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Identification of *Itea virginica* Cultivars by Using RAPD-PCR[1]

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Abstract

Itea virginica L., Virginia sweetspire, is a flowering shrub native to the eastern United States. It has become popular recently due to its multiple seasons of interest, ease of propagation, and relative lack of significant insect or disease problems. Several cultivars of *I. virginica* varying in fall color, growth habit, and inflorescence length are now commonly seen in the landscape. These cultivars can be difficult to distinguish morphologically and can be confused in the trade. We used randomly amplified polymorphic DNA (RAPD) markers to reliably identify four of six commonly grown cultivars: 'Sarah Eve', 'Saturnalia', 'Henry's Garnet', and 'Longspire'. The technique was also useful for identifying unknown or apparently mis-labeled cultivars. However, RAPD markers were not sensitive enough to separate the well-known *I. virginica* cultivars 'Sprinch' (Little Henry™) and 'Merlot'.

Index words: RAPD (randomly amplified polymorphic DNA) markers, cultivar identification.

Species used in this study: Virginia sweetspire (*Itea virginica* L.).

Chemicals used in this study: Tris(hydroxymethyl)aminomethane (Tris), borate (boric acid), ethylenediaminetetraacetic acid (EDTA).

Significance to the Nursery Industry

Morphological identification of Virginia sweetspire cultivars (*Itea* spp.) can be difficult, especially on newly propagated plants. Thus, mix-ups can occur in labeling so that the incorrect plant is shipped. This can be problematic for the customer desiring and purchasing a compact cultivar but not receiving the correct one due to misidentification. Randomly amplified polymorphic DNA (RAPD) markers were used to identify four ('Sarah Eve', 'Saturnalia', 'Henry's Garnet', and 'Longspire') of six commonly grown Virginia sweetspire cultivars. This technique was not able to reliably distinguish two compact-growing *Itea* cultivars 'Sprinch' (Little Henry™) and 'Merlot'.

Introduction

Itea virginica L. (Virginia sweetspire) is a woody landscape shrub that has recently gained much popularity in the landscape. Several cultivars of *Itea* have been selected for fall leaf color and plant habit (2). Distinguishing some of these cultivars by using morphological traits is extremely difficult, and confusion exists in the nursery trade (Dr. Michael A. Dirr, University of Georgia, personal communication).

RAPD (randomly amplified polymorphic DNA) markers have been successfully used to distinguish genotypes within several plant species (3, 4, 5, 6, 9, 13). The goals of our research effort were to a) find primers that yield polymorphic banding patterns for different *Itea* genotypes; b) determine if the polymorphic banding patterns produced for a given genotype are consistent enough to identify a specific genotype reliably when it is included within a group of coded unknowns; c) use the consistently banding polymorphism-yielding primers to determine if a 'Saturnalia' genotype from a given nursery was the 'Saturnalia' genotype from the original introduction. Because of difficulty in distinguishing 'Sprinch' and 'Merlot' with the initial primers we used, we added the goal of determining if any of the primers available to us could produce RAPD markers distinguishing 'Sprinch' and 'Merlot'.

Materials and Methods

Plant material. Cultivars of *I. virginica* were purchased from the following sources: 'Merlot', Forestfarm, Williams, OR; 'Sprinch', Woodlanders, Aiken, SC; 'Longspire', Heronswood Nursery, Kingston, WA; 'Sarah Eve', Arborvillage, Holt, MO; 'Saturnalia', Ridgecrest Nursery, Wynne, AR; 'Henry's Garnet', Greenleaf Nursery, Park Hill, OK. Four coded samples of *I. virginica* cultivars were obtained as unrooted cuttings from Longwood Gardens, Kennett Square, PA. Additional plant material of both 'Merlot' and 'Sprinch' was obtained from Griffith Propagation Nursery in Watkinsville, GA.

