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Use of Fluorescent-Amplified Fragment Length Polymorphism for Species Identification in the Genus *Pulmonaria*[1]

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Abstract

The species identity of 'Roy Davidson', a popular commercial cultivar in the genus *Pulmonaria*, is uncertain. To determine to which species 'Roy Davidson' is related to, we used fifteen primer combinations for Fluorescent-Amplified Fragment Length Polymorphism (F-AFLP) analysis. UPGMA dendrograms generated from AFLP markers obtained from the primer combinations showed that 'Roy Davidson' shares more genetic information with *P. longifolia* than with *P. saccharata*. Several factors that can affect F-AFLP profiles (DNA isolation from leaf tissue, optimization of F-AFLP procedures, and the use of this method for genotyping pulmonarias) were also evaluated; the results are discussed in this paper.

Index words: *Pulmonaria*, Lungwort, F-AFLP, AFLP, DNA fingerprinting, breeding.

Species used in this study: Lungworts (*Pulmonaria longifolia* 'Bertram Anderson'; *P. saccharata* 'White Wings' and 'Pierre Pure Pink'; *P. longifolia* (or *saccharata*) 'Roy Davidson'; *P. hybrida* 'Majeste'; *P. hybrida* 'Victoria Brooch').

Significance to the Nursery Industry

With F-AFLP we have demonstrated that 'Roy Davidson' is more likely a cultivar of *P. longifolia* than of *P. saccharata*. The application of this technique to pulmonarias (lungworts) can alleviate some of the confusion of cultivar identity associated with morphological characteristics. These results also indicate that F-AFLP can be used to determine the genetic relationships between cultivars and can estimate the genetic diversity among cultivars. Knowledge of the genetic diversity between cultivars is useful when making breeding decisions concerning lungworts and determining which accessions will contribute the most or least genetic diversity to a collection. Accurate identification of each accession in a collection would provide growers with a basis for decision-making to avoid redundancy in cultivar maintenance and propagation.

Introduction

Pulmonarias are important to the nursery industry. There are 15 species and many cultivars in this genus, many introductions are difficult to identify by morphological characteristics. For example, 'Roy Davidson', a popular cultivar in the industry, is listed in Ohio State University's Pocket Gardener (12) and by Michigan State University (10) as a *P. longifolia*; by Joy Creek Nursery (6) as a *P. saccharata*; and by D. Heim (5) and T. Avent (2) as a hybrid between *P. longifolia* 'Bertram Anderson' and *P. saccharata*.

Horticulturists identify lungworts by using phenotypic characteristics such as leaf spotting patterns, leaf shapes, flower characteristics, and stress tolerances. Since there is no distinct morphological standard that can be used to differentiate many species in this genus, classification is difficult. Biochemical or molecular markers can provide information that can be used for the determination of the genetic relationships between different genotypes and assist in the identification of *Pulmonaria* species.

DNA fingerprinting by AFLP is presently the most powerful method for distinguishing individual genotypes, for the estimation of differences among heterogeneous populations and for the development of molecular markers (1, 9, 13, 14). AFLP can be used to differentiate closely related organisms (15). Fluorescent-based AFLP (F-AFLP) in combination with fluorescent detection instrumentation can size automatically and accurately F-AFLP fragments and provide the data necessary to accurately identify individual plants.

In this study F-AFLP molecular markers were developed for several cultivars and species of pulmonarias with different phenotypic characteristics and their genetic relationships were assessed using these markers. Genetic marker-tagged information related to phenotypic traits is useful during the selection of parental plants for breeding and during the evaluation of progenies. Genetic markers are also valuable for true-to-type plant identification and for plant patent applications.

