 46 accessions. For 44 accessions, one or two primer combinations generated enough fragments to discriminate each of the accessions, and similar clades were produced by cluster analysis of the polymorphic fragments amplified by different primer combinations. All the scorable fragments were polymorphic, for one primer combination there were 148 markers from one image and the other produced 160 markers on two images. These results demonstrate that TRAP is an effective method for molecular characterization of ornamental collections.**

Pelargonium species are some of the most popular flowers in the world, and the Royal Horticultural Society (http://www.rhs.org.uk/) listed >3000 entries for distribution in 2004. In 2004, the combined wholesale value for all flats, hanging baskets, and pots of *Pelargonium* plants in the United States was over 206 million USD (Jerardo, 2006). There are over 280 species in the genus, and most are native to South Africa. Some species have been domesticated to take advantage of their distinct flower color, leaf shape, and scent (Van der Walt, 1977; Van der Walt and Vorster, 1988). The interest in breeding has led to many improved or novel cultivars. In fact, efforts have been made to improve cultivars through somaclonal variation (Ravindra et al., 2004) and genetic transformation (Hassanein et al., 2005; Winkelmann et al., 2005).

The United States' floricultural industry ranked *Pelargonium* as one of the three most important floral species for germplasm conservation (Tay, 2003), and in 2003, the OPGC (Ornamental Plant Germplasm Center, Columbus, OH) *Pelargonium* collection included ≈900 accessions representing ≈60 species. Plants were donated by Richard Craig of Penn State University and Charles Heidgen of Shady Hill Gardens. Because the *Pelargonium* cultivars require vegetative propagation, the germplasm can be more efficiently maintained at OPGC if only the most genetically dissimilar accessions are retained. This necessitates a large-scale molecular screening of the current collection to identify redundant cultivars and provide space for additional accessions representing the >200 remaining species.

Ornamental plants are often selected for their aesthetic qualities rather than their ability to survive in any particular environment. As a result, the genetic base of most modern flower cultivars risks loss of other important traits. Gene banks (like OPGC) serve an important function by maintaining populations with traits that could otherwise be lost before anyone knew their importance (Tanksley and McCouch, 1997). By preserving the diversity of ornamentals, OPGC is protecting consumers and breeders against loss of genetic diversity required for future breeding. Many ornamental plants also produce compounds that have potential use in agriculture and medicine. For example, the essential oils that scented geraniums (*Pelargonium* sp.) produce have been used as perfumes and food flavoring (Becker and Brawner, 1996; Ravindra et al., 2004), and their inhibitory effects on bacteria and nematodes have also been studied (Lis-Balchin et al., 1995). In addition, some *Pelargonium* accessions are resistant to arthropods, which a team at The Pennsylvania State University attributed to the anacardic acid composition of glandular exudates (Shultz et al., 1996).

Previous molecular systematics of *Pelargonium* species have focused on the use of chloroplast DNA (Bakker et al., 2000; James et al., 2004). Although maternal inheritance of chloroplast DNA can provide an advantage in studies of many plant species, this is not necessarily the case with *Pelargonium* species. Pollen can carry chloroplast DNA, providing for potential biparental inheritance (James et al., 2001).

We intend to determine the genetic similarity of the accessions through molecular characterization by target region amplified polymorphism (TRAP) markers (Hu and Vick, 2003). TRAP is a technique that combines the AT- and GC-rich primers of SRAP (sequence-related amplification polymorphism; Li and Quiros, 2001) with a third "fixed" primer that matches a gene of interest. The advantages of using this technique are that there is no need for extensive pre-PCR treatment of the DNA samples, that