DNA extraction and quantitation. Actively growing shoot tips and developing inflorescences were collected from *I. virginica* plants growing in the field during the early spring and summer. One-hundred-twenty mg (fresh weight) of shoot tip or inflorescence tissue were ground to a fine powder in liquid nitrogen with a mortar and pestle, and then the DNA was extracted (according to the manufacturer's protocols) from the tissue using a Qiagen DNeasy™ Plant Mini Kit (Valencia, CA). After extraction, the DNA was re-precipitated using ammonium acetate (one-half of the original extraction volume) and 100% ethanol (three times the extraction volume plus the ammonium acetate). The sample DNA pellet was later washed with 70% ethanol and resuspended in 50 µL of 1X Tris-EDTA (TE) buffer (composed of 10 mM Tris-HCL, pH 7.4, 1 mM EDTA).

A Hoechst dye from the Bio-Rad Fluorescent DNA Quantitation Kit (Hercules, CA) was used to stain the DNA found in a 5-µL sample of the template stock solution, and the DNA was then quantified using a Bio-Rad Versafluor™

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fluorometer. Using the same extraction procedure, DNA collected from shoot tips generally ranged from 50 to 1000 ng/µL, while DNA collected from the developing inflorescences generally ranged from 300 to 2500 ng/µL.

PCR reaction mixture composition. The PCR reaction mixtures were comprised of reagents from the PCR Core System II kit from Promega (Madison, WI). The reaction mixture consisted of Promega's 1X thermophilic DNA polymerase reaction buffer (500 mM KCL, 100 mM Tris-HCL at pH 9.0, and 1.0% Triton® X-100), 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM primer from Operon (Alameda, CA), 1.25 ng/µL of *Itea* template DNA, and 1.5 U per reaction volume of *Taq* DNA polymerase in a total reaction volume of 50 µL. Components were mixed in 0.5 mL thin-walled microcentrifuge tubes. A positive control supplied with the Promega PCR Core System II kit was included in all experiments.

To maximize banding consistency over time, PCR reaction mixtures were assembled in an ice bath as master mixes in which all components aside from the experimental primers and *Taq* DNA polymerase were mixed together and then dispensed, at the appropriate volume, into the 0.5 mL thin-walled microfuge tubes. Primers and *Taq* DNA polymerase were added to each sample immediately prior to placing the microfuge tubes in the thermocycler.

PCR reaction conditions. The PCR reactions were carried out in a Hybaid (Teddington, Middlesex, United Kindom) PCR Sprint thermocycler programmed to cycle through the temperature regime found in Table 1 (6). PCR reaction conditions and the PCR temperature regime were largely adopted from Levi et al. (6) who optimized a PCR protocol for woody plant species that utilized stringent conditions for annealing the primer oligonucleotides to the template DNA, yet maintained a high degree of amplification. Levi et al. (6) believed that this protocol resulted in less amplification products overall but ultimately improved band pattern repeatability over time while maintaining fragment amplification at levels detectable by gel electrophoresis.

Electrophoresis and visualization of PCR products. After the PCR reaction, 15 µL of each sample was transferred to 0.5 mL microcentrifuge tubes and mixed with 3 uL of loading buffer (bromophenol blue and orange G added to 25% Ficoll™). Then, 15 µL of the loading buffer-PCR product mixture was loaded into wells on a 1.25% agarose gel immersed in 1X Tris-borate-EDTA (TBE) running buffer (composed of 100 mM Tris base, pH 8.4, 90 mM Borate, and 1 mM $Na_2EDTA{\bullet}2H_2O$). The 1 Kb Plus DNA ladder (Life Technologies, Rockville, MD) was run alongside the samples so that the size (in base pairs) of DNA fragments could be estimated. The gel was run in a Bio-Rad (Bio-Rad, Hercules, CA) wide mini-sub cell horizontal electrophoresis system under 130 volts for ~3 hr. Following electrophoresis, the gels were stained with ethidium bromide for 30 minutes (ethidium bromide was not incorporated into the gel or running buffer) and digitally photographed using an Alpha Innotech ChemiImage gel documentation system (Alpha Innotech Corporation, San Leandro, CA).

