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Evaluation of AFLP in Poinsettia: Polymorphism Selection, Analysis, and Cultivar Identification

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Abstract. Fingerprinting using molecular markers is a highly effective method of cultivar identification that is a powerful aid to traditional methods based on morphology. Amplified fragment length polymorphism (AFLP) is a robust and reliable method for generating molecular markers that has been used to evaluate many crops for a variety of applications. In this study, AFLP was used to develop and validate robust genetic fingerprints for poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzch) cultivars. Polymorphism selection was completed to facilitate the identification of useful polymorphisms and minimize future fingerprinting costs and time. Poinsettia is a highly variable crop subject to genetic drift and variable cultivars. Validation of polymorphisms to remove those associated with intracultivar variation improved the reliability of the fingerprinting. The result was a poinsettia AFLP database that defines the genetic fingerprints of 104 cultivars. Cluster analysis illustrated differentiation of most poinsettia cultivars tested. Selection of a subset of AFLP polymorphisms resulted in clustering of cultivars according to known origin and breeding program. This method has applications not only for cultivar identification for cultivar protection, and maintenance of cultivar uniformity, but also has the potential application of developing markers for important traits.

Poinsettia is the most valuable potted plant in the United States, with a wholesale value of $256 million in 2001 (USDA, 2003). Poinsettias were introduced to the United States in the early 1800s, yet the first commercial-quality cultivars were not introduced until 1963. Today, more than 175 cultivars of cultivated poinsettia are available. Because of the ever-changing selection of cultivars and their valuable market share, breeders are under intense pressure to develop new cultivars. With this process comes the desire to protect the cultivars and breeder's rights. Evaluation of morphological characteristics, such as bract color, growth habit, and time to flower, has been the primary method of cultivar identification; however, there are several shortcomings to this method. Morphological characteristics may be similar between some cultivars, making differentiation difficult. These traits are also influenced by environmental conditions, which can cause variation in their appearance. Finally, morphological evaluation can be costly, as plants must be maintained for an entire growth cycle to score many of these traits.

Molecular techniques have distinct advantages over morphological evaluation for cultivar identification. They are not influenced by environmental factors, making them more reliable and stable. These methods can be applied at almost any stage of growth, reducing the time and cost of cultivar identification. Molecular techniques can provide more genetic information, since the number of molecular markers is virtually unlimited. A limited number of molecular studies have been done with floral crops. Fingerprinting of petunia (*Petunia hybrida* L.) has been reported using restriction fragment length polymorphism (RFLP) (Beyermann et al., 1992; Vainstein and Ben-Meir, 1994) and randomly amplified polymorphic DNA (RAPD) (Peltier et al., 1994). Microsatellites have been used for molecular analysis of rose (*Rosa hybrida* L.) (Esselink et al., 2003). Recently, methods such as RAPD (Ling et al., 1997), DNA amplification fingerprinting (DAF) (Starman and Abbitt, 1997), and arbitrary signatures from amplification profiles (ASAP) (Starman et al., 1999) have been applied to poinsettia to provide faster and more definitive methods of cultivar differentiation. However, these studies included a limited number of cultivars (9, 11, and 11, respectively) and did not assess the variability of (validate) each polymorphism, nor was the method tested across diverse as well as closely related cultivars.

Amplified fragment length polymorphism has distinct advantages over other molecular techniques. AFLP detects polymorphisms throughout the genome by selective amplification of restriction fragments, rapidly producing a large number of markers. AFLP is highly reproducible (Jones et al., 1997) and requires no prior genetic information (Vos et al., 1995). Exploitation of the AFLP technology could provide increased resolution capable of differentiating the more than 175 existing poinsettia cultivars. In addition, this database could be used to generate estimates of similarity between candidates and existing cultivars.

Research with other crops has shown the utility of AFLP for determining relationships between cultivars and for cultivar identification, including bermudagrass (*Cynodon* L.C. Rich.) (L.H. Zhang et al., 1999), eggplant (*Solanum* L.) (Mace et al., 1999), and lettuce (*Lactuca* L.) (Hill et al., 1996). Recently, AFLP has been applied to ornamental crops for the same purpose, including geranium (*Pelargonium* L'Hér.) (Barcaccia et al., 1999), peruvian lily (*Alstroemeria* L.) (Han et al., 1999, 2000), rose (*Rosa* L.) (D. Zhang et al., 1999), daylily (*Hemerocallis* L.) (Tomkins et al., 2001), and new guinea impatiens (*Impatiens hawkeri* W. Bull.) (Carr et al., 2003; J.H. Lyerly, unpublished data). Generally, these studies show that genetic marker data agree with available pedigree data (Carr et al., 2003).