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many fragments can be amplified in a single PCR reaction, and that previously reported genetic information has the potential to be used as the targeted primer. Each of the two arbitrary primers has a different fluorescent label, so the PCR-amplified DNA fragments can be detected by two different channels, each producing a separate image for one of the two fluorescently labeled primers on the same gel, using the LI-COR Global Genotyper (LI-COR Bioscience, Lincoln, NE). Although targeting a gene of interest can provide an advantage for finding markers to map that gene, it is important to remember the markers are not all directly linked to the gene of interest. Many of the fragments amplified with the fixed primer will be products of slight mismatches, because the first five cycles have a lower stringency. Also, some fragments are amplified between the arbitrary primers and would therefore not be linked to the gene of interest at all. Hu and Vick (2003) demonstrated that TRAP provides reproducible results by analyzing wheat from a recombinant inbred line and from an F_2 generation. They also reported that marker data generated from 16 perennial *Helianthus* species with two TRAP reactions by six primers (two arbitrary and one fixed in each TRAP reaction) produced a phylogenetic tree that had similar clustering as those produced using morphological characteristics.

The TRAP technique has been applied with various plants, including lettuce (*Lactuca sativa* L.), common bean (*Phaseolus vulgaris* L.), sugarcane (*Saccharum* species), wheat (*Triticum aestivum* L.), and sunflower (*Helianthus annuus* L.). Hu et al. (2005) amplified 107 polymorphic markers from 10 TRAP reactions, which discriminated the 53 lettuce cultivars analyzed and was fairly stringent in grouping the cultivars by horticultural types. Miklas et al. (2006) used a disease-resistance gene in the common bean and demonstrated that TRAP identified new markers linked to disease resistance. Alwala et al. (2006) used TRAP on sugarcane, which confirmed known systematics and also sorted the phylogeny according to the gene of interest. Yang et al. (2005) reported using TRAP to detect markers for quantitative trait loci (QTL) mapping in wheat. They observed that TRAP produced more markers but not in the targeted QTL regions. Liu et al. (2005) compared TRAP and SSR markers in wheat and determined that both could be used to generate genetic maps for QTL identification, but that TRAP produced more data from a single reaction. Chen et al. (2006) identified six TRAP markers linked to a male-sterility gene in sunflower, and by using SSR markers for comparison with the public linkage map, they mapped the gene to linkage group 10.

Wang et al. (2006) attempted to use TRAP to map the physical end of a linkage map by using a fixed primer for the telomere region of the chromosome to which a Hessian fly resistance gene was mapped, but the linked marker was not in the telomere region. Using the fixed primers derived from the *Arabidopsis*-type telomere sequences, Hu (2006) succeeded in defining 21 of the 34 linkage group ends of the sunflower linkage map. As for the fragments amplified by TRAP, Hu (2006) speculated from two experiments in cloning TRAP fragments, that the three-primer TRAP reaction (initial ratio of fixed primer to arbitrary primers, 30:1:1) could amplify six types of fragments with relative frequencies of 900:30:30:1:1:2. The most abundant fragment type has the fixed primer on both ends and is not observed because it is not fluorescently labeled. The next two types combine the fixed primer on one end with one of the two arbitrary primers on the other end, and they represent most of the observed fragments. The two least-frequent fragment types have the same arbitrary primer on both ends, and the final type has a different arbitrary primer at each end.

TRAP was chosen as an ideal method for analyzing the collection of a gene bank because it allows for evaluation of genetic variation that emphasizes specific traits of interest. As it had not yet been tested on *Pelargonium* species, our objective was to demonstrate that TRAP could provide markers that distinguish between *Pelargonium* species. Successful results will segregate according to known species designation of selections, indicating that our long-term goal of applying TRAP to evaluate the similarity of all the accessions in the OPGC collection should do the same.