Table 1. Thermocycler regime.

	Temperature (C)	Time	Cycles
Initial denaturation	94	4 min	
Initial annealing	48	70 sec	1
Initial extension	72	120 sec	
Denaturation	94	45 sec	
Annealing	48	70 sec	45
Extension	72	120 sec	
Final extension	72	5 min	1
Soak	4	Indefinite	1

Comparison of Itea cultivars and identification of unknowns using RAPD banding patterns. Two primers, OPA-2 and OPA-13, were used to generate banding patterns for 'Henry's Garnet', 'Sprinch', 'Merlot', 'Sarah Eve', 'Longspire', 'Saturnalia' from Larry Lowman (Ridgecrest Nursery, Wynne, AR), and 'Saturnalia' from another supplier. The PCR reaction was repeated once with both primers to determine pattern reproducibility for each taxon. Then, OPA-2 and OPA-13 were used to generate RAPD banding patterns for four numbered unknown tissue samples (19970942, 19971419, 19980411, and 19980683) sent to us from Longwood Gardens, Kennett Square, PA. The PCR products for all *Itea* taxa were assessed side-by-side via gel electrophoresis.

Bands were scored for presence or absence across all the taxa, and the resulting binary matrix was used to generate a symmetric matrix of distances between all pair combinations based on the Dice (1)/Nei and Li (8) similarity coefficient. A dendrogram was then generated approximating the relationships among the seven known taxa and the four unknown taxa derived from the genetic distance matrix data by using the sequential, agglomerative, hierarchical, and nested (SAHN) clustering module in NTSYSpc 2.1 to perform the unweighted pair group method (UPGMA) algorithm. To assess the goodness of fit of the cluster analysis to the data set, a symmetrical matrix of cophenetic (ultrametric) values was generated using the COPH (Cophenetic) module in NTSYSpc 2.1. This matrix was compared to the original similarity matrix using the MXCOMP (matrix comparison) module in NTSYSpc 2.1. The MXCOMP module utilizes the Mantel (7) test for matrix correspondence to measure the degree of relationship between the two matrices, expressed (for our purposes) as the cophenetic correlation (the ordinary product-moment correlation coefficient), r (11, 12).

Polymorphic primer identification and 'Saturnalia' comparison. A 'Saturnalia' plant obtained from Larry Lowman (Wynne, AR), who originally released the 'Saturnalia' cultivar, was compared using RAPD markers to a 'Saturnalia' plant obtained from another nursery. PCR reactions using all the primers in the Operon RAPD™ decamer primer kits A, B, C, D, and E (each kit consists of 20 randomly generated primers) were performed with template DNA extracted and purified as described from the two sample plants. PCR fragments generated from each test primer for the two sample plants were evaluated side-by-side via gel electrophoresis. Primers that yielded polymorphic banding patterns for the two taxa were used in two more reactions to confirm that the banding patterns were repeatable. PCR fragment bands that occurred in all three reactions with a specific primer were scored for band presence or absence (bands were not evalu-

Table 2. List of polymorphism generating primers for *I. virginica* cultivars.

Polymorphism generating primers	Nucleotide sequence (5' to 3')
OPA-2	TGCCGAGCTG
OPA-13	CAGCACCCAC
OPB-7	GGTGACGCAG
OPC-2	GTGAGGCGTC
OPC-5	GATGACCGCC
OPC-8	TGGACCGGTG
OPC-9	CTCACCGTCC
OPD-2	GGACCCAACC
OPD-8	GTGTGCCCCA

ated for intensity) across the two genotypes. Nine primers yielded consistent polymorphisms between the two taxa out of the 100 primers attempted. The reactions were repeated twice more with polymorphism-generating primers (Table 2), and bands that appeared in all three efforts were scored for presence or absence at each locus (Fig. 1). Loci were assigned a number based on decreasing molecular weight for each primer (OPA-2_1, OPA-2_2, OPA-2_3, etc.). Scored bands (present equals 1 and absent equals 0) were used to generate the Dice (1)/Nei and Li (8) pairwise similarity coefficient (9).