Materials and Methods

Pulmonaria longifolia 'Bertram Anderson', *P. saccharata* 'White Wings' and 'Pierre Pure Pink', *P. longifolia* (or *saccharata*) 'Roy Davidson', and *P. hybrida* 'Victoria Brooch' and 'Majeste' were evaluated using F-AFLP. All accessions were obtained from nurseries specializing in the production and development of new pulmonaria cultivars and maintained in a greenhouse until needed. For each accession, the genomic DNA was extracted from 100 mg of freshly collected young leaves. Fresh leaves were homogenized with 800 µl of CLS-VF and 200 µl PPS (FASTDNA Kit, B1010, Carlsbad, CA) using a FAST PREP FP120 homogenizer (Savant) set at speed setting #4, for 20 s. Homogenates were centrifuged at

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15,000 rpm on a Marathon 2100R centrifuge (Fisher Scientific, Pittsburgh, PA) for 10 min to remove debris. The supernatants were transferred to a Dneasy Plant Minikit (Qiagen, Valencia, CA) column and eluted with sterile ultrapure water preheated to 65C. Resulting DNA extracts were quantified by fluorometry (DyNA Quant 200 DNA fluorometer, Hoefer Scientific, San Francisco, CA) and stored at –20C until needed.

The AFLP system (AFLP Analysis System I and AFLP Starter Primer Kit) from GIBCOBRL Life Technologies (Rockville, MD) was used. Procedures for DNA restriction, ligation and pre-selective amplification reactions were performed according to the manufacturere's guidelines (IRDye Fluorescent AFLP Kit, LI-COR, Lincoln, NE). Genomic DNAs (250 ng) were digested with Mse I and EcoR I for 2 h at 37C and heated to 70C for 15 min for enzymes deactivation. DNA fragments were ligated to EcoR I adaptor and Mse I adaptor in a solution containing T4 DNA ligase and ligation adaptor for 2 h at 20C. Pre-amplifications were carried out in a PTC-100 programmable thermal controller (MJ Research Inc., Watertown, MA) in a 1:10 dilution of ligation template DNA, pre-amp primer mix, 1U Taq DNA polymerase (Qiagen) and 10 × PCR buffer. The following thermal cycling parameter was used for 20 cycles: 94C for 30 sec, 56C for 1 min, 72C for 1 min and cooled to 4C.

Selective amplification reactions were performed using the following primers: EcoR I 5'-labeled with IR-800 (LI-COR) and Mse I (GIBCOBRL Life Technologies). The three selective nucleotides for EcoR I were: E-AAC, E-AAG, E-ACA, E-ACC, E-ACG, E-ACT, E-AGC, E-AGG and for Mse I: M-CAA, M-CAC, M-CAG, M-CAT, M-CTA, M-CTC, M-CTG, M-CTT. Sixty-four EcoR I/Mse I primer combinations were evaluated. Selective restriction fragment amplifications were performed in an 11-ul solution containing 2 µl of a 1:40 dilution of pre-amplified solution, 0.8 µl labeled EcoR I primer, 2 µl unlabeled Mse I primers, and 6.1 µl of buffer containing 0.3 µl Taq DNA polymerase. PCR reactions were carried out in 0.2 ml natural PCR reaction tube strips (Fisher Scientific) in a Genius Thermal Cycler (Techne, Jepson Bolton's International). The cycle conditions were modified from Vos et al. (15). The first thermal cycle was: 94C for 30 s, 65C for 30 s, and 72C for 1 min. For the 12 following cycles, the annealing temperature was lowered by 0.7C following each cycle. The denature temperature was kept at 94C for 30 s, and the extension temperature was 72C for 1 min. The final 30 cycles were: 94C for 30 s, 56C for 30 s, and 72C for 1 min, and the final extension temperature was 72C for 10 min.

PCR products obtained from selected reactions were combined with 5 µl stopping solution (LI-COR) and heated to 94C for 3 min. A 1 µl aliquot of each sample and a 0.5 µl standard solution were loaded into each polyacrylamide gel wells (25-cm in length and 0.25-mm in thickness) prepared using 6.5% GB Plus Gel Matrix (LI-COR). Because AFLP profiles can be affected by running different products on different gels (7), all AFLP products from the same primer combinations with different plant materials were run at the same time on the same gel. The DNA molecular standard was LI-COR broad range (50–700 bps). It was loaded on each side of the AFLP products of the same primer combination.

Electrophoresis analyses were carried out on a LI-COR IR^2 DNA Sequencer Model 4200L-1. The buffer was 1×TBE and the running parameters were: 1500 V, 27 mA, 40 W, at 50C.