Materials and Methods

Plant material and TRAP primers. Forty-six *Pelargonium* accessions were selected from the 900 accessions available at OPGC in 2003, including some with known pedigrees to demonstrate clustering within a species and some representing the diversity of species in the OPGC collection to test for future applicability to the entire collection. Selected accessions included nine species and 22 cultivars (Table 1). The primers used here were selected as a result of Jinguo Hu's experience that these primers, which have been developed for other projects in his laboratory, produce acceptable TRAP results for most plant families. Because the goal is to evaluate the procedure rather than a specific gene of interest, these primers were ideal. The two arbitrary primers were Sa12–700 (TTCTAGGTAATCCAACAACATTCTAG GTAATCCAACAACA; Hu et al., 2005) and Ga5–800 (GGAACCAAACACATGAAGA GGAACCAAACACATGAAGA; Hu et al., 2005), and the fixed primers were QHA21B09a (TGTCATTCAATTCGGTGC, homolog to an *Arabidopsis thaliana* gene coding for an unknown protein At5g65840.1) and QHF6H21L (ACAGGAAAAGCCTGTCAC, homolog to an *A. thaliana* gene coding for the BEL1-like homeobox 1 protein). Information regarding both fixed primers was obtained from The Compositae Genome Project website (http://compgenomics.ucdavis.edu).

Pelargonium DNA extraction. This high-salt CTAB procedure was modified from techniques for cactus (Tel-zur et al., 1999) and for barley (Saghai-Maroof et al., 1984). *Pelargonium* leaf tissue (1 g) ground in liquid nitrogen was added to 8 mL extraction buffer (as in Tel-zur et al., 1999). The samples were centrifuged at 3300 rpm for 15 min, and the pellet was resuspended in 8 mL fresh extraction buffer. As in Tel-zur et al. (1999), this was repeated for a total of 3 washes. After the third wash, the pellet was suspended in a mixture of 1 mL extraction buffer and 5 mL high salt-CTAB buffer [from Tel-zur et al. (1999), with modifications: doubled Tris-HCl concentration, added sorbitol (25 $g\cdot L^{-1}$), 10% SDS, PVPP (Sigma P6755; 20 $g\cdot L^{-1}$), and proteinase K (0.002 $g\cdot L^{-1}$)], and incubated in a 55–65 °C water bath for 60 min. An equal volume of chloroform was mixed in by shaking vigorously for 35 s and then separated by centrifuging at 3300 rpm for 15 min. The supernatant was transferred to a new tube, precipitated with isopropyl alcohol/ sodium acetate, centrifuged (3300 rpm for 15 min), and washed in ethanol as in Tel-zur et al. (1999). After centrifuging again at 3300 rpm for 10 min, the pellet was air-dried for 10–15 min. The pellet was dissolved in 300 μL TE buffer with 10 μL RNase (10 $mg\cdot mL^{-1}$) and then incubated, extracted (twice), and precipitated according to Tel-zur et al. (1999), centrifuging when called for at 13,200 rpm for 10 min. The pellet was washed with 500 μL ice-cold ethanol (75%) and centrifuged at 13,200 rpm for 5 min. The pellet was air-dried for 15 min and was then dissolved in 50 μL TE before being stored at –20 °C.

TRAP amplification. The PCR reaction was conducted using 96-well plates holding 15 μL per well, ≈50 ng DNA, 1.5 μL Qiagen 10× buffer, 1.5 μL 25 mM $MgCl_2$, 1 μL 5 mM dNTPs, 0.3 pmol of each fluorescently labeled arbitrary primer, 1 pmol fixed primer, and 1.5 units of *Taq* polymerase. The reaction ran at 94 °C for 2 min; 5 cycles of 94 °C for 45 s, 35 °C for 45 s, 72 °C for 1 min; 35 cycles of 94 °C for 45 s, 50 °C for 45 s, 72 °C for 1 min; and finally 72 °C for 7 min. After loading dye was added to the TRAP products, the samples were loaded onto sequencing gels in LI-COR sequencers, which recorded digital images of the fluorescent banding patterns.