The objectives of this study were to optimize the use of AFLP as a fingerprinting tool for poinsettia and to determine the extent of variability detected with specific AFLP polymorphisms. In this study we have used AFLP to identify a set of polymorphic DNA fragments that are useful for poinsettia cultivar differentiation. The polymorphisms were selected based on their consistent presence or absence in replicates of selected cultivars. In addition,

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multiple statistical models were evaluated for use in generating similarity or dissimilarity indices that would facilitate genotype comparisons.

Materials and Methods

Plant material. One hundred and four poinsettia genotypes and two other *Euphorbia* L. species, *E. fulgens* Karw. ex Klotzch and *E. cornastra* (Dressler.) Radcl.-Sm., were included in the fingerprint analysis. For the validation study, 77 sources of nine genotypes, as shown in Table 1, were collected from locations worldwide and coded to remove analytical bias. Plant material was provided by breeders or collected from two poinsettia trials conducted at the Horticulture Field Laboratory, North Carolina State University.

Genomic DNA was isolated from fresh tissue using a modified benzyl chloride procedure (Zhu et al., 1993). The extraction protocol was adapted to small volume processing of 150 mg of leaf tissue in a microcentrifuge tube. Fully expanded leaves with midribs removed were selected from all cultivars for extraction.

AFLP analysis. The AFLP protocol was performed as described by Vos et al. (1995) using AFLP Analysis System I (Life Technologies, Gaithersburg, Md.) with the following modifications. The Life Technologies protocol was modified by extending the restriction digest incubation to overnight and increasing the ligation incubation to 6 h at 16 °C to improve reproducibility in the final AFLP result. ^{33}P was used to label the *Eco*RI primer for specific amplification. The products of the specific amplification were electrophoresed on a 6% denaturing acrylamide gel at 60 W for ≈2 h. The gel was fixed in 5% acetic acid/5% methanol and dried in a gel dryer (BioRad, Hercules, Calif.), then exposed to a phosphor screen (Molecular Dynamics, Sunnyvale, Calif.) overnight. The screen was then scanned on a phosphorimager (Molecular Dynamics) and the gel image was saved as a TIFF file.

Table 1. Poinsettia cultivars used to identify polymorphisms useful for fingerprinting and cultivar identification. Group 1 cultivars were used to identify AFLP primers useful for cultivar identification. Group 2 cultivars were used to evaluate intracultivar variation of the polymorphisms and to validate the poinsettia AFLP fingerprints.

Cultivar	No. of sources
Group1 cultivars	
Bonita	1
Freedom Jingle Bells	1
Freedom White	1
Gross Heirloom	1
Peterstar Jingle Bells	1
Peterstar Pink	1
Maren	1
Angelika Red	1
Freedom Red	3
Peterstar Red	1
Group 2 cultivars	
Angelika Red	7
Freedom Red	13
Peterstar Red	8
Hegg Dark Red	5
Supjibi	6
Lilo Red	5
Snowcap	8
Sonora Red	19
V-14 Glory Red	6

Data analysis. The gel image was analyzed using Pro-RFLP image analysis software (DNA ProScan, Nashville, Tenn.); ϕ-X174/*Hin*fI (Promega, Madison, Wis.) was used as the standard reference to size the AFLP fragments. Selected AFLP polymorphisms were sized and scored as present (1) or absent (0). The scored data was exported as 1 or 0 to a Microsoft Excel (Microsoft, Inc., Redmond, Wash.) spreadsheet. Coefficients of association, for both similarity and distance, were generated from the binary data using five different models, three that incorporate only positive matches (1/1), and two that incorporate both positive and negative matches (0/0). Distance was calculated using a model described by Lynch (1988). Similarity and all other analyses were calculated using four different coefficients in the statistical software package NTSYSpc 2.0 (Exeter Software, Setauket, N.Y.): 1) Dice, $S_{ij} = 2a/(2a + b + c)$, where S_{ij} is the similarity between two cultivars, *i* and *j*, a is the number of fragments shared by *i* and *j*, b is the number of bands present in *i* and absent in *j*, and c is the number of bands present in *j* and absent in *i* (Dice, 1945); 2) Jaccard, $S_{ij} = a/(a + b + c)$ (Jaccard, 1908); 3) simple matching, $S_{ij} = a + d/a + b + c + d$, where d is the number of bands absent in both *i* and *j* (Sokal and Michener 1958); and 4) unnamed coefficient 1, $S_{ij} = 2(a + d)/2a + b + c + 2d$. The similarity or distance matrices were then analyzed using four different SAHN clustering methods, UPGMA (unweighted pair-group method; Sokal and Michener, 1958), WPGMA (weighted pair-group method; Sneath and Sokal, 1973), CL (complete linkage; Lance and Williams, 1967), and SL (single linkage; Lance and Williams, 1967). Dendrograms were created from the clustered matrix using TREE. The COPH and MXCOMP programs calculated the goodness of fit of the clustering to the data matrix. Principal coordinates analysis was performed using DCENTER and EIGEN.

