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

David C. Zlesak1 • James M. Bradeen2 • Neil O. Anderson3

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 BAileam 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 ‘BAileam’ 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: 2n gamete; clonal integrity; diplospory, Hydrangea macrophylla, Lilium longiflorum, Rosa hybrida

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 (Debenet 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 (Douchkovskikh and Dodd 2003; Zhang et al. 1999) and genetic variability between different leaves and stems within even a single plant (Douchkovskikh 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

Received: 15 November, 2006. Accepted: 22 March, 2007.
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 (Bleas 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 (Vieilleux 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. 1998; 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* ‘BAleam’ (DayDreamTM, 2005 All-America Rose Selection winner; US Plant Patent No. 15,736) is reported to be a cross of ‘INTerlav’ (Lavender DreamTM; US Plant Patent No. 5,916) × ‘Henry Kelsey’ (Lim 2005). ‘BAleam’ 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 SummerTM; 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, ‘BAleam’ and ‘Bailday’, are very similar phenotypically 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.

---

Fig. 1 Representative flowers of rose cultivars ‘Henry Kelsey’, ‘INTerlav’, and their reported offspring ‘BAleam’ (A) and hydrangea cultivars ‘Bailmer’, ‘Variegata’, and ‘Bailday’ (B) (scale not shown).
MATERIALS AND METHODS

Plant materials

Two plants each of *H. macrophylla* ‘Bailday’, ‘Bailmer’, and ‘Variegata’ and *R. hybrida* ‘BAlcam’ 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® Plant Mini Plant Kit the optional centrifugation step was performed in order to have recoverable supernatant. Varying quantities of fresh lily leaf tissue for >1 bulb per grower bulb lot and one DNeasy® and CTAB extraction per plant) were digested 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 EcoRI (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 EcoR I primers were radioactively labeled with 32P. A 32P-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 EcoRI/MseI digestion only and undiluted digestion/ligation reaction and six EcoRI/MseI digestion/ligation primer pairs (E-AAC/M-CAC; E-AAC/M-CTT; E-AGC/M-CAC; E-AGC/M-CTT; E-ACG/M-CAC; E-ACG/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; 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 *EcoR* 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

![AFLP Steps](image)

**Fig. 2** AFLP protocol (Vos et al. 1995) with highlighted modifications used on lily, hydrangea, and rose.
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 ‘BAileam’, ‘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 smear 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 ‘BAileam’ differed by 21 polymorphic bands (Table 1) with 20 of the polymorphisms having the band present in ‘INTerlav’ and absent in ‘BAileam’ and the remaining polymorphism was the band present in ‘BAileam’ and absent in ‘INTerlav’ (Fig. 3). Importantly, there were no polymorphic bands that were present in both ‘Henry Kelsey’ and ‘BAileam’ and absent in ‘INTerlav’. Considering only polymorphic fragments, the Jaccard’s coefficient of similarity was 0.77 between ‘BAileam’ and ‘INTerlav’ and <0.03 between ‘Henry Kelsey’ with both ‘BAileam’ 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 macrophylla (‘Bailmer’, ‘Bailday’, and ‘Variegata’) and Rosa hybrida (‘BAileam’, ‘Henry Kelsey’, and ‘INTerlav’).

<table>
<thead>
<tr>
<th>Primer pair Total</th>
<th>Hydrangea Polymorphic</th>
<th>Distinguishing*</th>
<th>Total</th>
<th>Rose Polymorphic</th>
<th>Distinguishing</th>
</tr>
</thead>
<tbody>
<tr>
<td>E-AAC/M-CAC</td>
<td>84</td>
<td>34</td>
<td>0</td>
<td>64</td>
<td>46</td>
</tr>
<tr>
<td>E-AAC/M-CTT</td>
<td>103</td>
<td>20</td>
<td>0</td>
<td>57</td>
<td>17</td>
</tr>
<tr>
<td>E-AAG/M-CAC</td>
<td>49</td>
<td>15</td>
<td>0</td>
<td>23</td>
<td>10</td>
</tr>
<tr>
<td>E-AAG/M-CTT</td>
<td>76</td>
<td>22</td>
<td>0</td>
<td>70</td>
<td>35</td>
</tr>
<tr>
<td>E-AAC/M-CAC</td>
<td>65</td>
<td>31</td>
<td>1</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>E-AAC/M-CTT</td>
<td>93</td>
<td>35</td>
<td>0</td>
<td>64</td>
<td>30</td>
</tr>
<tr>
<td>Total</td>
<td>470</td>
<td>157</td>
<td>1</td>
<td>338</td>
<td>165</td>
</tr>
</tbody>
</table>