Gel analysis. The gel images were analyzed using Crosschecker (Buntjer, 1999). Any data points that were not clearly present or absent were manually designated as missing data to avoid biasing the results. The binary interpretation was transferred to NTSYSpc 2.11S (Rohlf, 2000), in which a matrix of "simple matching coefficients" was generated assigning a numerical value to the similarities between each pair of individuals. Then a dendrogram was generated using the UPGMA method of the SAHN function. All the dendrograms that could be produced from different combinations of tied similarity values were combined by majority rule into a consensus dendrogram with branch probabilities indicating the percentage of dendrograms that contain that subset. Using the cophenetic values and

Table 1. Pelargonium accession details.[z]

No.	Source	Species	Cultivar/breeding no. [parentage]	Ploidy/scent
511	Shady Hill	*P. ×fragrans*	Apple	(Scented)
529	Shady Hill	*P. ×fragrans*	Golden Nutmeg	(Scented)
542	Shady Hill	*P. ×fragrans*	Nutmeg	(Scented)
543	Shady Hill	*P. ×fragrans*	Old Spice	(Scented)
526	Shady Hill	*P. ×fragrans*	Snowy Nutmeg	(Scented)
346	R. Craig	*P. ×fragrans*		(Scented)
495	Shady Hill	*P. ×hortorum*	Frank Headley	2x
238	R. Craig	*P. ×hortorum*	Juliette syn. Risque [80–191–3 × Honseler's Glorie Rot]	4x
548	Shady Hill	*P. ×hortorum*	Madame Salleron	2x
503	Shady Hill	*P. ×hortorum*	Petals	2x
505	Shady Hill	*P. ×hortorum*	Wilhelm Langguth	2x
240	R. Craig	*P. ×hortorum*	203 syn. 81–344–4 [Honseler's Glorie Rot × Karminball]	4x
218	R. Craig	*P. ×hortorum*	60–58–5 [G 9–18]	4x
265	R. Craig	*P. ×hortorum*	71–17–7	2x
221	R. Craig	*P. ×hortorum*	78–125–4	4x
219	R. Craig	*P. ×hortorum*	78–51–5	2x
222	R. Craig	*P. ×hortorum*	79–32–5 [Stadtbern × Wilhelm Langguth]	2x
223	R. Craig	*P. ×hortorum*	79–33–6 [(Stadtbern × Wilhelm Langguth) × Berlin]	2x
226	R. Craig	*P. ×hortorum*	80–167–26	4x
234	R. Craig	*P. ×hortorum*	81–18–2 [Stadtbern]	2x
285	R. Craig	*P. ×hortorum*	82–127–6 [80–196–1 × 80–210–45]	4x
283	R. Craig	*P. ×hortorum*	83–185–32	2x
287	R. Craig	*P. ×hortorum*	83–186–11	2x
282	R. Craig	*P. ×hortorum*	83–186–4	2x
338	R. Craig	*P. ×hortorum*	837	4x
256	R. Craig	*P. ×hortorum*	86–106–15 [Jean Billes]	4x
273	R. Craig	*P. ×hortorum*	86–33–17 [82–107–1]	4x
244	R. Craig	*P. ×hortorum*	86–54–26	4x
337	R. Craig	*P. ×hortorum*	87–16–1	2x
284	R. Craig	*P. ×hortorum*	Jubilee syn. 175 83–16–3 [Jean Billes × Honseler's Glorie Lila]	4x
264	R. Craig	*P. ×inquinans*	86–22–1 syn. G 630	2x
258	R. Craig	*P. caylae*	Caylae	4x
417	R. Craig	*P. caylae*	Steu 2198	4x
550	Shady Hill	*P. cotyledonis*		
531	Shady Hill	*P. crispum*	French Lace	(Scented)
523	Shady Hill	*P. crispum*	Lemon	(Scented)
551	Shady Hill	*P. dasycaule*		
514	Shady Hill	*P. denticulatum*	Balsam	(Scented)
516	Shady Hill	*P. graveolens*	Chocolate Mint	(Scented)
519	Shady Hill	*P. graveolens*	Peppermint Rose	(Scented)
524	Shady Hill	*P. graveolens*	Snowflurry	(Scented)
549	Shady Hill	*P. sidoides*		
512	Shady Hill	*P.* sp.	Apple Cider	(Scented)
518	Shady Hill	*P.* sp.	Cloves	(Scented)
527	Shady Hill	*P.* sp.	Lavender Lad	(Scented)
535	Shady Hill	*P.* sp.	Pine	(Scented)

[z]Accessions are numbered with OPGC identification numbers; source refers to where OPGC acquired the accession; the cultivar column after the species identification includes cultivars in single quotes, breeding numbers as provided by the source, and parentage as provided by the source.

matrix comparison modules of NTsys, the cophenetic correlation was generated and the *r* value was reported as an estimate of the cluster's "goodness of fit."