Results

Selection of polymorphisms. A two-level screening strategy was used to determine which AFLP primer pairs were the most appropriate for fingerprinting poinsettia cultivars. Initially, all 64 possible primer combinations in AFLP Analysis System I were used to amplify DNA from four poinsettia genotypes (C1, C17, C27, and Selection 119) that were the non-grafted progenitors of three major cultivar groups. The phytoplasma-free genotypes were selected to insure that the polymorphisms were poinsettia in origin, and to span the diversity of poinsettia cultivars. Primer combinations were ranked based on the number, intensity, and reproducibility of polymorphisms. Polymorphisms were selected for analysis if they were present in at least one phytoplasma-free cultivar, easily scored on the AFLP image in terms of intensity and separation from other fragments, and reproducible in at least two independent amplifications. A preliminary evaluation of the four best primer combinations on a larger number of cultivars, listed as 1-4 in Table 2, did not result in a high level of differentiation.

Table 2. AFLP primer combinations selected for fingerprint analysis of poinsettia cultivars. Average includes the total number of fragments ranging from 60 to 750 bp. Scored polymorphisms are all polymorphisms scored prior to validation. Cultivar-linked polymorphisms are validated polymorphisms used in the final analysis. E = GACTGCGTACCAATTC and M = GATGAGTCCTGAGTAA.

Primer combination	Avg no. of fragments	Scored polymorphisms (no.)	Cultivar-linked polymorphisms (no.)
1 E-AAG/M-CTA	99	10	5
2 E-AAG/M-CTG	91	18	9
3 E-ACA/M-CTC	95	8	4
4 E-ACA/M-CTT	109	10	4
5 E-ACA/M-CTA	88	11	4
6 E-ACA/M-CTG	70	6	2
7 E-AGC/M-CAC	55	21	7
8 E-AGC/M-CTA	71	14	6
Total		98	41

A second evaluation of the 30 best primer combinations from the initial screen was completed with a set of 12 cultivars spanning a broad range of similarities, including three sources of the same cultivar from different sources for detecting intracultivar variation, listed as Group 1 in Table 1. The primer combinations that generated the most useful polymorphisms were scored and analyzed individually, and prioritized according to their ability to detect polymorphisms between closely related cultivars without detecting intracultivar variation. The new primers were then analyzed in combinations with the original four primers according to priority, until the similarities for the set of cultivars were optimized; this balanced differentiation of cultivars with clustering of related cultivars. Four additional primer combinations were selected, listed as 5-8 in Table 2. A total of 98 AFLP polymorphisms were selected for further analysis, resulting in Jaccard similarities ranging from 0.27 to 0.98.

Reproducibility of polymorphisms. To further validate the reproducibility and reliability of the AFLP fingerprints, the polymorphisms were evaluated. In a preliminary study to test the hypothesis that some polymorphisms were in regions of the genome associated with intracultivar variation, we analyzed plants from multiple sources of five cultivars: 'Freedom Red' (seven sources), 'Hegg Dark Red' (two sources), 'Peterstar Red' (two sources), 'Lilo Red' (two sources), and 'V14 Glory Red' (one source). The results of this test demonstrated the presence of intracultivar variation among the polymorphisms.

