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The Use of AFLP Markers to Resolve Clonal Origin and Integrity in Rose, Hydrangea, and Lily

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ABSTRACT

Amplified fragment length polymorphism (AFLP) is a reliable and robust marker system which has been useful for various genetic studies including clonal integrity studies differentiating genetically similar germplasm. AFLP was evaluated for its effectiveness to resolve clonal origin and integrity questions in three clonally-propagated, ornamental species. The origins of the rose cultivar BAIeam and the hydrangea cultivar Bailday, and relationships among intraclonal selections of Easter lily 'Nellie White' were investigated. A standard AFLP protocol provided repeatable and consistent fingerprints for rose and hydrangea, while repeatable AFLP fingerprints could not be obtained for Easter lily despite exploring modifications to DNA extraction, digestion, preamplification, selective amplification, and polyacrylamide electrophoresis. AFLP data suggest that 'BAIeam' may be an apomictic seedling of the maternal parent 'INTerlav' resulting from diplospory, and that 'Bailday' is not a sport out of 'Bailmer', as suspected, but differs from the phenotypically similar cultivar 'Variegata' by only one AFLP fragment. AFLP analysis worked well to differentiate genetically similar germplasm for rose and hydrangea. For some organisms like Easter lily, however, factors such as large genome size (~77pg/2C nucleus) and highly repetitive DNA complicates AFLP analysis. Optimization to obtain repeatable, consistent, and scorable fingerprints may not be possible using AFLP to assess genetic variation in species with large genome sizes such as lily.

Keywords: 2*n* gamete; clonal integrity; diplospory, *Hydrangea macryphylla*, *Lilium longiflorum*, *Rosa hybrida*
Abbreviations: AFLP, amplified fragment length polymorphism; **L**, undiluted ligation mixture, **LD**, diluted ligation mixture; **PD**, preamplification dilution; **SN**, selective nucleotides

INTRODUCTION

The ornamental horticulture market has a strong incentive for breeding programs to rapidly introduce cultivars to gain a competitive advantage with new, high-demand products (Anderson 2004). Such products are frequently "fast-tracked" through the domestication process (Anderson *et al*. 2006). The origin of a potential cultivar may be unclear due to apomixis, incomplete or inaccurate records – any of which could jeopardize the ability to file protective US Plant Patents or European Plant Breeder's Rights (Aguirre 2006; www.uspto.gov; www.upov.int). Additionally, within established clonally-propagated cultivars intraclonal lines may differ from each other due to originating from different ortets, which themselves may trace back to different mutation events and different lineages of accumulated mutations. Accurately identifying different intraclonal lines in order to characterize intraclonal variation for intellectual property rights and for identification and marketing purposes is of considerable economic value (Hale *et al*. 2005). When clonal identity or integrity requires resolution, the objectivity of DNA fingerprinting can be very useful, especially since plant phenotype can be strongly influenced by the environment (Veilleux and Johnson 1998).

Amplified Fragment Length Polymorphism™ (AFLP) has been a valuable marker technique useful for clonal integrity studies where there may be high genetic similarity among the genotypes in question. For instance, AFLP has been able to differentiate between somaclonal variants generated *in vitro* (Vendrame *et al*. 1999; Prado *et al*. 2005) and between sports and the original cultivars (Debener *et al*. 2000; Scott *et al*. 2000; Vosman *et al*. 2004). In the limited germplasm base of commercial *Dendrobium* orchids, Hong *et al*. (2005) using AFLP proposed a genetic distance threshold of <0.09 useful for suggesting individuals are siblings and a genetic distance of <0.01 for original clones and their sports. In addition, AFLP has been successfully used to characterize clonal distribution and diversity of plant populations consisting of clonal mixtures (Douhovnikoff and Dodd 2003; Zhang *et al*. 1999) and genetic variability between different leaves and stems within even a single plant (Douhovnikoff and Dodd 2003).

AFLP combines the specificity of restriction enzyme analysis with the sensitivity and ease of detection of the polymerase chain reaction (PCR) (Vos *et al*. 1995). Advantages of AFLP include generation of large numbers of fragments, a generally high rate of polymorphism across genotypes, and high reproducibility and repeatability (Blears *et al*. 1998; Mueller and Wolfenbarger 1999). AFLP is a versatile marker system due to the need for relatively little *a priori* genome knowledge and sampling of DNA fragments across the genome (Mueller and Wolfenbarger 1999). In addition to clonal integrity questions, AFLP fingerprints have been useful for many other applications including intellectual property rights, studying genetic diversity, developing linkage maps, and finding markers which co-segregate with traits of interest for marker assisted selection and positional cloning (Blears *et al*. 1998; Mueller and Wolfenbarger 1999).

Although the standard AFLP protocol first described by Vos *et al*. (1995) has been used in most AFLP applications, optimizations have been necessary, especially to accommodate species with genome sizes at the extremes of AFLP capability (Blears *et al*. 1998; Han *et al*. 1999; Mueller and Wolfenbarger 1999; Suazo and Hall 1999; van der Wurff *et al*. 2000; Fay *et al*. 2005). Common protocol modifications

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Fig. 1 Representative flowers of rose cultivars 'Henry Kelsey', 'INTerlav', and their reported offspring 'BAIeam' (A) and hydrangea cultivars 'Bailmer', 'Variegata', and 'Bailday' (B) (scale not shown).

include restriction enzyme manipulation (e.g. number of restriction enzymes, size and composition of the recognition sequence, methylation-sensitive/insensitive restriction enzymes; Mueller and Wolfenbarger 1999; Suazo and Hall 1999; van der Wurff *et al*. 2000) and the number of selective nucleotides added to the 3′ end of PCR primers during pre-amplification and selective amplification, with a greater number of selective nucleotides yielding generally fewer amplified fragments (Blears *et al*. 1998; Han *et al*. 1999; Mueller and Wolfenbarger 1999; Fay *et al*. 2005).

AFLP is a favorable marker system for clonal integrity research because it has a generally low (<2%) inter-laboratory error rate (Mueller and Wolfenbarger 1999). A low error rate is critical because relatively few spurious fragments could strongly impact biological interpretations, especially with genetically similar germplasm where very few, if any, fingerprint differences can be detected (Vielleux and Johnson 1998). AFLP has been used successfully to answer multiple clonal integrity questions, necessitating judicious attention to repeatability and replication to ensure observed polymorphisms are the result of genomic differences and not procedural artifacts (Zhang *et al*. 1999; Leitão 2001; Imazio *et al*. 2002; Douhovnikoff and Dodd 2003; Hale *et al*. 2005).

The objective of this study was to test the effectiveness of AFLP to resolve cultivar integrity questions in three commercially grown, clonally-propagated species representing different clonal origin or integrity questions and different genome sizes: *Rosa hybrida* L. (triploids ~1.8 and tetraploids 1.9 to 2.3 pg/2C nucleus; Yokoya *et al*. 2000), *Hydrangea macrophylla* (Thunb.) Ser. (3.9 to 4.3 pg/2C nucleus; Cerbah *et al*. 2001), and *Lilium longiflorum* Thunb. (77.1 pg/2C nucleus; Lim *et al*. 2001).