Results and Discussion

In evaluating TRAP's potential to discriminate a large population of *Pelargonium* accessions, two issues were considered. The first was isolation of high-quality DNA for TRAP reactions, as many *Pelargonium* species produce phenolic compounds. After several rounds of testing, high-quality *Pelargonium* DNA was produced by combining the multiple washes of an existing procedure by Tel-zur et al. (1999) with increased antiphenolic chemicals from a procedure by Saghai-Maroof et al. (1984). The second issue was whether TRAP markers could distinguish *Pelargonium* accessions and group them according to phenotypes. Appropriate gel images were obtained from most of the 46 accessions using two fixed TRAP primers that have already been used in other plant species (Hu et al., 2005). Two dendrograms from separate data sets placed *P. ×hortorum* with related accessions and formed a separate group including scented species and cultivars. Our results demonstrate that most of the *Pelargonium* accessions analyzed could be differentiated from each other using the TRAP markers and that groups formed based on similar results from TRAP were consistent with what was previously known about the accessions.

Optimization of DNA extraction method for TRAP reaction. A basic CTAB method was adequate for many *Pelargonium* accessions; however, it failed with almost every cultivar that was not a zonal type *Pelargonium*, especially the scented species. In tests of several methods, a procedure by Saghai-Maroof et al. (1984), which was in use in our laboratory for rice DNA extractions, produced DNA from some plants for which the basic CTAB method failed. Because there were still many accessions from which DNA could not be extracted, increased the concentrations of antiphenolic chemicals were used along with the multiple-wash technique used by Tel-zur et al. (1999). In comparison with the procedure by Tel-zur et al. (1999), our procedure's most significant differences were the addition of sorbitol, SDS, PVPP, and proteinase K directly to the high-salt CTAB solution, in which the concentration of Tris-HCl was doubled. In extractions that excluded the multiple-wash technique, some samples still failed despite the extra antiphenolic chemicals. We determined that both high levels of antiphenolic compounds and multiple washes were essential for DNA extraction from scented-leaved *Pelargonium* accessions.

All of the DNA used for these TRAP analyses was produced using the above technique. However, the DNA concentration was sometimes too low, so additional extractions were completed using three times the leaf tissue and extraction buffer for the first three washes. In those cases, the amount of both buffers was doubled for the CTAB step. To reach a higher throughput, we took advantage of the recommendation of Tel-zur et al. (1999) that the DNA could be stored at –20 °C before continuing the procedure. This provided an opportunity to complete the procedure up to that point multiple times and then complete the remainder of the procedure with all those individual samples. The volumes used in the second half of the procedure allow more samples to be processed simultaneously, so the partially processed samples could be collected in the freezer up to the maximum number for the second half of the procedure. In most cases, eight samples at a time were processed before we completed the procedure with 16–32 separate samples collected from two to four repetitions of the first half of the procedure.

TRAP analysis of 46 Pelargonium *accessions.* A sample image of the TRAP amplification is shown in Fig. 1. This image was generated by a fixed primer (QHF6H21L) designed against a sunflower EST and an arbitrary primer Sa12–700. Of the 46 samples shown in this gel image, most of those in the left half of the gel are similar accessions from the same species (*P. ×hortorum*), and those on the right half of the gel include additional *Pelargonium* species. This image demonstrates that many fragments were amplified in a single run and that most of the accessions could be