* Number of fragments which distinguish the most similar hydrangea (‘Bailmer’ and ‘Bailday’) and rose (‘INTerlav’ and ‘BAileam’) cultivars.
AFLP for resolving clonal integrity questions. Zlesak et al.

‘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 dendrogram 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 to 158 (grower bulb lot 1-plant...
Fig. 5 Sections of AFLP autoradiograms for hydrangea with primer pair E-AGC/M-CAC showing polymorphisms between ‘Bailday’ 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.

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-CTA.

<table>
<thead>
<tr>
<th>Lily</th>
<th>DNA extraction</th>
<th>Reaction No.</th>
<th>L/LD</th>
<th>E-A/M-C</th>
<th>E-A/M-CT</th>
<th>Mean ± SD</th>
<th>R1</th>
<th>R2</th>
<th>R1</th>
<th>R2</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5 CTAB</td>
<td>156</td>
<td>156</td>
<td>158</td>
<td>155</td>
<td>156 ± 1.3</td>
<td>112</td>
<td>113</td>
<td>98</td>
<td>103</td>
<td>106.5 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>154</td>
<td>157</td>
<td>125</td>
<td>135</td>
<td>142.8 ± 15.3</td>
<td>125</td>
<td>106</td>
<td>126</td>
<td>121</td>
<td>119.5 ± 9.3</td>
<td></td>
</tr>
<tr>
<td>DNeasy®</td>
<td>132</td>
<td>99</td>
<td>122</td>
<td>92</td>
<td>111.3 ± 18.9</td>
<td>74</td>
<td>65</td>
<td>63</td>
<td>57</td>
<td>64.8 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>131</td>
<td>141</td>
<td>129</td>
<td>124</td>
<td>131.3 ± 7.1</td>
<td>70</td>
<td>63</td>
<td>70</td>
<td>71</td>
<td>68.5 ± 3.7</td>
<td></td>
</tr>
<tr>
<td>2-7 CTAB</td>
<td>130</td>
<td>149</td>
<td>139</td>
<td>134</td>
<td>138.0 ± 8.2</td>
<td>110</td>
<td>127</td>
<td>115</td>
<td>102</td>
<td>113.5 ± 10.5</td>
<td></td>
</tr>
<tr>
<td>DNeasy®</td>
<td>136</td>
<td>127</td>
<td>107</td>
<td>144</td>
<td>128.5 ± 15.9</td>
<td>111</td>
<td>108</td>
<td>101</td>
<td>118</td>
<td>109.5 ± 7.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>146</td>
<td>142</td>
<td>130</td>
<td>150</td>
<td>146.0 ± 4.0</td>
<td>106</td>
<td>110</td>
<td>84</td>
<td>96</td>
<td>99.0 ± 11.6</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>127</td>
<td>119</td>
<td>123</td>
<td>145</td>
<td>128.5 ± 11.5</td>
<td>61</td>
<td>61</td>
<td>74</td>
<td>91</td>
<td>64.3 ± 6.5</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>135.0 ± 16.7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>93.2 ± 23.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*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.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-5</td>
<td>158</td>
<td>123</td>
<td>108</td>
<td>96</td>
<td>85</td>
<td>92</td>
<td>93</td>
</tr>
<tr>
<td>3-7</td>
<td>158</td>
<td>116</td>
<td>97</td>
<td>103</td>
<td>86</td>
<td>84</td>
<td>132</td>
</tr>
<tr>
<td>4-9</td>
<td>133</td>
<td>130</td>
<td>103</td>
<td>99</td>
<td>104</td>
<td>91</td>
<td>119</td>
</tr>
<tr>
<td>Average</td>
<td>149.7</td>
<td>123.0</td>
<td>102.7</td>
<td>99.3</td>
<td>91.7</td>
<td>89.0</td>
<td>114.7</td>
</tr>
</tbody>
</table>