Origination of new rose and hydrangea cultivars and distinguishing between intraclonal selections of *L. longiflorum* 'Nellie White' are the critical clonal integrity questions to be addressed in this study using AFLP marker analysis. *Rosa hybrida* 'BAIeam' (DayDream™; 2005 All-America Rose Selection winner; US Plant Patent No. 15,736) is reported to be a cross of 'INTerlav' (Lavender Dream™; US Plant Patent No. 5,916) x 'Henry Kelsey' (Lim 2005). 'BAIeam' is difficult to distinguish morphologically from its female parent (**Fig. 1A**): both roses produce semi-double, lavender flowers on plants having a similar growth habit. In a stock plant block of *H. macrophylla* 'Bailmer' (Endless Summer™; US Plant Patent No. 15,298) at Bailey Nurseries Inc. (St. Paul, Minnesota) a single plant with variegated foliage was found (Bailey Nurseries Inc., unpublished data). It was assumed to be a sport of 'Bailmer' and was named and released as 'Bailday' (Light-O-day™ = Eclipse™). The floral structure of 'Bailday', however, is vastly different than 'Bailmer' (mop head inflorescence; large infertile florets throughout the inflorescence ranging in color from blue to pink) and difficult to distinguish from the variegated cultivar 'Variegata' (lace cap inflorescence; small, fertile, purple florets in the center of the inflorescence and large, white, infertile florets around the perimeter) (**Fig. 1B**). The recent cultivars, 'BAIeam' and 'Bailday', are very similar phenoltypically to 'INTerlav' and 'Variegata', respectively. AFLP analysis was used to ascertain the origin of these new cultivars and their potential relationship with the cultivars they most resemble.

One >60 year old Easter lily (*L. longiflorum*) cultivar, 'Nellie White', predominates the North American potted flowering Easter lily market and is independently propagated and distributed by <10 field bulb producers for greenhouse finishers primarily in the US and Canada (Zlesak and Anderson 2003). Bulb producers perform periodic intraclonal selection (~1x/10 yrs.) by choosing ≥1 genotype with a superior phenotype to propagate for commercial production (Zlesak and Anderson 2003). Quantitative differences in forcing characteristics of 'Nellie White' selections have been documented among bulb growers, and are attributed, in part, to differences in individual bulb size, bulb maturity and dormancy, variability in virus titer, and possibly mutation accumulation and genetic divergence (Zlesak and Anderson 2007). If AFLP markers can distinguish intraclonal selections, it will provide a useful tool to identify superior intraclonal selections for production verification and intellectual property rights as well as insight into genetic divergence and relationships between intraclonal selections. This study was designed to document the utility of AFLP to ascertain clonal origin or integrity in phenotypically similar germplasm and to explore the effects of modification of the AFLP protocol for adaptation to the large genome organism Easter lily.

MATERIALS AND METHODS

Plant materials

Two plants each of *H. macrophylla* 'Bailday', 'Bailmer', and 'Variegata' and *R. hybrida* 'BAIeam' and 'Henry Kelsey' were obtained from Bailey Nurseries Inc. (St. Paul, MN), and two plants of *R. hybrida* 'INTerlav' were obtained from Sam Kedem Nursery (Hastings, MN). *Lilium longiflorum* 'Nellie White' plants representing 12 different bulb lots (n≥7 growers; n=12 or 15 bulbs/lot) previously characterized in a phenotypic screen (Zlesak and Anderson 2007) were available for this study. The bulbs were obtained from major Easter lily bulb growers in Smith River, CA and Brookings, OR and their sources were coded and blinded from the authors (Zlesak and Anderson 2007).

DNA extraction

Young leaf tissue (~100 mg/extraction, unless noted) was used for all DNA extractions. DNA extractions for hydrangea, lily and rose were performed using a CTAB method (Haymes 1996) with modifications. The volume of extraction buffer and chloroform/isoamyl was 500 µl. The chloroform/isoamyl purification was performed twice and the DNA pellet was resuspended in 100 µl of nuclease-free water. For lily, 1 ml of -20°C 95% ethanol was used for DNA precipitation. DNA extracted using the CTAB method for all three species was further purified using phenol and chloroform (Ausubel *et al*. 1992). Independent of CTAB extractions, lily DNA was also extracted using the DNeasy® Plant Mini Kit (Qiagen Inc.; Valencia, CA, USA). Within step four of the DNeasy® Mini Plant Kit the optional centrifugation step was performed in order to have recoverable supernatant. Varying quantities of fresh lily leaf tissue (10, 25, 50, 75, and 100 mg) were used to determine whether DNA yield with the DNeasy® extraction method was affected by amount of starting tissue. All DNeasy® DNA samples were suspended in 100 µl of AE buffer. One independent CTAB DNA extraction was performed per plant of rose and hydrangea (two extractions/cultivar), and one DNeasy® DNA extraction was performed for every Easter lily bulb and one CTAB DNA extraction for ≥1 bulb per grower bulb lot. All DNA samples were quantified by visual comparison with DNA standards of a known concentration following electrophoresis through a 1.2% agarose TAE gel, ethidium bromide staining, and exposure to UV light.

To gauge DNA purity, four DNA samples from two randomly selected Easter lily plants (each plant from a different grower bulb lot and one DNeasy® and CTAB extraction per plant) were digested using *Eco*R I (Invitrogen™ Life Technologies; Carlsbad, CA, USA) and compared to undigested genomic DNA (600 ng CTAB extraction method or 20 ng DNeasy® extraction method) following electrophoresis through a 1.2% agarose TAE gel with staining and visualization as above. Digestions (25 µl total) were performed using 600 ng (CTAB extraction method) or 20 ng (DNeasy® extraction method) of DNA, 1.25 µl *Eco*R I (10 u/µl), 2.5 µl REact® 3 buffer (Invitrogen™ Life Technologies), and 1.25 µl water. Reactions were incubated for 2 hrs at 37°C followed by 70°C for 15 min to heat inactivate the enzyme.

AFLP analyses

AFLP reactions were conducted using the AFLP® Analysis System I kit (Invitrogen™ Life Technologies) with several modifications (**Fig. 2**). All reaction volumes were reduced to one quarter of the recommended size and ~60 ng (CTAB) or 2 ng (DNeasy®) starting template DNA was used for each reaction. For all reactions, selective amplification *Eco*R I primers were radioactively labeled with ^{33}P. A ^{33}P-labeled 30-330 bp AFLP DNA ladder (Invitrogen™ Life Technologies) was used to reference fragment size on 5.1% polyacrylamide gels. Dried gels were exposed to X-Ray film (Kodak Biomax MR Film, Rochester, NY) for two or three days.

For rose and hydrangea, preamplification was performed independently on the diluted (standard protocol; 1:10 dilution) and undiluted digestion/ligation reaction and six E+3/M+3 selective amplification primer pairs (E-AAC/M-CAC; E-AAC/M-CTT; E-ACG/M-CAC; E-ACG/M-CTT; E-AGC/M-CAC; E-AGC/M-CTT) were used. Two replications were performed per cultivar/primer pair and each replication traces back to a unique CTAB DNA extraction from a separate plant of each cultivar.