Attempt to distinguish between 'Sprinch' and 'Merlot'. 'Sprinch' and 'Merlot' were indistinguishable using primers OPA-2 and OPA-13. These two cultivars were tested with all the primers in the Operon RAPD™ decamer kits A, B, C, D, and E that produced banding patterns for 'Saturnalia' to find primers that could distinguish the two cultivars. Banding data were processed as previously described for the data produced from the 'Saturnalia' comparison.

Results and Discussion

Comparison of Itea *cultivars and identification of unknowns by using RAPD banding patterns.* OPA-2 and OPA-13 produced 23 loci that generated unique banding patterns for all the *Itea* taxa in this study except for 'Sprinch' and 'Merlot', which could not be distinguished from each other with these two primers. The banding patterns produced by OPA-2 and OPA-13 for the coded unknowns were compared to the banding patterns produced by 'Henry's Garnet', 'Sprinch', 'Merlot', 'Sarah Eve', 'Longspire', 'Saturnalia' (from Larry Lowman), and 'Saturnalia' (from another supplier). Based on the comparison of the banding patterns, we concluded that 19970942 was 'Henry's Garnet', 19971419 was 'Saturnalia' (from Larry Lowman), and 19980411 was 'Longspire' (Fig 2). The banding patterns for the 'Saturnalia' from the alternate supplier were indistinguishable from the banding patterns for 'Longspire' and 19980411, so we concluded that this cultivar was actually 'Longspire' rather than 'Saturnalia' (Fig. 2).

The banding pattern for 19980683 was indistinguishable from 'Sprinch' and 'Merlot', so we believed that it was one of these two. However, we could conclude nothing until we identified two primers that seemed to distinguish between the two cultivars. Based on these primers (discussed more in the section on 'Sprinch' and 'Merlot'), we concluded that 19980683 was 'Merlot'. We reported our findings to Longwood Gardens, and their records corroborated our conclusions (Dr. Tomasz Anisko, personal communication).

The dendrogram derived from our data summarizes the relationships between the *Itea* cultivars in this study (Fig. 2). The cophenetic correlation of 0.8735 suggests that the cluster analysis correctly described the trends within the data (12). The most divergent groups of *Itea* taxa were still 90% similar, but this degree of similarity was to be expected since all the evaluated taxa are within the same species and, conceivably, some taxa may share common pedigrees. While we believe that the dendrogram correctly describes our data and suggests possible relationships among the *Itea* cultivars, we concede that any relationships implied as a result of this study are of a preliminary nature due to the low number of polymorphic primers (with a correspondingly low number of loci) used. Clearly, the determination and use of more polymorphic primers will add credence to the perceived relationships