*Two replications were run per PD/primer pair and all replications per PD/primer pair had identical banding patterns.
pies of one retrotransposon (Smyth et al., 1989). Due to repetitive DNA in

frequent, difficult-to-score bands) of polymorphic regions of high copy number complicating detection (weakly amplification number. 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 fingerprin ts, 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. henryi 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 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

![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 number) and using EcoR I only digestion and a pair of E/E selective amplification primers (E-A*/E-AAC; * radioactive label).](image)

(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-CA(G(A)) did not show the same trend in band numbers 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 (77.1 pg/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.

Table 1:

<table>
<thead>
<tr>
<th>Plant</th>
<th>Replication</th>
<th>R1</th>
<th>R2</th>
<th>R1</th>
<th>R2</th>
<th>R1</th>
<th>R2</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lily</td>
<td>1-5</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3-7</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4-9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<500 bp

>500 bp

![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.](image)
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 lily leaf enzymes (max. 100 mg) slowly 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, Mose 77.1 pg/2C nucleus; Lim et al. 2001).

In order to reduce fragment number and fingerprint complexity in lily, digestion with only EcoRI (the infrequent cutter) was investigated. Digestion using EcoRI alone was reported and useful for AFLP analysis in honey bee (Suazo and Hall 1999). Restriction digests with EcoRI alone yielded generally large (>500 bp), faint bands which were not scorable (Fig. 6). When the standard restriction enzyme mixture of EcoRI and MspI selective amplification primers are used, no or very few faint bands were observed. This may be due to EcoRI / EcoRI fragments with EcoRI digestion only containing internal MspI sites. Digestion with EcoRI 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 misamplification 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. (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 allow for increased 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 CTAD and DNeasy® extraction method (Table 2) could 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 incomplete digestion/ligation mixtures. In this study the two primer sets with varying selective nucleotide 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 expected if the four selective 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’ and ‘Variegata’ did not share a common parent. Sequencing of unusual fragments, 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’ ones 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.
(21 polymorphisms) which eliminates the possibility of ‘BAIeam’ being an apomictic seedling of ‘INTerlav’ via adventitious embryony or apospory. 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 the original ‘INTerlav’ being a hybrid of ‘BAIeam’. In diplospory, a megaspore mother cell develops into the embryo directly or after partial or modified meiosis (Koltunow 1993). Diplospory with meiotic recombination can lead to 2n 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 2n gamete formation and rate and sites of crossovers, different levels of parental heterozygosity can be transmitted via 2n gametes. For first division restitution (FDR) and second division restitution (SDR) 2n gametes with crossing over, ~80% and ~40% of parental heterozygosity is transmitted, respectively (Peeters et al. 1983). ‘BAIeam’ shares 99% of the AFLP markers with ‘INTerlav’, suggesting a higher than average level of transmission of heterozygosity than expected for FDR 2n gametes. High transmission rates of parental heterozygosity via suspected FDR 2n 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 chiasma and recombination, thereby resulting in relatively higher transmission of parental heterozygosity in FDR 2n 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 reduce the transmission of AFLP markers by 25%. The frequency of fragment loss can appear less than ~25% for reasons including repetitive sequence across homologous 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 not derived from the original variety. 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 sports and sports, five polymorphic bands were identified. Between the garden rose and its two seedlings, ~26.5% polymorphisms were found (Debever 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).

The Act of 1991 was amended by the Jaccard’s coefficient of ~0.95 (considering all bands and not just polymorphic ones) in 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 bands 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 hyrangea 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 bands and low sensitivity to 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.

ACKNOWLEDGEMENTS

Scientific Journal Series No. 061210173 of the Department of Horticultural Science, University of Minnesota. Supported, in part, by the Minnesota Agricultural Experiment Station and grants from the University of Minnesota Grant in Aid and the Fred C. Gloeckner Company.