For lily, multiple modifications to the AFLP protocol of Vos *et al*. (1995) were explored and included modifying the restriction digestion and pre- and selective amplification steps (**Fig. 2**). The restriction digestion step was modified to evaluate the effects of eliminating the frequent cutting enzyme (4 bp recognition sequence;

AFLP Steps	Modifications for:	Hydrangea/Rose	Lily
Genomic DNA extraction.	DNeasy® and CTAB		X
Genomic DNA digestion (*Eco*R I and *Mse* I).	*Eco*R I digestion only		X
	Digesting twice with *Eco*R I/*Mse* I		X
Ligation of DNA adapters of known sequence with one having a complementary sticky ends to *Eco*R I (E) and the other to *Mse* I (M). Ligation mixture is diluted ten-fold.	Ligation mixture not diluted	X	X
Preamplification of fragments using primers complementary to adaptors along with one selective nucleotide (E-A and M-C). Preamplification mixture is diluted fifty-fold.	E-A and M-CT preamplification primer pair		X
Selective amplification of fragments using primers complementary to adaptors along with two additional selective nucleotides for a total of three selective nucleotides for each primer (E-A_ _ and M-C_ _).	E and M primers with four selective nucleotides		X
	Selective amplification with two E primers and no M primers		X
Fragment visualization and scoring.	3.6% polyacrylamide gel		X
	Ran gel 4 hours (60 watts)		X

Fig. 2 AFLP protocol (Vos *et al*. 1995) with highlighted modifications used on lily, hydrangea, and rose.

Mse I) on fingerprint complexity. CTAB extracted lily DNA from three lily plants representing three different bulb lots (grower bulb lot-plant number; 4-9, 6-9, and 7-8), was independently subjected to standard (*Eco*R I + *Mse* I) and to modified (*Eco*R I only) digestion (two replications per lily/restriction enzyme combination). For each sample, selective amplification was performed using the primer pair E-AC*/E-AAC (asterisk indicates radioactive label). Appropriate concentrations of dNTPs (200 µM each) were included in the selective amplification reactions. Subsequently, selective amplifications were carried out on four pre-amplification dilutions (1:50) derived from *Eco*R I only digestion of four lilies, each from a different bulb lot (grower bulb lot-plant number; 1-5, 2-7, 3-7, and 4-9). Primers for these reactions were E-A/E-AAC*; E-A/E-ACGC*; E-AC/E-AAC*; E-AC/E-ACGC* (asterisk denotes radioactive label; dNTP concentrations were appropriately adjusted to give 200 µM of each in the selective amplification). For these reactions, the extension time was increased from one to two minutes per cycle for both preamplification and amplification steps to account for anticipated larger AFLP fragments. Also to account for increased fragment size, polyacrylamide gel electrophoresis was modified. Two polyacrylamide gel concentrations (5.1% and 3.6%) and longer run times (4 hrs at 60 watts) were explored.

Digestion for lily was also modified by repeating the digestion step resulting in using twice the amount of enzyme and running the reaction twice as long. The digestion mixture contained 2 µl DNA, 1.5 µl water, 1 µl 5x reaction buffer, and 0.5 µl enzyme (1.25 u each/µl). The reaction was run for 2 h at 37°C without 70°C denaturing. Next, 0.25 µl 5x reaction buffer, 0.5 µl enzyme, and 0.5 µl water were added and the reaction ran for an additional 2h at 37°C and then the enzyme was heat denatured at 70°C for 15 min. Four lily plants from grower 2 were used with two replications per lily ran per the standard digestion and modified digestion comparison. The dilution step of the digestion/ligation before preamplification was omitted and the selective amplification primer pair used was E-AAC and M-CAC.

Two modifications to the preamplification step were considered (**Fig. 2**). First, the preamplification was performed independently on the standard diluted (1:10 dilution) and undiluted digestion/ligation reaction. Second, the standard preamplification M-primer (M-C) and a preamplification M-primer plus an extra selective nucleotide (M-CT) were compared. These two preamplification modifications were conducted in a complete factorial with two lilies (grower bulb lot-plant number; 1-5 and 2-7) using CTAB and DNeasy® lily DNA extracted from each lily plant. In addition, two digestion/ligation reactions were conducted per lily/DNA combination and two preamplifications were conducted per diluted and undiluted digestion/ligation mixture for replication. Selective amplifications were performed using primer pair E-AAC/M-CTAA.

Modification to the selective amplification step entailed manipulation of primer composition. The effect of number of selective nucleotides for both the E and M selective amplification primers on fingerprint complexity was evaluated. For these experiments, preamplification reactions were generated using standard (E-A/M-C) preamplification primers. They originated from a CTAB DNA extraction from three different lily plants, each from a different lot (grower bulb lot-plant number; 1-5, 3-7, 4-9). Two different E/M (3+3, 3+4, 4+3, and 4+4) primer sets were used [E-AGC(A)/M-CTA(A); E-ACG(C)/M-CAG(A)]. The E+4 and M+4 primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) and were prepared as specified by Vos *et al*. (1995).

AFLP fragments were visually scored from X-ray films as present (1) or absent (0). Statistical analyses were performed using the software NTSYSpc version 2.11c (2002) and included cluster analysis of Jaccard's coefficients of similarity (only polymorphic fragments across genotypes are used for calculations) via the unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis (PCA). In addition, band number across AFLP modifications in lily were compared using Student's *t*-test.

Ploidy determination of rose

Chromosome counts were conducted for *R. hybrida* 'BAIeam', 'INTerlav', and 'Henry Kelsey'. Root tip squashes were used to determine chromosome number and were performed according to Zlesak *et al*. (2005).

RESULTS

DNA quantification and digestion

A DNA band of >20 kb was observed for all species and extraction methods on agarose gels (data not shown) indicating large genomic DNA fragments. DNA yield was ~30-100 ng/µl and ~1 ng/µl using the CTAB (all three species) and DNeasy® (lily only) DNA extraction methods, respectively. Reducing the starting quantity of fresh lily leaf tissue (100 to 10 mg) for DNeasy® extractions had no effect on overall DNA yield (data not shown). Restriction digestion of lily DNA, regardless of extraction method, yielded uniform smears on agarose gels, consistent with complete and efficient digestion (data not shown).

Rose

Consistent DNA fingerprints were obtained across replications for genotype/primer pair combinations. The undiluted digestion/ligation mixture versus diluted digestion/ligation mixture did not alter AFLP fingerprint scoring, although in some cases the band intensity was marginally stronger in the undiluted mixture (data not shown). Across the three rose cultivars, the six selective amplification primer pairs yielded 338 total bands (23 to 70/primer pair), of which 165 (10 to 46/primer pair) were polymorphic (**Table 1**). 'INTerlav' and 'BAIeam' differed by 21 polymorphic bands (**Table 1**) with 20 of the polymorphisms having the band present in 'INTerlav' and absent in 'BAIeam' and the remaining polymorphism was the band present in 'BAIeam' and absent in 'INTerlav' (**Fig. 3**). Importantly, there were no polymorphic bands that were present in both 'Henry Kelsey' and 'BAIeam' and absent in 'INTerlav'. Considering only polymorphic fragments, the Jaccard's coefficient of similarity was 0.77 between 'BAIeam' and 'INTerlav' and <0.03 between 'Henry Kelsey' with both 'BAIeam' and 'INTerlav'. PCA (data not shown) was in agreement with the UPGMA dendogram constructed using Jaccard's coefficient of similarity (**Fig. 4**). Root tip squashes revealed that 'Henry Kelsey' is tetraploid ($2n=4x=28$), while both

Table 1 Total, polymorphic, and distinguishing numbers of DNA fragments from six AFLP primer pairs used on *Hydrangea macryphylla* ('Bailmer', Bailday', and 'Variegata') and *Rosa hybrida* ('BAIeam', 'Henry Kelsey', and 'INTerlav').