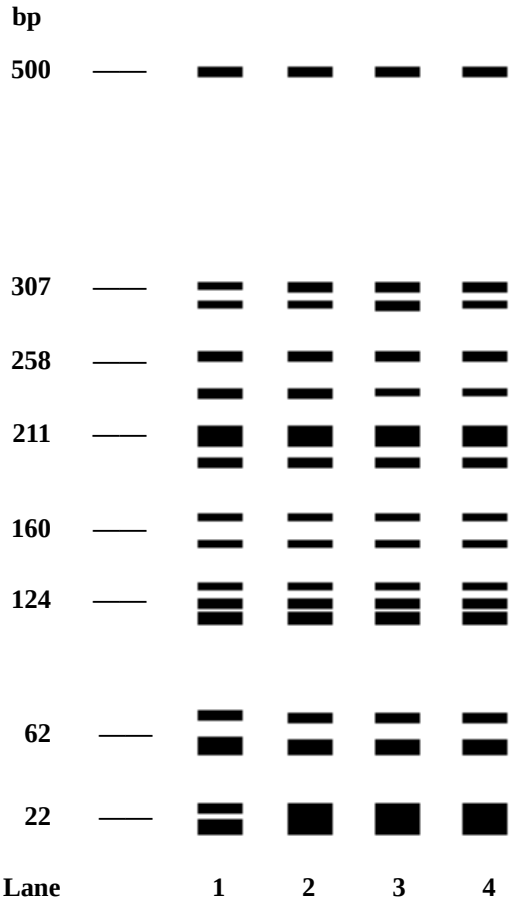

Fig. 1. The banding pattern of the same AFLP product running in different lanes of the same gel. The picture shows the composite gel developed using the Gene ImagIR Database Manager software (Version 4.02, Scanalistics).

Image data (16-bits) were automatically collected and recorded during electrophoresis. AFLP fragments were scored, analyzed and converted into numerical data using the Gene ImagIR software (Version 4.02, Scanalistics). The band detection parameter was set at 8% of maximum intensity. Following band filtration (>50 bp), all markers were binned with a 0.5% tolerance, and scored as dominant markers. The similarity between different accessions was measured using the similarity index (percentage of shared fragments in the total number of fragments). The genetic distances between plant selections were estimated according to Neil and Li (11) and a dendrogram was produced using UPGMA clustering (TreeCon Package, Scanlistics).

Results and Discussion

Establishment of F-AFLP procedures for pulmonarias. The first step in this experiment was to establish reliable F-AFLP procedures for *Pulmonaria* spp. Previous authors have reported that many factors can affect the reliability of this method (3). AFLP profiles are affected by for DNA preparation protocols, handling of the AFLP products and by the analysis (4, 6, 8, 14). We also found that the quality of the DNA isolated has a detrimental effect on the F-AFLP banding pattern of *Pulmonaria* spp. Because pulmonaria leaves have high sugars and phenols content, the DNA was of poor quality and yellowish in color when isolated using the Dnease

Table 1. The Diversity of F-AFLP bands of *P. longifolia* 'Bertram Anderson', *P. saccharata* 'White Wings', *P. saccharata* 'Pierre Pure Pink', and *P. longifolia* (*saccharata*) 'Roy Davidson'.

Similarity index	Bertram Anderson (1)	Roy Davidson (2)	White Wings (3)	Pierre Pure Pink (4)
1		0.145 (45/309)[z]	0.0528 (13/246)	0.0537 (16/298)
2			0.0985 (33/335)	0.0906 (35/386)
3				0.159 (44/276)

[z]Stands for the bands shared/total bands for the two genotypes.

Plant Minikit columns (Qiagen). This DNA isolation was recalcitrant in response to the EcoRI/MseI enzymatic digestion and produced incomplete digestion products. When these products were used for F-AFLP analysis only a few fragments were observed on the gel profiles. However, when the lysis buffer was replaced with the buffer from the FASTDNA Kit (No. B10101), high quality DNA was obtained. This DNA produced good smear profiles following enzyme digestion and yielded F-AFLP fragments ranging from 50 to 500 bps.