In a larger test designed to identify the polymorphisms that should be excluded from the analysis, we used 77 sources of nine different cultivars as listed in Table 1. The amount of intracultivar variation of the polymorphisms was different for each of the cultivars. 'Angelika Red' showed the least variation with differences noted in 10 of the 98 polymorphisms. Sources from four of the validation cultivars, 'Freedom Red', 'V14 Glory Red', 'Snowcap', and 'Hegg Dark Red', showed the most variation. One source of each of these cultivars was very different from all other sources of the cultivar; differences were seen in as many as 33 of the 98 polymorphisms. Polymorphisms that were variable among sources of a given cultivar were likely to be variable among sources of one or more other cultivars; 22 varied in one cultivar, and 35 varied in more than one. Of the 98 polymorphisms, eight were consistent in all sources of all cultivars. In total, 57 of the 98 polymorphisms were found to be highly variable, varying in more than one source of a particular cultivar or cultivars. These highly variable polymorphisms were eliminated from AFLP analysis. The 41 validated polymorphisms were used to create a poinsettia AFLP database for poinsettia genotypes and two outgroup species, *E. cornastra* and *E. fulgens*. This database includes 81 commercially released cultivars as well as 23 unnamed, unreleased genotypes of poinsettia.

Data analysis. To determine which statistical methods would yield the most accurate representation of the relationships between the cultivars in this study, comparisons were made between five similarity or distance coefficients and four clustering methods. The dendrograms constructed using the various association and clustering methods were examined and the cophenetic correlation coefficients of each were compared (Table 3), to test the goodness of fit of the association coefficient to its respective dendrogram. The association coefficients that gave the highest cophenetic correlation coefficients were those that incorporated only positive matches (1/1). UPGMA clustering gave the highest correlation coefficients of the clustering methods, from 0.813 to 0.877, indicating a good fit of the similarity matrix to the dendrogram. The coefficients that incorporated only positive matches, Dice, Lynch, and Jaccard, yielded the same cultivar group clusters in the same orientation as shown in the dendrogram of 81 named cultivars and two outgroup species (Fig. 1). The two coefficients that incorporated positive and negative matches, SM and UN1, also clustered the cultivar groups, but the orientation of the clusters on the dendrogram differed from that of the other three methods. Jaccard's similarity coefficient, when clustered with the UPGMA technique, gave the highest of all the correlation values, 0.877, indicating the best fit to the data.

There was a full range of Jaccard similarity coefficients. Between poinsettia cultivars, similarity coefficients ranged from 1 (identical), between 21 different cultivar pairs, and 0.219 (least similar), between 'Airbrush' and 'Freedom Rose'. The similarities between related cultivars ranged from 1 to 0.615, with the lowest similarity being between 'Freedom Red' and its color sport 'Freedom Rose'. *E. conrastra* had much lower similarities to the poinsettia cultivars, with coefficients ranging from 0.032 to 0.217. *E. fulgens* also had lower similarities to the poinsettia cultivars, ranging from 0.152 to 0.433. The similarity between these two outgroup species was 0.095.

A small set of the cultivar pairs could not be differentiated with this set of AFLP polymorphisms. The 21 identical pairwise comparisons involved 16 cultivars from three main cultivar groups; one comparison involved 'Lilo', seven were from 'Freedom', and 13 were from 'Angelika'/'Peterstar'. All of the cultivar pairs with identical coefficients were related; some of these cultivars are color sports resulting from either natural or induced mutations. Seven comparisons involved color variants of a cultivar and seven others were color variants of related cultivars. Four comparisons involved cultivars with a leaf variegation mutation. One comparison was between a cultivar and a selection of the same cultivar. The remaining two comparisons were also pairs of derivative cultivars, and had similar coloring of bracts and leaves.

Figure 1 shows the dendrogram of the 81 commercially released cultivars and the two *Euphorbia* outgroup species. A critical value of ≈0.63 resulted in five major clusters on the dendogram: 'Lilo'/'Hegg', 'Cortez'/'Sonora', 'Peterstar'/'Angelika', 'Celebrate'/'V14 Glory', and 'Freedom'. The most distant poinsettia cultivar was 'Xenia Red Deluxe', which branched from the dendrogram at ≈0.42. *E. fulgens* and *E. cornastra* made up the outermost branches at ≈0.31 and 0.12, respectively.

Principal coordinates analysis gave further support to the clusters created by UPGMA clustering. The first three eigenvectors explain 56.93%, 9.88%, and 4.09% of the total variation, cumulatively accounting for 70.91% of the variation. The three-dimensional PCO plot shows the same clusters found with SAHN analysis, 'Lilo'/'Hegg', 'Cortez'/'Sonora', 'Peterstar'/'Angelika', 'Celebrate'/'V14 Glory', and 'Freedom'.

Cultivar group analysis. The genetic lineages of all cultivars were not available to provide direct evidence of a correlation

Table 3. Cophenetic correlation coefficients for the five similarity or distance coefficients and four clustering methods applied to the final AFLP data set containing the 81 commercial poinsettia cultivars and two outgroup species.