	No. of fragments					
	Hydrangea			Rose		
Primer pair	Total	Polymorphic	Distinguishing[a]	Total	Polymorphic	Distinguishing
E-AAC/M-CAC	84	34	0	64	46	7
E-AAC/M-CTT	103	20	0	57	17	0
E-ACG/M-CAC	49	15	0	23	10	0
E-ACG/M-CTT	76	22	0	70	35	3
E-AGC/M-CAC	65	31	1	60	27	6
E-AGC/M-CTT	93	35	0	64	30	5
Total	470	157	1	338	165	21

[a] Number of fragments which distinguish the most similar hydrangea ('Bailmer' and 'Bailday') and rose ('INTerlav' and 'BAIeam') cultivars.

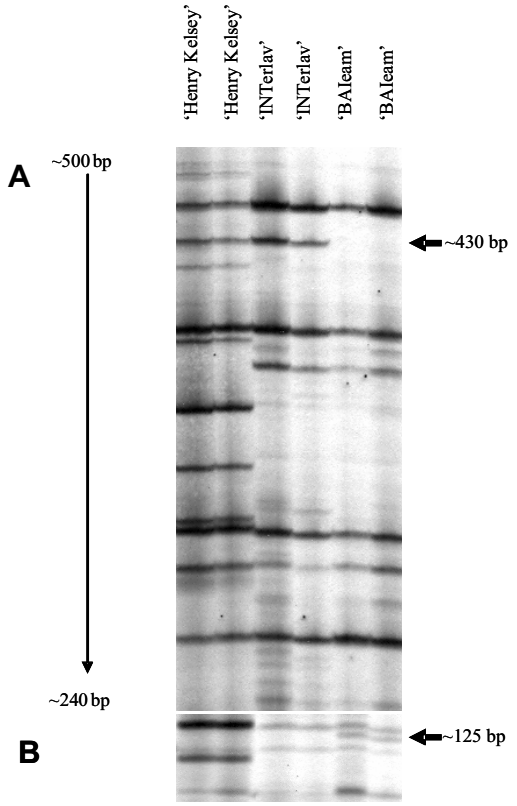

Fig. 3 Sections of AFLP autoradiograms for rose using primer pair E-ACG/M-CAC (A) and E-AGC/M-CAC (B). Fragments distinguishing rose cultivars 'BAIeam' and 'INTerlav' are highlighted with arrows and estimated size.

'INTerlav' and 'BAIeam' are triploid ($2n=3x=21$).

Hydrangea

Consistent DNA fingerprints were obtained across replications for genotype/primer pair combinations. The undiluted digestion/ligation mixture versus diluted digestion/ligation mixture did not alter AFLP fingerprint scoring, although in some cases the band intensity was marginally stronger in the undiluted mixture (data not shown). Among the three hydrangea cultivars, the six selective amplification primer pairs yielded 470 bands total (49 to 103/primer pair) of which 157 (15 to 35/primer pair) were polymorphic (**Table 1**). 'Bailday' and 'Variegata' differed by only one polymorphic band, while these two cultivars differed from 'Bailmer' by 156 polymorphic bands (**Table 1, Fig. 5**). Considering only polymorphic fragments, the Jaccard's coefficient of similarity was 0.99 between 'Bailday' and 'Variegata' and <0.01 between 'Bailmer' and both 'Bailday' and 'Variegata' (**Fig. 4**). PCA (data not shown) was in agreement with the UPGMA dendogram constructed using Jaccard's coefficient of similarity (**Fig. 4**).

Lily

In order to test if AFLP markers can distinguish intraclonal selections in lily, consistent AFLP fingerprints are needed across replications of the same genotype and primers. Consistent fingerprints could not be obtained even after exploring several modifications to the AFLP protocol (**Fig. 2**). For instance, using a single DNA extraction per lily (CTAB method) and the same selective amplification primers across modifications and replications expected to result in the same AFLP fingerprint, band number within plant was quite variable and ranged from 92 to158 (grower bulb lot 1- plant

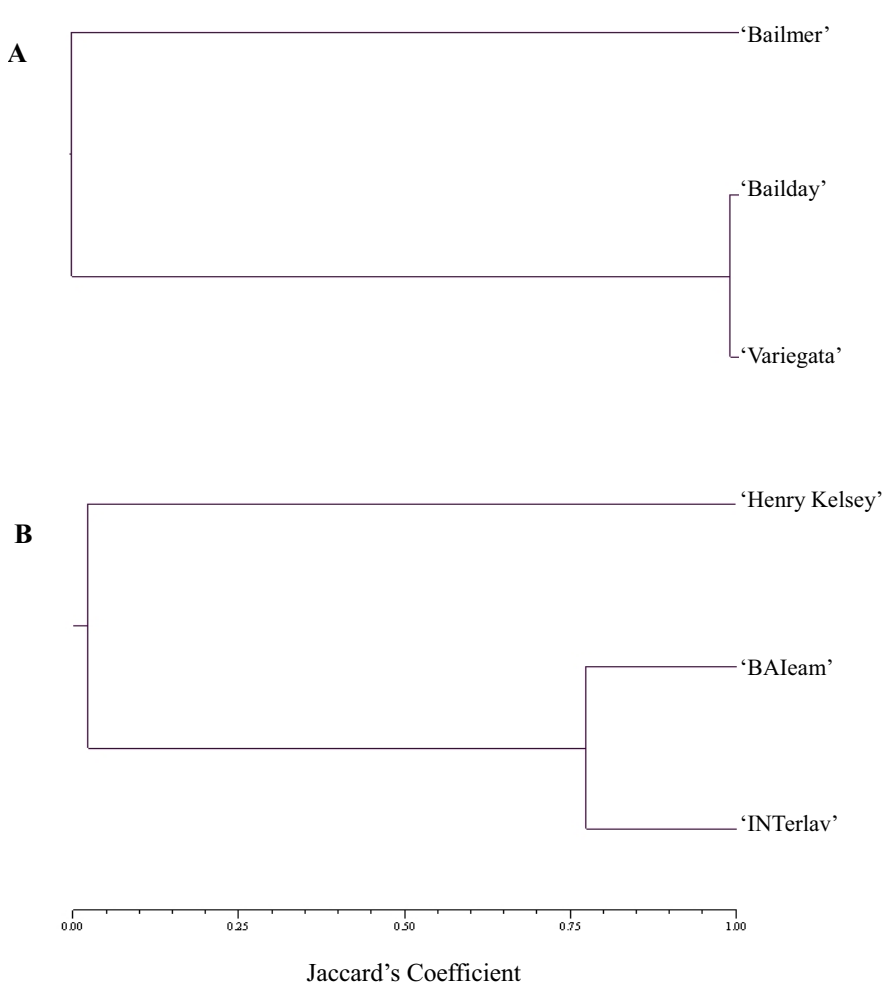

Fig. 4 UPGMA dendrograms constructed from Jaccard's coefficients of similarity calculated from AFLP data for hydrangea (157 AFLP markers) (A) and rose (165 AFLP markers) (B).