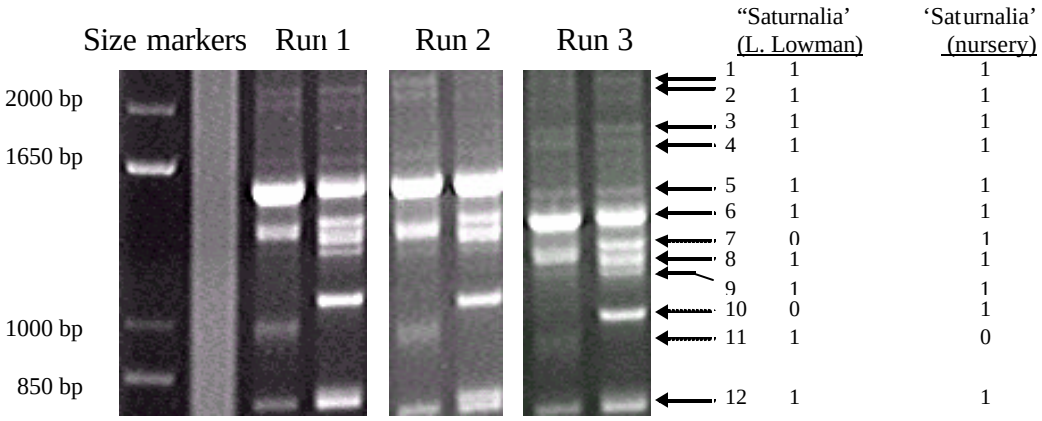

Fig. 1. An example of the RAPD banding patterns generated from PCR with Operon primer A2 (OPA-2) for the 'Saturnalia' genotype from Larry Lowman, Ridgecrest Nursery, (left lane) and 'Saturnalia' from another nursery (right lane). Arrows indicate loci (dashed arrows indicate polymorphic loci). Only bands clearly discernible in all three PCR runs are scored for presence or absence of bands. Present bands are denoted as '1', and bands present in one genotype but not in the other are denoted as '0' for the genotype in which they are absent. Molecular size marker fragments are from the 1 Kb Plus DNA ladder (Life Technologies, Rockville, MD).

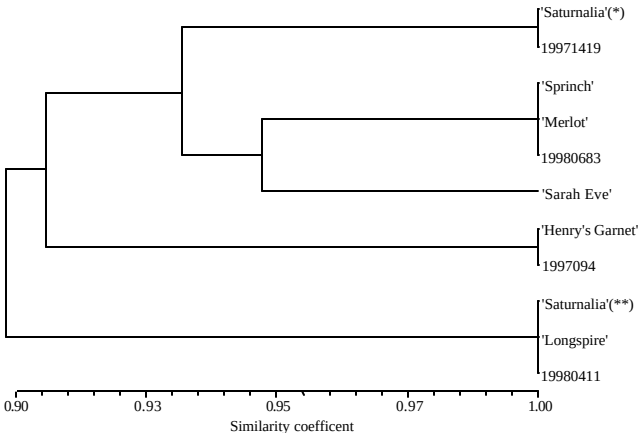

Fig. 2. Dendrogram of UPGMA cluster analysis for 11 *Itea* taxa. 'Saturnalia' (*) is from Larry Lowman, Ridgecrest Nursery, and 'Saturnalia' (**) is a plant received from another nursery. 19970942, 19971419, 19980411, and 19980683 are coded unknown *Itea* taxa provided by Longwood Gardens. The cophenetic correlation (ordinary product-moment correlation coefficient) r = 0.8735.

between these *Itea* cultivars. Even so, we were able to distinguish four of the six cultivars and accurately identify four coded unknown taxa and one misidentified taxon by using only two primers.

Polymorphic primer identification and 'Saturnalia' comparison. Nine primers, yielding a total of 68 loci, were identified that produced consistent banding patterns with some loci being polymorphic between the two 'Saturnalia' taxa. The other 91 primers were excluded from analysis because of one or more of the following: less than three loci were detected, all bands amplified with a primer were monomorphic for the two taxa, or the majority of bands were not detected again when the PCR reaction was repeated. Also, bands generated by polymorphism-generating primers that were not seen each time that the PCR reaction was repeated were excluded from analysis. The Dice (1) coefficient for the two taxa was 0.81, and we concluded that although the two taxa were very similar, they were distinct taxa. Accordingly, we believe that the alternate supplier had provided an *Itea* genotype erroneously identified as 'Saturnalia'.

