HRI's Mission:

To direct, fund, promote and communicate horticultural research, which increases the quality and value of ornamental plants, improves the productivity and profitability of the nursery and landscape industry, and protects and enhances the environment.

Armstrong (12% by high rate both years and 9% by low rate 1987), Blue Pacific (16.7% by high rate in 1991), Hetz Blue (23.1% by high rate in 1987), and Grey Owl (27.1% by high rate in 1987 and 10.9 and 17.4% by the low and high rates respectively in 1991) junipers indicating that visual ratings may not be the best measure of Basagran's effect on these species.

Sasanqua Camellia, Burford Holly, Rotunda Holly, Yaupon Holly, Hetz Blue Chinese Juniper, Blue Pacific Juniper and Japanese Spurge were not visually injured by either rate any year. However, growth was reduced with the high rate for Rotunda Holly and Blue Pacific Juniper one of the two years. It appears that growth can be slowed by Basagran applications even though injury may not be obvious for some species. This growth reduction may be more critical in container production of landscape plants than when the plants are in the landscape. Variable results were obtained for the remaining species in the two studies.

Several species in this study were tolerant to topical applications of Basagran without damage or only temporary injury symptoms. Basagran is considered a contact herbicide with little translocation in plants. Therefore, when applied as a directed spray Basagran may cause minimal injury for most of the species evaluated. *Cotoneaster dammeri*, *Juniperus conferta* 'Blue Pacific', *Nandina domestica*, and *Rhododendron satsuki* 'Amagasa' are registered for applications of Basagran T/O as a directed spray. However, caution should be used when directed applications are made to these very sensitive plants or in areas where visible damage can not be tolerated.

(*Ed. note*: This paper reports the results of research only and does not imply registration of a pesticide under amended FIFRA. Before using any of the products mentioned in this research paper, be certain of their registration by appropriate state and/or federal authorities).

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Use of Single-Primer DNA Amplifications for the Identification of Red Maple (*Acer rubrum* L.) Cultivars[1]

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Abstract

Positive cultivar identification is often difficult or impossible based solely on morphological traits. A technique ensuring reliable, repeatable, and unique cultivar identification is needed. The use of molecular markers offers such a technique, allowing assessment of fine levels of variation directly at the DNA level. In this study, RAPD (Random Amplified Polymorphic DNA) markers were investigated for their utility to identify red maple cultivars. Three out of nineteen primers tested resulted in unique banding patterns for all the maples tested, including 9 red maple clones, 5 silver maple seedlings, and 4 purported interspecific cultivars. The red maple cultivars 'Red Sunset' and 'October Glory', which are almost indistinguishable morphologically as young trees, were clearly distinguished using RAPD markers. RAPD markers provide a consistently reliable technique for red maple cultivar identification.

Index words: RAPD (Random Amplified Polymorphic DNA) markers, cultivar identification, patent, trademark, and royalty protection.

Species used in this study: Red maple (*Acer rubrum* L.); silver maple (*Acer saccharinum* L.); *Acer* × *freemanii*.

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Significance to the Nursery Industry

Patent protection rights can only be enforced if the patented plant is uniquely identifiable. Traditionally, morphological traits have been relied upon for cultivar identification. However, characteristic morphological traits are often altered by environmental conditions or stage of plant development. Recent technology now permits the assessment of differences at the DNA level. Since DNA is unaffected by either environmental conditions or stage of plant development, molecular markers offer a potentially powerful and reliable tool for the identification of cultivars. In this study, one type of molecular marker (RAPD or Random Amplified Polymorphic DNA marker) was used to uniquely identify 9 red maple clones, 5 silver maple seedlings, and 4 purported interspecific hybrid cultivars.