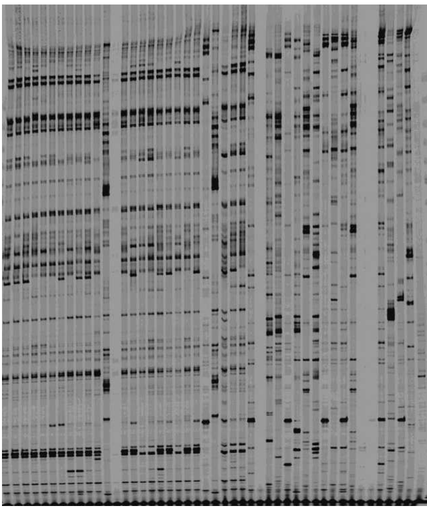

Fig. 1. Sample TRAP image generated by the primer combination w3a–700 (QHF6H21L + Sa12–700). Most of the samples on the left half of this image are from *P.* ×*hortorum*, while the right half represents several different species (from left to right: 218, 219, 221, 222, 223, 226, 234, 238, 240, 244, 256, 258, 264, 265, 273, 282, 283, 284, 285, 287, 337, 338, 346, 417, 495, 503, 505, 511, 512, 514, 516, 518, 519, 523, 524, 526, 527, 529, 531, 535, 542, 543, 548, 549, 550, 551). The LI-COR DNA size standard (50–700 bp) is loaded in the last lane on the right.

differentiated on the basis of those fragments. The images were scored by two separate researchers to generate binary data sets by scoring the presence/absence of individual bands across the 46 accessions for further analysis with NTSYSpc2.

To compare the consistency of the polymorphic patterns generated by different primer combinations with these 46 accessions, two dendrograms were constructed (Fig. 2). One was based on 148 bands amplified by the primer combination w3a–700 (QHF6H21L + Sa12–700) (Fig. 2A), and the second was based on 160 bands amplified by two primer combinations, w1–700 (QHA21B09a + Sa12–700) and w1–800 (QHA21B09a + Ga5–800) (Fig. 2B). The population being analyzed was intentionally diverse in order to evaluate the applicability of this procedure to as much of the population as possible. This had the added effect of producing some polymorphism in every marker scored. In the data set that combined w1–700 and w1–800 data, 107 of the 160 markers were present in more than one individual. In the other data set, every scorable marker was present in multiple individuals. The dissimilarity coefficient for the first branch point of both dendrograms was 92% (see the scales in Fig. 2A and B), indicating that the TRAP markers easily differentiated the most diverse *Pelargonium* accessions. In addition, most of the accessions that clustered together were differentiated. For some accessions, the dissimilarity coefficient is 8% on at least one of the two trees, implying that those accessions are very similar. Defining how similar accessions must be to be considered redundant will be an important step to applying this technique to sort the OPGC collection. Similarities between the two dendrograms suggest that analysis of the whole collection will be possible.

Because the two dendrograms were produced from the same accessions, similar groupings of the accessions could be expected. In fact, many similarities between the two dendrograms were observed. All 24 *P.* ×*hortorum* accessions grouped together with the exception of 548, which was the last individual in the *P.* ×*hortorum* section on one tree and mixed in with the scented accessions on the other tree (Fig. 2). Within the *P.* ×*hortorum* cluster, individuals with some shared parentage (based on pedigrees from Richard Craig) clustered together. For example, accessions 222 and 223 are both derived from cultivars Stadtbern and Wilhelm Langguth, and on both dendrograms they group together. The dendrogram from