	Dice	Jaccard	SM	UN1	Lynch
UPGMA	0.870	0.877	0.839	0.813	0.867
WPGMA	0.855	0.861	0.807	0.775	0.817
CL	0.759	0.791	0.775	0.733	0.751
SL	0.785	0.778	0.754	0.726	0.781

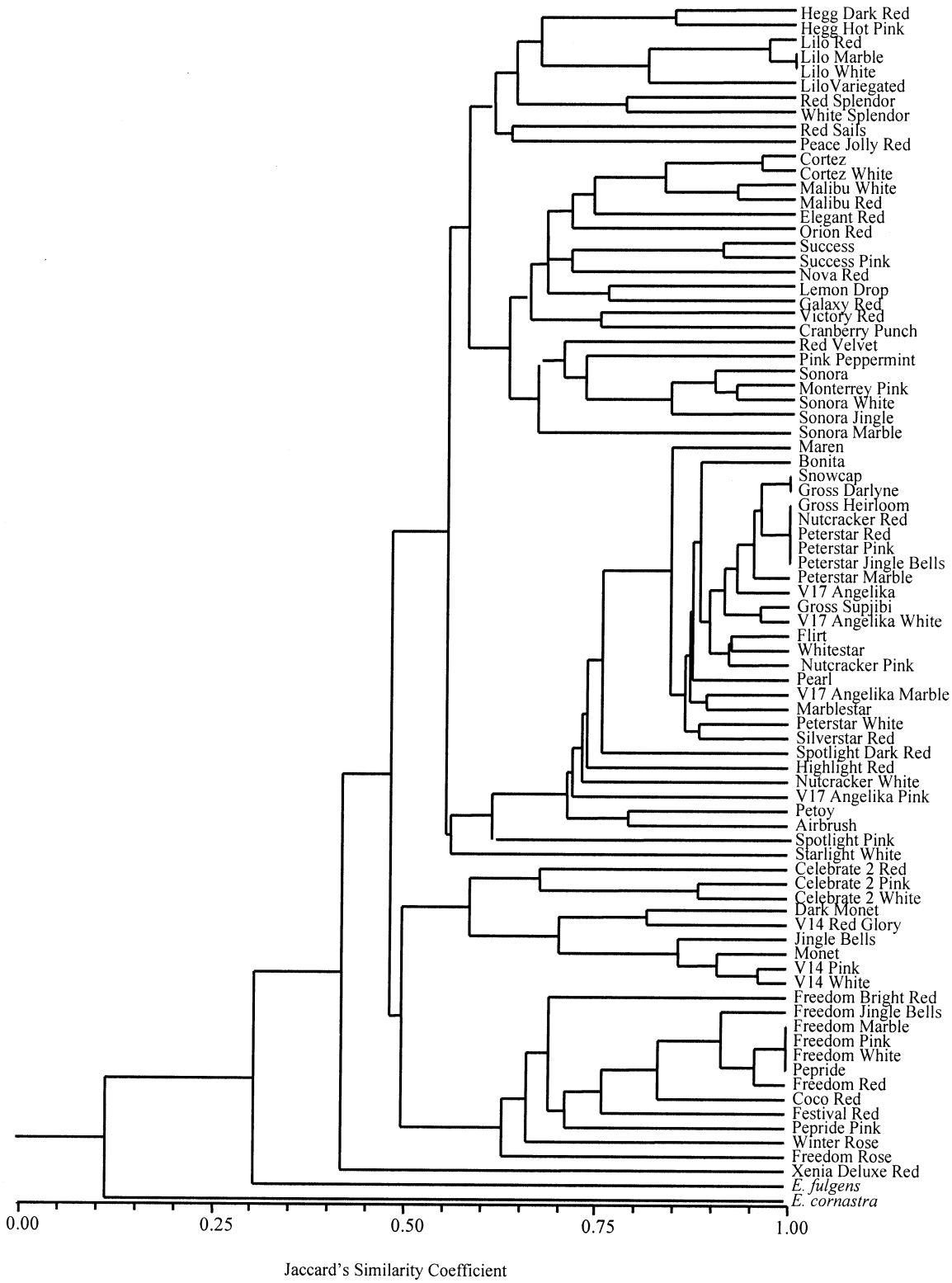

Fig. 1. Dendrogram of 81 commercial poinsettia cultivars and two outgroup species using 41 AFLP polymorphisms, generated using Jaccard's similarity coefficient and UPGMA clustering.