Introduction

Red maple (*Acer rubrum* L.) is indigenous to North America with a wide native range extending from Newfoundland to Florida and west to Minnesota, Iowa, Oklahoma, and Texas (1). Red maple is the number one shade tree produced in the U.S. both in terms of numbers propagated and revenue generated (J. Frank Schmidt, Schmidt Nursery, Boring, Oregon. 1991 pers. comm.). The popularity of red maple is due to its great beauty (especially its brilliant fall coloration), wide range of adaptability, and general ease of culture. Due to the extensive native range and concomitant phenotypic and physiological variability oiver 35 cultivars (10) have been selected for traits such as cold hardiness, branching habit, leaf size and shape, growth rate, and fall color. Red maple and silver maple (*Acer saccharinum* L.), a closely allied species, hybridize in nature and several of the "red" maple cultivars are purportedly interspecific hybrids (*Acer* × *freemanii*). Although presently all commercially available red maple cultivars are simply selections from either the wild or cultivated situations, Dr. Alden Townsend of the U.S. National Arboretum, Glenn Dale, Maryland is currently evaluating several new selections which are the result of controlled crosses.

Although several red maple cultivars are patented and/or trademarked, thousands of dollars in royalty fees are lost to patent holders due to "pirating" of cultivars by unlicensed growers. A basic stumbling block to the enforcement of plant variety protection laws is the difficulty of positive identification of specific cultivars. Traditionally, morphological features have been used almost exclusively for cultivar identification. However, morphological traits are often greatly affected by environmental conditions and stage of plant development and thus may be unreliable for positive cultivar identification. Several red maple cultivars, specifically 'Red Sunset' and October Glory', are virtually indistinguishable morphologically as young, nursery-grown trees. In cases where identity is difficult to establish based solely on morphological characters, the utility of isozymes and more recently, DNA markers is being investigated for cultivar identification. Tobolski and Kemery (12) recently investigated the use of isozymes for the identification of red maple cultivars. Seven of the 14 enzyme systems tested produced well-resolved and polymorphic banding patterns for the cultivars sampled. Employing these seven enzymes, singly or in combination, Tobolski and Kemery were able to distinguish 15 out of 18 red maple cultivars. However, three cultivars, 'Armstrong', 'Red Sunset', and 'October Glory' could not be distinguished from one another using these enzyme systems.

One of the distinct advantages offered by the use of DNA markers is the ability to assess variation directly at the DNA level, rather than at the level of expression as with either morphological traits or isozyme banding patterns. One type of genetic marker, restriction fragment length polymorphisms or RFLPs, has proven useful for cultivar identification in a wide range of taxa, including: rice (6), blackberry and raspberry (9), apple (10), avocado (8), dogwood (5), rose (7, 13), gerbera (13), and carnation (13, 15). Recently, a DNA polymorphism assay has been developed based on the amplification of segments of genomic DNA using single primers of arbitrary sequence. The primers employed for RAPD marker analysis consist simply of an arbitrarily chosen sequence of 10 nucleotides. In the PCR process the primers anneal to and amplify sites in the genome defined solely by the primer used. The segments of genomic DNA amplified using primers of arbitrary sequence are visualized as discrete bands known as Random Amplified Polymorphic DNA or RAPD markers (14). We chose to test RAPD markers for red maple cultivar identification because for our purposes the technique offers several advantages over other DNA fingerprinting techniques. These include, elimination of the necessity for either prior knowledge of the target DNA sequence or cloned and characterized probes and avoidance of the use of radioactive material, making the use of RAPD markers less laborious and time-consuming than other DNA fingerprint techniques. RAPD markers also offer the distinct advantage for cultivar identification of detecting finer levels of variation than RFLPs (14). RAPD markers have been successfully employed to identify cultivars of dogwood (*Cornus florida* L.), cultivars of several turfgrass species including, *Zoysia japonica* Steud., *Eremochloa ophiuroides* (Munro) Hack., *Cynodon dactylon* (L.) Pers., and *Buchloe dactyloides* (Nutt.) Engelm. (2), and cultivars of soybean (3).