A

218 *P.×hortorum*
221 *P.×hortorum*
219 *P.×hortorum*
273 *P.×hortorum*
222 *P.×hortorum*
223 *P.×hortorum*
226 *P.×hortorum*
285 *P.×hortorum*
338 *P.×hortorum*
234 *P.×hortorum*
238 *P.×hortorum* 'Juliette' syn 'Risque'
240 *P.×hortorum*
244 *P.×hortorum*
284 *P.×hortorum* 'Jubilee'
264 *P. inquinans*
265 *P.×hortorum*
337 *P.×hortorum*
282 *P.×hortorum*
287 *P.×hortorum*
503 *P.×hortorum* 'Petals'
283 *P.×hortorum*
505 *P.×hortorum* 'Wilhelm Langguth'
256 *P.×hortorum*
495 *P.×hortorum* 'Frank Headley'
548 *P.×hortorum* 'Mme. Salleron'
258 *P. caylae*
417 *P. caylae*
514 *P. denticulatum* 'Balsam'
519 *P. graveolens* 'Peppermint Rose'
516 *P. graveolens* 'Chocolate Mint'
524 *P. graveolens* 'Snowflurry'
551 *P. dasycaule*
346 *P.xfragrans*
511 *P.xfragrans* 'Apple'
518 *P.* sp. 'Cloves'
526 *P.xfragrans* 'Snowy Nutmeg'
529 *P.xfragrans* 'Golden Nutmeg'
543 *P.xfragrans* 'Old Spice'
527 *P.* sp. 'Lavender Lad'
549 *P. sidoides*
550 *P. cotyledonis*
523 *P. crispum* 'Lemon'
531 *P. crispum* 'French Lace'

0.08 0.29 0.50 0.71 0.92
Coefficient of Similiarity

B

218 *P.×hortorum*
219 *P.×hortorum*
221 *P.×hortorum*
222 *P.×hortorum*
223 *P.×hortorum*
238 *P.×hortorum* 'Juliette' syn 'Risque'
287 *P.×hortorum*
338 *P.×hortorum*
495 *P.×hortorum* 'Frank Headley'
226 *P.×hortorum*
240 *P.×hortorum*
265 *P.×hortorum*
337 *P.×hortorum*
244 *P.×hortorum*
273 *P.×hortorum*
284 *P.×hortorum* 'Jubilee'
285 *P.×hortorum*
256 *P.×hortorum*
282 *P.×hortorum*
283 *P.×hortorum*
505 *P.×hortorum* 'Wilhelm Langguth'
503 *P.×hortorum* 'Petals'
234 *P.×hortorum*
346 *P.xfragrans*
511 *P.xfragrans* 'Apple'
518 *P.* sp. 'Cloves'
543 *P.xfragrans* 'Old Spice'
514 *P. denticulatum* 'Balsam'
551 *P. dasycaule*
527 *P.* sp. 'Lavender Lad'
549 *P. sidoides*
526 *P.xfragrans* 'Snowy Nutmeg'
529 *P.xfragrans* 'Golden Nutmeg'
548 *P.xhortorum* 'Mme. Salleron'
258 *P. caylae*
417 *P. caylae*
550 *P. cotyledonis*
516 *P. graveolens* 'Chocolate Mint'
519 *P. graveolens* 'Peppermint Rose'
524 *P. graveolens* 'Snowflurry'
523 *P. crispum* 'Lemon'
531 *P. crispum* 'French Lace'

0.08 0.29 0.50 0.71 0.92

Fig. 2. (**A**) Dendrogram from the w3a–700 primer set. *P.* ×*hortorum* accessions are encased in a box on the dendrogram. Within that box, dotted circles indicate diploids and solid circles indicate tetraploids; in the list, the same plants are boxed according to shared parentage. Remaining clusters that are consistent between the two dendrograms are circled on the dendrogram. The branch probabilities are indicated in a box for each branch. Cophenetic correlation: $r = -0.91163$. (**B**) Dendrogram from w1–700 and w1–800 primer sets. *P.* ×*hortorum* accessions are encased in a box on the dendrogram. Clusters, ploidy levels, and branch probabilities are indicated in the same manner as in (**A**). *Note:* Accession 264 is not included because it did not have sufficient data, and an extra box is drawn around the *P.* ×*hortorum* accession that is separated from the others. Cophenetic correlation: $r = -0.89712$.

the w3a–700 primer set also clusters accession 234 nearby; it has parentage from 'Stadtbern'. Accession 505, with parentage traced to 'Wilhelm Langguth', is separate from that cluster in both trees even though it shares some parentage. Both dendrograms have accessions related to 'Honseler's Glorie Rot' or 'Jean Billes' clustered somewhat closely (see related accessions identified in Table 1). Only accession 256 escaped from that cluster on the dendrogram from the w3a–700 primer set, but it clustered with the rest of the group (238, 240, 244, 284, 285, 338) on the other dendrogram. Accession 256, a descendant of a colchicine induced tetraploid, is also the only tetraploid to cluster in the primarily diploid section of the dendrogram from the w3a–700 primer set.