Attempt to distinguish between 'Sprinch' and 'Merlot'. The primer set that produced consistent banding for 'Sprinch' and 'Merlot' for differences was the same as the one used for the 'Saturnalia' comparison except that OPB-5 (5'-TGCGCCCTTC-3') was included in the analysis for 'Sprinch' and 'Merlot', but was not included in the 'Saturnalia' comparison due to inconsistent banding. Also, OPC-8 was excluded from the 'Sprinch' and 'Merlot' analysis because of an excessive number of inconsistent bands. All primers that exhibited polymorphisms for the 'Saturnalia' taxa failed to show polymorphisms for 'Sprinch' and 'Merlot' except for OPC-5. OPB-5, interestingly, seemed to generate two polymorphic bands, and OPC-5 generated one polymorphic band. These two primers were inconsistent from one PCR reaction to the next, but since they were the only primers that seemed to show any differences, a total of six PCR reactions was carried out to determine if the polymorphic bands were reproducible. Out of the six attempts, three showed no banding while three showed banding with the polymorphic bands. Since the polymorphisms were seen in three PCR amplification efforts, OPB-5 and OPC-5 were included in the analysis. The Dice (1) similarity coefficient for 'Sprinch' and 'Merlot' was 0.98 with 70 loci.

We rejected the implied null hypothesis that 'Sprinch' and 'Merlot' were the same and failed to reject the alternate hypothesis that the taxa were distinct, but we are uncertain if this distinction is useful to the nursery industry for two reasons. First, we are uncertain if the perceived differences are real. The PCR products used in RAPD analysis are extremely sensitive to slight variations in thermocycler conditions, template DNA, and *Taq* DNA polymerase resulting in banding pattern variations from reaction to reaction. The primers that seemed to produce polymorphisms for 'Sprinch' and 'Merlot' appear to be especially susceptible to this type of variation (10). Conceivably, the perceived polymorphisms for these two taxa may be nothing more than artifacts that cannot be reproduced in other laboratories. We are more confident about the differences in the other *Itea* taxa because many more than three polymorphisms were observed. Second, if the polymorphisms are real and indicate genotypic differences, they may not be associated with genes that are expressed phenotypically. The two genotypes may, for all practical intents and purposes, be indistinguishable in appearance.

During the course of this study, primers were identified that yielded consistent banding patterns for *Itea*. Nine primers were found to be polymorphic for the 'Saturnalia' from the original release and the mislabeled 'Saturnalia' (which we now believe to be 'Longspire') from an alternate supplier. The Dice (1) similarity coefficient for the two 'Saturnalia' taxa was originally calculated to be 0.81. When the two 'Saturnalia' taxa were included in the statistical analysis with the other *Itea* taxa using only two primers, the similarity coefficient between the two was 0.90. We believe this anomaly is a result of only using OPA-2 and OPA-13 for the comparison of six *Itea* taxa and that using the other polymorphic primers to evaluate the six *Itea* taxa will probably result in the similarity coefficients for all between taxa comparisons being lowered slightly. Our intention was simply to show that RAPD-PCR can consistently exhibit differences between the *Itea* cultivars and not necessarily to establish a hypothesis on the phylogenetic relationships between the taxa.

We have also successfully identified coded unknown *Itea* genotypes, illustrating that RAPD-PCR can potentially be used to sort out mislabeled cultivars in the nursery industry. We were not able to distinguish between 'Sprinch' and 'Merlot'; although, we suspect, based on what seems to be two polymorphic loci generated by OPB-5 and on one polymorphic locus generated by OPC-5, the two may be distinct cultivars. Attempting to optimize PCR reaction components and the thermocycler regime for more consistent amplification of the OPB-5 fragments, or testing 'Sprinch' and 'Merlot' with another 100 random primers, might identify a more consistent polymorphism-generating primer. However, a more profitable approach might be to attempt more sensitive and reliable techniques such as amplified restriction fragment polymorphism (AFLP), DNA amplification fingerprinting (DAF), arbitrary signatures from amplification profiles (ASAP), or sequence characterized amplified region (SCAR) DNA (10, 14).

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