The specific objectives of this study were to test the efficacy of RAPD markers to: (1) identify red maple cultivars, (2) verify hybridity of cultivars of known parentage, (3) verify or refute purported interspecific (red × silver) hybridity of some "red" maple cultivars, and (4) evaluate differences in banding patterns between red maple cultivars and a limited number of silver maple samples.

Materials and Methods

Plant material of the *Acer rubrum* and *Acer* × *freemanii* cultivars was generously donated by A. McGill and Son Nursery in Fairview, Oregon and Schmidt Nursery in Boring, Oregon. *Acer rubrum* 'Tim' was donated by Trailridge Nursery in Dehgan, Florida. Red maple accessions NA 59906 ('Red Sunset' × 'Autumn Flame') and NA 61016 ('October Glory' × 'Autumn Flame') were kindly contributed by Dr. Alden Townsend of the U.S. National Arboretum, Glenn Dale, Maryland. Silver maple samples were collected from seedling alley trees in Evanston, Illinois (Table 1).Genomic DNA was extracted from young, newly expanded leaves of a single plant by the CTAB technique of Saghai-Maroof et al. (4). Nineteen primers, each 10 nucleotides in length, were used singly to amplify segments of genomic DNA from nine red maple clones, four purported interspecific (*Acer* × *freemanii*) cultivars, and five silver maple seed-

Table 1. Plant identity and species.

Identity	Species
'Autumn Flame'	*A. rubrum*
'Karpick'	*A. rubrum*
NA 59906	*A. rubrum*
NA 61016	*A. rubrum*
'Northwood'	*A. rubrum*
'October Glory'	*A. rubrum*
'Redskin'	*A. rubrum*
'Red Sunset'	*A. rubrum*
'Tim'	*A. rubrum*
'Armstrong'	*A.* × *freemanii*
'Autumn Blaze'	*A.* × *freemanii*
'Indian Summer'	*A.* × *freemanii*
'Scarlet Sentinel'	*A.* × *freemanii*
Silver #1	*A. saccharinum*
Silver #2	*A. saccharinum*
Silver #3	*A. saccharinum*
Silver #4	*A. saccharinum*
Silver #5	*A. saccharinum*

lings. The primers used were originally developed by researchers at the duPont Company in Wilmington, DE, and subsequently manufactured by the Department of Chemistry, University of Georgia. The nucleotide sequence of each primer was chosen arbitrarily while meeting the imposed constraints of 50–80% G + C content and containing no palindromic sequences: APa:5'-TGGTCACTGA-3'; APb:5'-TCGTCACTGA-3'; APc:5'-TGCTCACTGA-3'; APd:5'-TGGACACTGA-3'; APf:5'-TGGTCTCTGA-3'; APg:5'-TGGTCAGTGA-3'; APh:5'-TGGTCACAGA-3'; APi:5'-TGGTCACTCA-3'; APj:5'-TGGTCACTGT-3'; C7:5'-GTCCCGACGA-3'; C13:5'-AAGCCTCGTC-3'; 101:5'-GCGGCTGGAG-3'; 123:5'-GTCTTTCAGG-3'; 124:5'-ACTCGAAGTC-3'; 125:5'-GCGGTTGAGG-3'; 126:5'-CTTTCGTGCT-3'; 127:5'-ATCTGGCAGC-3'; 128:5'-GCATATTCCG-3'; 129:5'-GCGGTATAGT-3'.

Reaction mixtures for PCR (25μl) contained 10 mM Tris-Cl pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 0.001% gelatin, 100 μM each of dATP, dCTP, dGTP, and dTTP, 0.2 μM primer, 15 ng genomic DNA, and 1 unit of *Thermus aquaticus* (*Taq*) DNA polymerase. The amplifications were carried out in a Cetus/Perkin-Elmer thermal cycler using the following conditions: 92°C (198°F) for 1 minute (denaturing), 35°C (95°F) for 1 minute (annealing), 72°C (162°F) for 2 minutes (extension), for 40 cycles. Amplification products were analyzed by electrophoresis in 2.0% agarose gels, followed by staining with ethidium bromide and viewing under UV radiation. Bands were scored as present (+) or absent (−) and band-sharing analysis was conducted using the PAUP (Phylogenetic Analysis Using Parsimony) computer program to generate a dendrogram depicting relationships among the maple cultivars and accessions. A heuristic search using the branch-swapping/tree bisection option was used to generate the dendrogram.