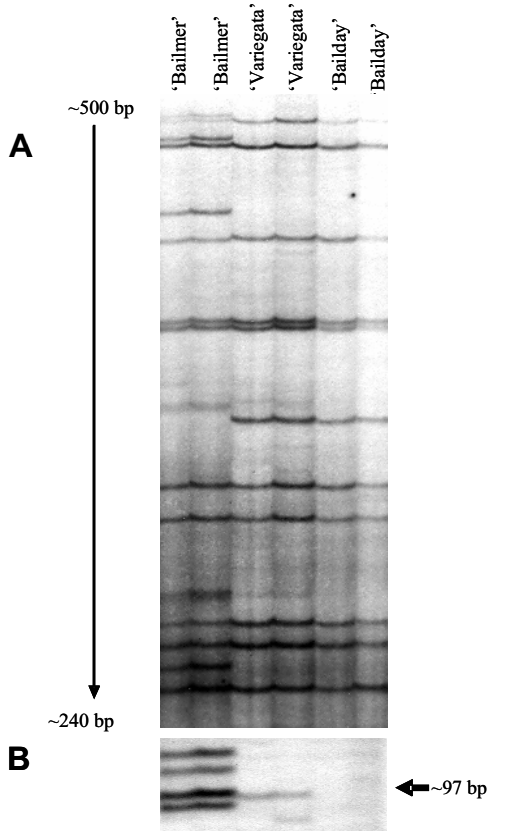

Fig. 5 Sections of AFLP autoradiograms for hydrangea with primer pair E-AGC/M-CAC showing polymorphisms between 'Bailmer' and both 'Bailday' and 'Variegata' (A) and one polymorphic fragment distinguishing 'Bailday' and 'Variegata' (B). Fragment distinguishing hydrangea cultivars 'Bailday' and 'Variegata' is highlighted with an arrow and estimated size.

5) and 107 to 150 (grower bulb lot 2- plant 7) (**Table 2**).

Although the data generated from AFLP analysis could not be used to distinguish intraclonal selections of 'Nellie White' due to procedural difficulties, modifications to the AFLP protocol showed differential effects on repeatability and consistency. The CTAB extraction method resulted in significantly more bands (t statistic = 4.0; df = 61; P <0.001) with a smaller standard deviation (126.8 ± 19.1, pooled) than the DNeasy® extraction method (100.3 ± 31.8, pooled) (**Table 2**). Using *Eco*R I digested DNA and two E primers for the selective amplification resulted in relatively large fragments (>500 bp) which were faint and difficult to score (**Fig. 6**). Decreasing the polyacrylamide percent from 5.1 to 3.6% and running the gel for 4 hrs instead of 2 hrs both resulted in slightly better separation of fragments, but did not change the fact that bands were faint and unable to be confidently scored. Using two E primers with DNA digested with *Eco*R I and *Mse* I relative to *Eco*R I only, resulted in much fewer, faint bands which were also relatively large (>500 bp) and unable to be confidently scored (data not shown). Digesting the lily DNA twice also did improve fingerprint consistency relative to a single digestion (data not shown).

Across each undiluted digestion/ligation mixture there were significantly more bands (t statistic = 8.1; df = 61; P <0.001) and a lower standard deviation (135.0 ± 16.7, pooled data) and generally greater consistency in banding pattern than the diluted digestion/ligation mixture (93.2 ± 23.4, pooled) (**Table 2; Fig. 7**). Modification of the preamplification primer pair by adding an additional selective nucleotide on the M primer (111.0 ± 29.0, pooled) did not significantly differ (t statistic = 0.7; df = 61; P = 0.47) from the standard preamplification primer pair (116.4 ± 29.6, pooled) (**Table 2**).

Variable numbers of selective nucleotides added to the E and M selective amplification primers using the standard digestion endonucleases (*Eco*RI and *Mse*I) resulted in different band numbers depending on primer set and selective nucleotide number on E and M primers within a primer set (**Table 3**). Primer set E-AGC(A)/M-CTA(A) generated the greatest number of bands with the E+3/M+3 primer pair

Table 2 Number of AFLP bands comparing DNA extraction method (CTAB and DNeasy®) and the standard (E-A/M-C) and a modified (E-A/M-CT; P2) preamplification primer pair for each of two *Lilium longiflorum* 'Nellie White' plants (bulb lot-plant number) using two digestion/ligation reactions (without/with dilution; L/LD) and two replications (R1 and R2) per extraction method/preamplification primer pair/L versus LD combination. The selective amplification primer pair used was E-AAC/M-CTAA.

			L					LD				
		L/LD	Preamplification primer pairs					Preamplification primer pairs				
	DNA	Reaction	E-A/M-C		E-A/M-CT			E-A/M-C		E-A/M-CT		
Lily	extraction	No.	R1	R2	R1	R2	Mean ± SD	R1	R2	R1	R2	Mean ± SD
1-5	CTAB	1	156	156	158	155	156.3 ± 1.3	112	113	98	103	106.5 ± 7.2
		2	154	157	125	135	142.8 ± 15.3	125	106	126	121	119.5 ± 9.3
	DNeasy®	3	132	99	122	92	111.3 ± 18.9	74	65	63	57	64.8 ± 7.0
		4	131	141	129	124	131.3 ± 7.1	70	63	70	71	68.5 ± 3.7
2-7	CTAB	5	130	149	139	134	138.0 ± 8.2	110	127	115	102	113.5 ± 10.5
		6	136	127	107	144	128.5 ± 15.9	111	108	101	118	109.5 ± 7.0
	DNeasy®	7	146	142	[a]	150	146.0 ± 4.0	106	110	84	96	99.0 ± 11.6
		8	127	119	123	145	128.5 ± 11.5	61	61	74	61	64.3 ± 6.5
Mean ± SD							135.0 ± 16.7					93.2 ± 23.4

[a]Reaction was not successful.

Table 3 Number of AFLP bands using two selective primer pairs with varying selective nucleotide (SN) number on both the E and M-primers (E-/M-) using preamplification dilutions (PD) from each of three different *Lilium longiflorum* 'Nellie White' plants (CTAB DNA extraction method; bulb lot-plant number) and an undiluted digestion/ligation.

	Selective primer pairs							
	E-AGC(A)/M-CTA(A)				E-ACG(C)/M-CAG(A)			
	SN No. (E-/M-)				SN No. (E-/M-)			
Lily	3/3	4/3	3/4	4/4	3/3	4/3	3/4	4/4
1-5	158[a]	123	108	96	85	92	93	123
3-7	158	116	97	103	86	84	132	115
4-9	133	130	103	99	104	91	119	106
Average	149.7	123.0	102.7	99.3	91.7	89.0	114.7	114.7

[a]Two replications were run per PD/primer pair and all replications per PD/primer pair had identical banding patterns.