The other 22 accessions included 18 accessions that were divided into distinct clusters related to the species of scented cultivars. Those clusters were consistent in both dendrograms, demonstrating that the polymorphic patterns generated by different primer combinations represent the same phylogenetic relationships. One of these clusters contained primarily *P.* ×*fragrans* accessions, which were divided into two subsections on both dendrograms. There was also a pair of *P. crispum* accessions that clustered together; one cluster included the two *P. caylae* accessions; and another cluster had three *P. graveolens* accessions. The highly similar results in both dendrograms suggest that the unidentified plants may represent the same species in each respective cluster. For example, the 'Cloves' cultivar is grouped close to *P.* ×*fragrans* accessions in both dendrograms. These results suggest that TRAP will be helpful with the classification of the other unidentified accessions in our collection.

Three of the 46 samples (accessions 512, 535, and 542) did not amplify well as they consistently had only a few faint bands. The only *P. inquinans* accession (264) in this data set only had sufficient amplification to be considered a practical part of the w3a–700 dendrogram. In the TRAP reactions with the w1–700 and w1–800 primer sets, insufficient amplification produced faint and limited bands from 264 just like the other three. The first three were removed from the analysis in both data sets, and 264 was removed from the w1–700/w1–800 data set. Because so many of our accessions were hybrids, some overlap is visible in Fig. 2B. One of the *P.* ×*hortorum* accessions (548) is in the part of the dendrogram filled with scented species. This is not too surprising because 548 is also the very last accession to cluster with the *P.* ×*hortorum* accessions in Fig. 2A, and hybrids are also present in the scented accessions. An analysis of how well the dendrograms produced from those data sets represent the data used to generate them was completed through the cophenetic correlation. Because both dendrograms had *r* values approaching 1, they were both closely fit to their data sets.

The first five cycles of the TRAP PCR reaction are less stringent, so there does not have to be a perfect match for a fragment to be produced with the fixed primer. The abundant fragments produced in combination between the fixed primer and one of the fluorescently labeled arbitrary primers provide sufficient data to differentiate the individuals in a population with only one or two reactions. This is a significant advantage when the population to be analyzed includes many individuals. In addition, the results can be even more useful when the target of the fixed sequence is a gene relevant to the population. The results using a fixed gene targeting a specific gene of interest in combination with a segregating population contain some fragments that can be used as markers for that gene of interest. For example, the telomere-specific primers Hu (2006) used to map the ends of linkage groups. This gives TRAP a significant advantage over other methods for mapping purposes because there is an increased chance (over methods using random markers) of finding markers for the gene targeted by the fixed primer.

In summary, we have optimized the DNA extraction method and established the TRAP protocols for *Pelargonium* species. The two dendrograms generated from two different data sets were similar, suggesting that only a few TRAP amplifications could be enough to generate a sufficient number of markers to classify the OPGC collection of *Pelargonium* species. The grouping of these *Pelargonium* accessions was consistent with the species of the individuals. Because OPGC has many accessions of *Pelargonium* species to be classified, TRAP will be a useful tool for efficiently accomplishing this task. Using TRAP results from the entire OPGC *Pelargonium* population, TRAP's effectiveness in species delineation could be compared with the classic *Pelargonium* systematics of accessions of identified species. TRAP results could then provide an initial categorization for unknown/unidentified accessions clustering with accessions of identified species. In addition, TRAP's potential for identifying markers that are specific to the gene of interest should allow screening of the population with primers for horticulturally relevant traits, such as pest and disease resistance or differences in essential oil composition.

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