between polymorphic profile similarity and genealogy; furthermore, many cultivars are derived from natural or induced mutations arising from existing genotypes. Alternatively, an iterative strategy was derived to determine the extent that the measure of similarity of polymorphic profiles reflected genetic relationships and thus the probability that a cultivar originated from a specific breeding program. The strategy consisted of obtaining genetic information from available plant patents and grouping cultivars based on known origin and breeding program. A subset of 14 of the 41 polymorphisms present in all seven of the 'Freedom'-derived cultivars was initially selected as the basis of a strategy to identify the breeding program or origin of the genotypes. When this set of polymorphisms was used to generate a dendrogram, the 'Freedom'-derived cultivars clustered together with a similarity of 1, with other cultivar groups clustering in a similar manner to that on the dendrogram that included all of the AFLP polymorphisms. Examination of nine cultivar groups revealed within-group consistency of five of the 14 polymorphisms; within the 'V14 Glory', 'Freedom', Celebrate', 'Cortez', 'Sonora', 'Peterstar', 'Angelika', 'Lilo', and 'Hegg' cultivar groups, the polymorphisms were either consistently present or absent. In addition, two more that were absent in all 'Freedom' cultivars were consistently present or absent in the other nine cultivar groups. Using these seven polymorphisms, a new dendrogram was generated, which placed all of the genotypes sharing a common origin into clusters with similarities of 1 (Fig. 2). The cophenetic correlation of this dendrogram was 0.869, showing high correlation of the clustering to the data. Principal coordinates analysis supported the same clusters, with the first three eigenvectors explaining a total of 68.67% of the variation.

To test the significance of the subset of AFLP polymorphisms, a random set of seven polymorphisms was generated from the 41, similarities were calculated, and a dendrogram was generated. The majority of cultivar groups were only partially clustered, and clustering of completely unrelated cultivars occurred with this set of polymorphisms. This shows the selection of the set of AFLP polymorphisms for cultivar group analysis was not due to random chance; clustering was dependent on the particular polymorphisms and was not an artifact of the small number of polymorphisms used in the analysis.

Discussion

The dendrogram of 81 commercially released cultivars constructed using the entire set of 41 validated polymorphisms is consistent with known pedigrees of poinsettia according to breeder and cultivar patent information. 'Hegg', 'Lilo', and 'Splendor' cultivars share a common ancestry, and they form a unique cluster on the dendrogram. 'Cortez' resulted from a cross of 'Lilo' and an unknown cultivar, and it occupies a cluster adjacent to 'Lilo'. 'Malibu Red' was developed from a cross involving 'Cortez', and clusters close to it. Mutation or natural breeding from 'Angelika Red' developed a number of cultivars that cluster in the center of the dendrogram. The three 'Celebrate' cultivars form the next cluster on the dendrogram. Cultivars that share a background of 'V-14', 'Jingle Bells', and 'Monet' form a distinct cluster. The 'Freedom' cluster includes the 'Freedom Red' color sports developed by mutation and natural breeding, as well as 'Coco Red', 'Festival Red', and 'Pepride', all developed from 'Freedom Red'. Finally, the outgroup species *E. fulgens* and *E. cornsatra* occupy the outmost branches of the dendrogram.

The set of 41 AFLP polymorphisms was able to differentiate all but 21 pairs of cultivars out of 3240 pairwise comparisons of the 81 cultivars. Although all cultivars appeared to be differentiated using the full set of 98 AFLP polymorphisms, removal of polymorphisms that were hypervariable reduced the resolution of the test, causing the 21 pairs of cultivars to no longer be differentiated. All but two of the cultivars that could not be differentiated with the set of polymorphisms are easily separated by morphological traits; 'Nutcracker Red' and 'Peterstar Red' were the only morphologically similar cultivars that could not be differentiated with this set of AFLP data. All other undifferentiated cultivars were either color sports of one another or had different leaf variegation. Most of those cultivars that are the most difficult to distinguish morphologically, such as the numerous red, white, and pink cultivars in the study, were differentiated with AFLP fingerprinting. This demonstrates the importance of validating polymorphisms, as some that appear to discriminate between sports of some poinsettia cultivars could potentially cause false identification of a cultivar if used in fingerprinting. Sports may be difficult to distinguish genetically (Weising et al, 1995), and a more sensitive method may be necessary to fully differentiate the cultivars.