The process of gel preparation and sample loading are the two most important steps necessary for consistent F-AFLP profiles. During gel preparation, we found that any mechanical disturbances or gel residues left in loading wells would affect the relative migration of molecular markers and AFLP products within the gel. Running AFLP products on the same gel yielded the same AFLP banding patterns (Fig. 1). To compare the difference among the different cultivars, the AFLP products from the same primers were loaded on the same gel in this experiment.

AFLP markers and +3 primer screening. Sixty-four AFLP primer combinations were initially used for analysis of all pulmonaria accessions. Fifteen primer combinations (E1M2, E1M4, E1M7, E2M3, E2M4, E3M4, E3M5, E3M7, E3M8, E4M2, E5M2, E5M4, E6M4, E6M5, E7M5) resulted in good AFLP profiles with bands that could be scored. Clear band separation began with the 50 bps fragments; the sizes of amplified bands ranged from 50 to 500 bps. AFLP fragments were distributed across the entire range with major distributions between 50 and 300 bps. With few accessions, hardly any bands over 400 bps were generated.

Genetic divergence analysis. When 'Roy Davidson' was compared with *P. longifolia* 'Bertram Anderson', *P. saccharata* 'White Wings', and *P. saccharata* 'Pierre Pure Pink', not many bands were shared among the accessions. Only 44 out of 276 bands were shared between the two cultivars of *P. saccharata* (Table 1) and fewer bands were shared among other accessions. The similarity index of 'White Wing' and 'Pierre Pure Pink' was similar to that of 'Bertram Anderson' and 'Roy Davidson'. The least similarity obtained was between 'Roy Davidson' and *P. saccharata* cultivars ('White Wing' and 'Pierre Pure Pink'). *Pulmonaria longifolia* 'Bertram Anderson' had a higher similarity index with *P. saccharata* than with 'Roy Davidson' and 'Roy Davidson' had a higher similarity index with *P. saccharata* than with *P. longifolia* 'Bertram Anderson'. From these results, it appears that 'Roy Davidson' is not directly related to *P. saccharata.*

When all sets of bands were used for the estimation of genetic distances (11), the dendrogram produced using UPGMA clustering analysis indicated that *P. hybrida* 'Majeste' was the most distantly related accession (Fig. 2). This accession was followed by *P. hybrida* 'Victoria Brooch'. The most closely related accessions were (A) *P. saccharata* 'White Wings' and *P. saccharata* 'Pierre Pure Pink'. (B) *P. longifolia* 'Bertram Anderson' and *P. longifolia* 'Roy Davidson'. It appears that the origin of 'Roy Davidson' is a somatic variant of *P. longifolia* 'Bertram Anderson', or an intra-species hybrid of *P. longifolia*. 'Roy Davidson' should be classified as a cultivar of *P. longifolia*.

The AFLP bands observed from DNA analysis of the accessions tested were very diverse; this indicates that there is substantial molecular variation and genetic diversity in this genus as well as within species. We found that 'Majeste', a cultivar not belonging to leaf-spotted types, produced specific bands in its AFLP profile. This information suggest that it might be possible to map genes that code for leaf spotting since in many species it is possible to tag biological and morphological characters with AFLP markers. We also found AFLP markers that are shared by 'Roy Davidson' and 'Bertram Anderson'. Avent (2) suggested that 'Roy Davidson' inherited stress resistance from 'Bertram Anderson'. Based on this assumption, it could be possible that studies with these co-dominant markers could lead to the discovery of molecular tags for stress resistance. Since pulmonarias have a wide natural mutant gene pool, genotyping this genus could result in the discovery of molecular markers and tags for other specific phenotypic characteristics. Once identified, these markers and tags could be used for selecting parental plant materials for breeding and for indexing their offspring.

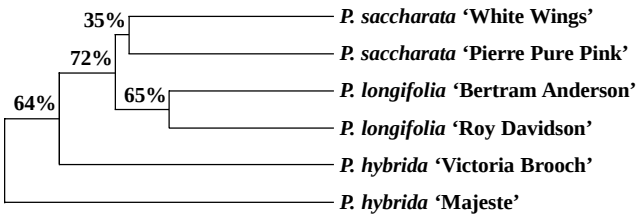

Fig. 2. UPGMA dendrogram of six genotypes of *Pulmonaria*. Bootstrap values are indicated for 100 UPGMA searches.

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