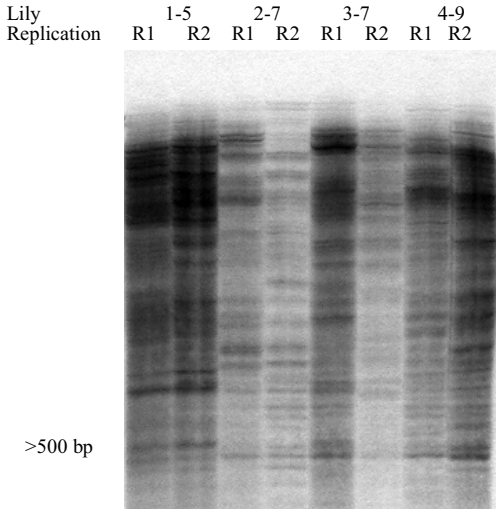

Fig. 6 An AFLP autoradiogram of *Lilium longiflorum* 'Nellie White' (CTAB DNA extraction technique) using two replications of a single preamplification dilution from each of four lilies (grower bulb lot-plant number) and using *Eco*R I only digestion and a pair of E/E selective amplification primers (E-A*/E-AAC; * radioactive label).

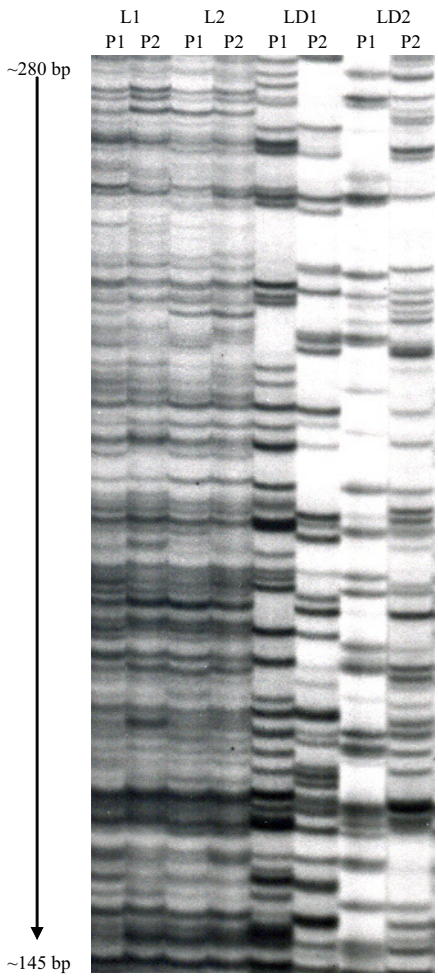

Fig. 7 An AFLP autoradiogram of *Lilium longiflorum* 'Nellie White' (bulb lot 1 plant 5; CTAB DNA extraction technique) comparing two non-diluted digestion/ligations (L1 and L2) and their corresponding standard dilutions (LD1 and LD2) and the standard (E-A/M-C; P1) and a modified preamplification primer pair (E-A/M-CT; P2). The selective amplification primer pair was E-AAC/M-CTAA.

(mean = 149.7) and the least with the E+4/M+4 primer pair (mean = 99.3) (**Table 3**). The E+4/M+3 (mean=123.0) and E+3/M+4 (mean = 102.7) primer pairs had an intermediate band number (**Table 3**). The other primer set (E-ACG(C)/ M-CAG(A)) did not show the same trend in band number across primer pair length and resulted in greatest band numbers for the E+3/M+4 (mean = 114.7) and E+4/M+4 (mean = 114.7) primer pairs and the fewest bands with the E+4/M+3 primer pair (mean=89.0) (**Table 3**). The same three preamplification dilutions (one from each of three different lilies) were used across primer pairs for both primer sets. Replications of each specific primer pair/pre-amplification dilution resulted in identical banding patterns and suggests the variability in the AFLP fingerprints in 'Nellie White' are being introduced before the preamplification dilution (i.e. digestion, ligation, and/or preamplification).

DISCUSSION

AFLP is a robust marker system and offered consistent and repeatable data for clonal differentiation in rose and hydrangea. However, using the standard protocol and several modifications, AFLP could not be optimized for Easter lily. The underlying reason is likely the very large genome size of Easter lily (77.1pg/2C nucleus; Lim *et al*. 2001). In contrast, *R. hybrida* (triploids ~1.8 and tetraploids 1.9 to 2.3 pg/2C nucleus; Yokoya *et al*. 2000) and *H. macrophylla* (3.9 and 4.3 pg/2C nucleus; Cerbah *et al*. 2001) have relatively smaller genomes, ~1/40 and ~1/20 and the size of Easter lily, respectively. Using the same primer pairs, hydrangea yielded more total bands (470) than rose (338) (**Table 1**). Greater band number in hydrangea is consistent with hydrangea having a larger genome, although other factors such as frequency and distribution of restriction enzyme sites could differ between species and influence fragment number.

Fay *et al*. (2005) proposed that genomes with >30 pg/2C nucleus (less than half of Easter lily) may prevent interpretable AFLP fingerprints, due to repetitive DNA in high copy number complicating detection (weakly amplified, difficult-to-score bands) of polymorphic regions of low copy number. Lily contains significant levels of repetitive DNA. For example, in *L. henryii* there are >13,000 copies of one retrotransposon (Smyth *et al*. 1989).

Even though AFLP markers are typically robust, reliable, and consistent, little or no AFLP variation has typically been noted between intraclonal selections or cultivars and their sports, consistent with a close genetic relationship (Veilleux and Johnson 1998; Hale *et al*. 2005). For instance, in roses Debener *et al*. (2000) found five polymorphic AFLP markers between a garden rose and its sports, but could not identify polymorphic markers among cut rose cultivars and their sports. In potato (*Solanum tuberosum*) 'Russet Norkotah' fifteen unique intraclonal selections have been identified which differ significantly in phenotype (Miller *et al*. 2004). Hale *et al*. (2005) used 112 AFLP primer pairs on six intraclonal 'Russet Norkotah' selections, but were unable to find any AFLP markers which could distinguish them from each other or the original 'Russet Norkotah' clone. Given low overall genetic variation in these studies between clonal variants, minimizing technical error and obtaining repeatable AFLP fingerprints across replications is essential in order to identify real and rare polymorphisms.

DNA purity can also affect the AFLP procedure. In *Allium*, van Treuren (2001) reported greater consistency in fingerprints across replications using the DNeasy® extraction method compared with the method of Fulton *et al*. (1995). van Treuren (2001) attributed the improvement to increased DNA purity. In the current study CTAB and DNeasy® extraction methods were used for lily and the DNeasy® extraction method contributed to greater variability (higher standard deviation) in band number (100.3 ± 31.8) across runs, using the same primers, than the CTAB method (126.8 ± 19.1) (**Table 2**). Since DNA yield was low (~1 ng/µl) from the DNeasy® extraction method, it is

unclear if the relatively lower and more variable band number is due to the low template DNA concentration instead of potential differences in DNA purity. Low yield from DNeasy® may be due to contaminates in lily such as proteins or polysaccharides which may be interfering with DNA recovery. In contrast to our results, the DNeasy® kit suggests yields of 38, 83, and 216 ng/µl in *Arabidopsis*, *Hordeum*, and *Nicotiana*, respectively. Reducing starting lily leaf material from 100 mg sequentially to 10 mg did not affect DNA yield. Samples from all starting material quantities were viscous and did not contain visible supernatant prior to optional centrifugation, although samples using less starting material were progressively less viscous. In spite of DNeasy® extractions containing little DNA (~1 ng/µl), 2 ng (2 µl) of DNA per reaction still allows for the lily genome to be represented at ~26x/reaction (*L. longiflorum* is ~77.1 pg/2C nucleus; Lim *et al*. 2001).