Additional selection of polymorphisms facilitated reliable classification of the cultivars into clusters indicative of genetic background and the breeding program or origin of the cultivar. The core set of seven AFLP polymorphisms selected using color variants places the cultivars in tight clusters of cultivar groups. Obvious cultivar groups with shared names clustered as expected, into groups with a similarity of 1. Other groups on the dendrogram with a similarity of 1 appear to cluster several cultivar groups together, 'Angelika' and 'Peterstar' groups for example. Further investigation into the breeding history of these cultivar groups revealed common ancestry; clustering using the core set of polymorphisms was a good predictor of breeding history of the cultivars. This cultivar group AFLP analysis could be used as a preliminary identification tool to place cultivars in the correct cultivar group, or it could be used to identify breeding group for cultivar protection purposes.

Other AFLP studies (Carr et al., 2003; Han et al., 2000) have focused on scoring a large number of polymorphisms to explain expected relatedness. However, this study shows that it is the quality as well as quantity of information that the polymorphisms contain, and not the quantity of polymorphisms themselves, which explains relationships and creates AFLP fingerprints. Selection of appropriate polymorphisms through validation leads to a robust system for analysis of relationships and creation of an AFLP fingerprint. The process is similar to validation of polymorphisms for any set of complex traits; here it is cultivar identification. Optimization of the set of polymorphisms can provide any level of differentiation that is needed. To help determine the breeding origin of a cultivar, the group-specific data can be used, and to differentiate, or fingerprint, a particular cultivar, the entire set of polymorphisms should be used. In addition, minimizing the number of primer combinations reduces the cost and time required to fingerprint cultivars. A high percentage of the expected relatedness can be explained by selecting the optimal primer combinations. In one other example, Ellis *et al.* (1997) found that by selecting the six best primer combinations, more than 80% of the expected relatedness could be explained. This study selected the best eight primer combinations from 64 possible, so it is possible to conclude that this study has found the majority of expected relatedness.

Other AFLP studies to date, especially those in ornamentals,

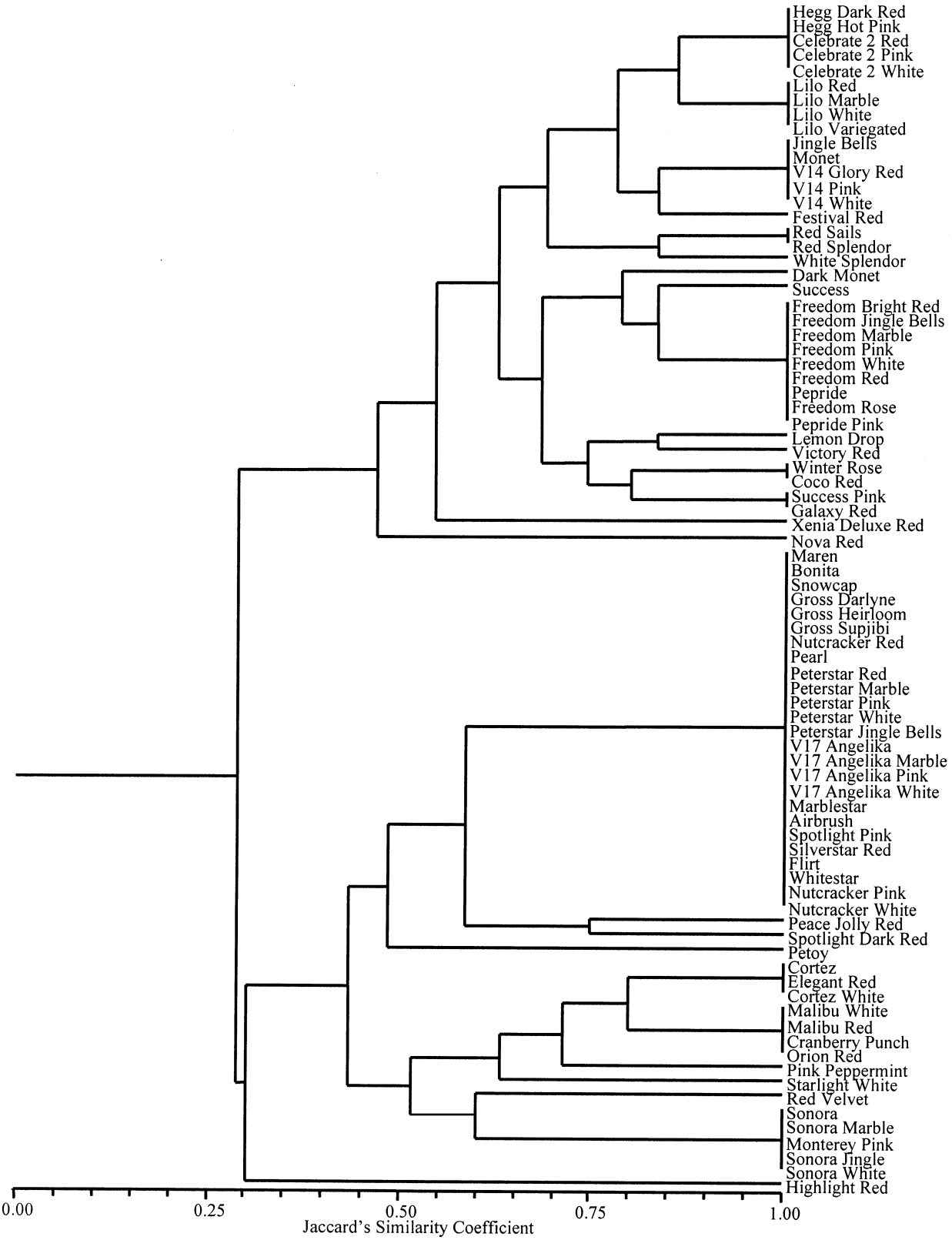

Fig. 2. Dendrogram of 81 commercial poinsettia cultivars using seven cultivar group specific AFLP polymorphisms, generated using Jaccard's similarity coefficient and UPGMA clustering. Those cultivars with a similarity of 1, represented in the dendrogram with a vertical line, denote cultivar groups.

have not addressed intracultivar variation of polymorphisms (Carr et al., 2003; Ling et al., 1997; Starman and Abbitt, 1997; Starman et al., 1999; D. Zhang et al., 1999). However, this study shows that analysis of the intracultivar variation of AFLP polymorphisms is vital to determine the degree of variation in the species of interest. Poinsettia is a vegetatively propagated crop that must be selected annually to maintain crop uniformity, demonstrating the variability that exists in this crop. Once the degree of intracultivar variation is established, some method to compensate for it should be implemented to establish a more robust fingerprint. The variation of specific AFLP polymorphisms in several different cultivars suggests that some of them likely originate from highly variable regions of the genome, and should be eliminated from fingerprint analysis. Discriminating between those polymorphisms that are reflective of this heterogeneity and those that are stable and connected with the distinct nature of that cultivar is essential to generating a reliable fingerprint. In addition, the high degree of intracultivar AFLP variation in poinsettia suggests that a molecular tool such as AFLP would be valuable in maintaining homogeneity of cultivar when used for marker-assisted breeding.

An additional important factor that determines reproducibility is complete restriction digestion. Many floral crops, including poinsettia, require optimization of extraction and digestion protocols for preparation of high-quality DNA requisite for reproducible polymorphic profiles (Barcaccia et al., 1999; Carr et al., 2003; J.H. Lyerly, unpublished data). Incomplete digestion can be detected in the AFLP pattern by loss of small AFLP fragments, along with gain of larger fragments (Life Technologies, Gaithersburg, Md.). Therefore, commonly occurring monomorphic fragments in the AFLP pattern, particularly those 100 bp and smaller, can serve as controls for complete restriction digest. Reliability and reproducibility of the AFLP technique is additionally insured by the stringent primer annealing conditions known as "touch-down PCR," which minimizes mispriming, and thus greatly reduces aberrant PCR products.

The repeatability of the AFLP banding patterns coupled with the validation of the polymorphisms by testing of multiple sources of various cultivars provides credibility to the AFLP fingerprints and the relationships concluded from them. Additional support of the data comes from the consensus of the different methods of analysis. The similar clustering in the dendrograms generated using different association coefficients and clustering methods verifies that the clusters are distinct (NTSyspc 2.0, Exeter Software, Setauket, N.Y.). Likewise, the clustered groups in the principal coordinates analysis gives further support to the clusters.

Information in this study has shown that validation of polymorphisms is essential for the AFLP technique to be an effective and robust tool for identifying and differentiating poinsettia cultivars, as well as for determining breeding relationships. AFLP analysis of poinsettia and other floral crops provides valuable information that will facilitate the use of molecular methods for cultivar protection, support in breeding programs, and the potential to develop markers for desirable characteristics.

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