In order to reduce fragment number and fingerprint complexity in lily, digestion with only *Eco*R I (the infrequent cutter) was investigated. Digestion using *Eco*R I only was reported and useful for AFLP analysis in honey bee (Suazo and Hall 1999). Restriction digestions with *Eco*R I alone yielded generally large (>500 bp), faint bands which were not scorable (**Fig. 6**). When the standard restriction enzymes (mixture of *Eco*R I and *Mse* I) and E/E selective amplification primers are used, no or very few faint bands were observed. This may be due to *Eco*R I/ *Eco*R I fragments with *Eco*R I digestion only containing internal *Mse* I sites. Digestion with *EcoR* I only may yield DNA fragments in lily which are too large to consistently amplify and separate on polyacrylamide gels without additional modification.

The extension of the preamplification M primer from M-C to M-CT would, ordinarily, allow for both the preamplification and selective amplification primers to introduce only two selective nucleotides at each amplification (assuming an M+4 selective primer). This may allow for greater PCR specificity and less misannealing of primers relative to introducing three selective nucleotides (M+4) in the selective amplification when using the standard preamplification M primer (M-C). van Heusden *et al*. (2002) modified the Vos *et al*. protocol (1995) by altering the preamplification primers (E-A + M-CT) to obtain repeatable AFLP fingerprints in Asiatic lilies (*L. elegans*). They did not report a comparison of the modified preamplification primer pair with the standard primer pair (E-A + M-C), but had used an identical approach previously for AFLP analysis of large genome *Allium* species (van Heusden *et al*. 2000). For lily, adding an extra selective nucleotide to the M preamplification primer did not aid in obtaining repeatable band numbers or banding patterns across replications when compared to the standard preamplification primers (**Table 2; Fig. 7**).

The modification having the most impact on consistency in lily was omitting the ten-fold dilution of the digestion/ligation mixture (**Fig. 7**). It is unclear why a concentrated digestion/ligation mixture in both the CTAB and DNeasy® DNA extraction methods (**Table 2**) would improve consistency of AFLP fingerprints in lily. Perhaps there is stochastic competition among fragments and an undiluted digestion/ligation mixture allows for more template DNA and amplification consistency for the lower copy number or otherwise less competitive fragments. Likewise, there may be incomplete digestion and/or ligation. These factors, in combination with a large genome may lead to inconsistent fingerprint patterns, although running digestion products on agarose gels suggests complete digestion. Another possibility for increased consistency for undiluted digestion/ligation mixtures may involve adapter ratio for *Mse* I and *Eco*R I restriction sites. Perhaps the ratio of *Mse* I and *Eco*R I restriction sites in lily is different than for other plants and the standard ratio of adapters within the AFLP kit is limiting for one restriction site.

Modifying the selective nucleotide number during the selective amplification reaction can alter final band number, allow for easier scoring, and/or increased data from each AFLP run (Han *et al*. 1999; van Treuren 2001; Fay *et al*. 2005). In addition, fewer bands reduce the probability that a polymorphism might be "masked" by a monomorphic fragment of the same size. Fifty to 100 bands are usually targeted per primer pair and selective nucleotide number for each primer is typically increased or decreased from the standard 3+3 (AFLP® Analysis System II kit; Invitrogen™ Life Technologies), based on genome size (Blears *et al*. 1998; Fay *et al*. 2005). Organisms with larger genomes (>30 pg/2C nucleus) can be especially challenging to obtain high quality AFLP fingerprints (high band number and weakly amplifying bands), even when using 3+4 or 4+3 selective nucleotides (Blears *et al*. 1998; Fay *et al*. 2005). Theoretically, the addition of each selective nucleotide would decrease band number four-fold, assuming equal representation and random genome distribution of each nucleotide, but this is not typically observed (Han *et al*. 1999). In this study the two primer sets with varying selective nucleotides numbers (3+3, 3+4, 4+3, and 4+4) yielded opposite trends in band number from 3+3 to 4+4 primer pairs. For primer set E-AGC(A)/M-CTA(A), there was a reduction in band number from the 3+3 primer pair (149.7) to the 4+4 primer pair (99.3) (**Table 3**), although not to the magnitude theoretically expected if the four nucleotides are represented equally in the genome (16-fold reduction). One possibility is that a 16-fold reduction in fragment amplification is truly occurring, but cannot be detected due to many different fragments of the same size or vast amounts of repetitive DNA across the genome appearing as a single band (Fay *et al*. 2005; Han *et al*. 1999). The other general primer set (E-ACG(C)/M-CAG(A)) produced an unexpected result in that there was a trend for an increase in band number going from 3+3 to 4+4 primers. Fay *et al*. (2005) reported a ~two-fold increase in band number for *Damasonium alisma* from E+3/M+3 to E+3/M+4 primer pairs and attributed this unexpected result to many weakly amplifying bands previously unscorable (E+3/M+3) becoming darker and scorable (E+3/M+4) due to reduced competition during PCR. Another possibility for increased band number in the E+4/M+4 primer pair is that as primer length increases, there may be an increased tolerance for 3' mismatches. The effect of increasing selective nucleotide number in lily was variable, and depended on primer set and specific primer pair.

Rose and hydrangea had consistent and repeatable AFLP fingerprints, which provided reliable data useful for biological inference, typical of crop and other plant species of similar genome sizes. Based on our analyses, it is highly unlikely that 'Bailday' is a sport of 'Bailmer' since of 470 AFLP markers, 156 (33.2%) were polymorphic. In contrast, 'Bailday' and 'Variegata' had very similar AFLP fingerprints with only one, relatively faint, polymorphic band between them (**Table 1** and **Fig. 5**). The almost identical AFLP fingerprint suggests 'Bailday' may be a sport of 'Variegata' or may be 'Variegata' itself. It has been documentted that contamination of plant tissue used for DNA extraction with prokaryotes or other organisms may introduce unique AFLP fragments (Dyer and Leonard 2000) or may be due to a mutation event which may or may not result in a detectable phenotypic change. Sequencing of unusual fragments, e.g. the polymorphic fragment distinguishing 'Variegata' and 'Bailday' (**Table 1** and **Fig. 5**), may aid in determining if the fragment is due to a contaminant organism. Further investigation can help confirm the relationship between 'Variegata' and 'Bailday' and could include sequencing the polymorphic fragment, using additional AFLP primer pairs, and making phenotypic comparisons.

Without bands present in both 'BAIeam' and 'Henry Kelsey' roses but absent in 'INTerlav', it is highly unlikely that 'Henry Kelsey' is the paternal parent of 'BAIeam'. Out of the 76 bands present in 'Henry Kelsey' and absent in 'INTerlav', ~50% (38/76) would be expected in 'BAIeam' if 'Henry Kelsey' was the paternal parent. 'BAIeam' and 'INTerlav' are clearly different in their AFLP fingerprints

(21 polymorphisms) which eliminates the possibility of 'BAIeam' being an apomictic seedling of 'INTerlav' via adventitious embryony or apospermy. Seedlings with such forms of apomixis are expected to have the identical genotype as the maternal parent (Koltunow 1993). Having 20 of the polymorphisms being bands present in 'INTerlav' and absent in 'BAIeam' and both 'INTerlav' and 'BAIeam' are triploid is consistent with the possibility of diplospory apomixis, with 'BAIeam' originating as a 2*n* egg of 'INTerlav'. In diplospory, a megaspore mother cell develops into the embryo directly or after partial or modified meiosis (Kultunow 1993). Diplospory with meiotic recombination can lead to 2*n* gametes with duplication of some regions of the parental genome and deletion of others. Duplications and deletions in a dominant marker system like AFLP can lead to the absence of some AFLP markers in the apomict relative to the parent and has been suggested to explain the AFLP fingerprint patterns of two parent-offspring sets in rose (Crespel *et al*. 2001). Depending on the mode of 2*n* gamete formation and rate and sites of crossovers, different levels of parental heterozygosity can be transmitted via 2*n* gametes. For first division restitution (FDR) and second division restitution (SDR) 2*n* gametes with crossing over, ~80% and ~40% of parental heterozygosity is transmitted, respectively (Peloquin 1983). 'BAIeam' shares 92.1% of the AFLP markers with 'INTerlav', suggesting a higher than average level of transmission of heterozygosity than expected for FDR 2*n* gametes. High transmission rates of parental heterozygosity via suspected FDR 2*n* gametes in rose have been reported using AFLP markers and have ranged from 84.3 to 100% in diploid rose H61 (Crespel *et al*. 2002). Since 'INTerlav' is triploid, pairing of each chromosome with its homolog may be hindered, resulting in bivalents and some univalents, in addition to unpaired chromosome arms in trivalents. Unpaired chromosomes or chromosome regions impede chiasmata and recombination, thereby resulting in relatively higher transmission of parental heterozygosity in FDR 2*n* gametes. The unique band present in 'BAIeam' and absent in 'INTerlav' (**Fig. 3**) can be sequenced to help ascertain its source- it may be from a contaminant organism (Dyer and Leonard 2000) or may be due to a recombination event that brought restriction sites on two separate homologous chromosomes together and allowed for a unique AFLP band in 'BAIeam'. Another possibility besides apomixis that can explain the highly similar AFLP fingerprints of 'BAIeam' and 'INTerlav' is that 'BAIeam' may be a self-fertilized seedling of 'INTerlav'. However, this is unlikely because one generation of selfing typically leads to ~50% homozygosity and would theoretically result in about a 25% reduction in AFLP fragments. The frequency of fragment loss can appear less than ~25% for reasons including repetitive sequence across homeologous chromosomes and the genetic constitution of the particular gametes which participated in fertilization. However, diplospory apomixis is more likely due to both 'BAIeam' and 'INTerlav' being triploid and having high band similarity comparable to other possible diplospory apomicts in rose (Crespel *et al*. 2001).

The International Union for the Protection of New Varieties of Plants (UPOV) has a provision for essentially derived varieties (EDVs) which was added in the Act of 1991 (Aguirre 2006; www.upov.int). A breeder can claim the same rights on an essentially derived variety as their original, protected variety. The ways in which EDVs can arise include spontaneous sports, varieties having a transgene, or other means (sexual or asexual) by which the new variety is predominantly derived from the initial variety (www.upov.int). Debener *et al*. (2000) compared AFLP fingerprints of two greenhouse cut flower roses (cut rose cultivars have a relatively limited gene pool) and one garden rose cultivar to their sports and sexual seedlings in order to determine if AFLP is useful in identifying EDVs in rose. They could not identify repeatable polymorphisms between the cut rose varieties and their sports, but found ~11% polymorphic bands between the cut rose varieties and two of their seedlings (one seedling each) (Debener *et al*. 2000). For the garden rose and sports, five polymorphic bands were identified. Between the garden rose and its two seedlings, ~26.5% polymorphisms were found (Debeber *et al*. 2000). In *Dendrobium* orchids, AFLP was also found to be a useful marker to help clarify genetic relationships and help distinguish which individuals are most likely seedlings versus sports (Hong *et al*. 2005).

Vosman *et al*. (2004) suggested a Jaccard's coefficient of $\geq$0.95 (considering all bands and not just polymorphic among the set of germplasm compared) is a useful threshold to distinguish EDVs (sports) from other rose germplasm. The Jaccard's coefficient between 'INTerlav' and 'BAIeam' was 0.77 when considering only polymorphic bands and 0.92 when all bands are considered. When considering only polymorphic bands among a set of germplasm in the calculation of the Jaccard's coefficient, as is common in recent literature, the coefficient reported can underestimate the true similarity in a pairwise comparison and be variable based on the other germplasm included. The more genotypes which are tested the greater the likelihood of finding additional polymorphic bands, which can increase the Jaccard's coefficient for specific pairwise comparisons if at least one of the individuals compared contains the new band(s) in question.

Possible diplospory apomicts reported in rose by Crespel *et al*. (2001) were from breeding lines and not from cultivars, and 'BAIeam' may represent a unique situation where a possible diplospory seedling with a very similar phenotype to its maternal parent was commercially introduced. Ultimately, it will be up to litigation courts to decide if a rose like 'BAIeam' or a hydrangea like 'Bailday' should be classified as an EDV of 'INTerlav' and 'Variegata', respectively. In the United States, the plant patent act does not include stipulations for EDVs and allows the discoverer of sports to own and patent them (Aguirre 2006; www.uspto.gov). Since there are unique bands between 'BAIeam' and 'INTerlav' and one unique band between 'Variegata' and 'Bailday', there is molecular evidence of differences between these two sets of cultivars, although obvious phenotypic differences have not been detected.

AFLP is a very useful and robust molecular marker for clonal integrity studies when the genome size is not large. Its reliability, high band number, and genome-wide representation make it a favorable marker system to detect differences even among genetically similar germplasm. The effectiveness of AFLP to distinguish highly similar, yet genetically distinct, germplasm relies, in part, on the probability of choosing primers that selectively amplify relatively rare polymorphic fragments and low error rates. AFLP analysis resolved clonal integrity questions for rose and hydrangea, species of modest genome size, by providing consistent AFLP fingerprints. For Easter lily, however, the large genome size and/or repetitive DNA compromised repeatable, consistent, and scorable AFLP fingerprints despite the numerous optimizations performed. Perhaps following up on the modifications that led to greater consistency and exploring additional modifications may lead to a reduced enough error rate to make AFLP practical for clonal integrity questions studies with very large genome crops such as Easter lily. While other molecular techniques will be investigated to assess clonal variation, they may be less stringent and useful than AFLPs since the lily genome has not been characterized and no classical or molecular linkage maps exist.

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