Results and Discussion

The use of agarose gels and ethidium bromide staining methodologies produced clear resolution of both major and minor bands with consistent reproducibility of banding patterns. Seven of the primers employed resulted in either poorly resolved or monomorphic banding patterns and were discarded. The other twelve primers (APa, APb, APd, APg, APh, APi, APj, C13, 123, 124, 127, 129) resulted in well-resolved and polymorphic banding patterns.

For each primer evaluated, one to thirty DNA segments were amplified from a given sample of genomic DNA. Repeated amplifications of the same DNA samples gave good reproducibility and DNA samples of the same cultivar from different sources resulted in identical banding patterns.

Abundant polymorphism was detected among the red maple clones, the purported interspecific hybrid cultivars, and the silver maple samples using RAPD markers. All thirteen red and interspecific maple clones were readily distinguished. Several of the primers including, APb, APh, and C13 produced unique banding patterns for all thirteen cultivars and the five silver maple seedlings (Figure 1). Thus, it was not necessary to combine banding patterns produced using different primers to separate the cultivars. The banding patterns were scored and used to generate the PAUP dendrogram (Figure 2).

The hybridity of NA 59906 ('Red Sunset' × 'Autumn Flame') and NA 61016 ('October Glory' × 'Autumn Flame') was verified using RAPD markers. The dendrogram depicts both NA 59906 and NA 61016 placed between their respective parent cultivars (Figure 2). Generally the banding patterns for NA 59906 and NA 61016 are a combination of the banding patterns of both parents. However, some few bands are unique to the hybrids. These bands represent additional sequences recognized by the primer due to recombination in the hybrids. However, the PAUP program was not designed to be used for fingerprinting or clustering analysis. In addition, hybridization has undocumented effects on PAUP results. In our study the PAUP program generated twelve trees with only minor rearrangements. In all twelve trees the hybrids NA 59906 and NA 61016 always placed between their respective parents, indicating that PAUP may be useful for hybrid documentation. We encourage other researchers to investigate PAUP's utility for hybrid analysis.

After the initial branching of the dendrogram depicting the placement of the purported interspecific cultivar 'Scarlet Sentinel', the dendrogram splits into two main clusters, one containing all the silver maples plus two purported interspecific cultivars and the other cluster containing all the red maple cultivars and one purported interspecific cultivar (Figure 2).

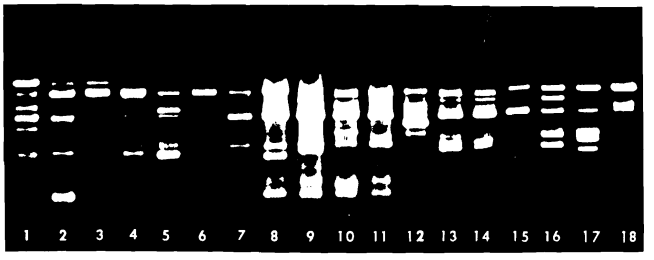

Fig. 1. Genomic DNA from 9 red maple clones, 4 interspecific (red × silver maple) cultivars, and 5 silver maple samples amplified with arbitrary primer C13:5'-AAGCCTCGTC-3'. Lane 1: 'Red Sunset'; lane 2: NA 59906; lane 3: 'Autumn Flame'; lane 4: NA 61016; lane 5: 'October Glory'; lane 6: 'Indian Summer'; lane 7: silver #4; lane 8: 'Armstrong'; lane 9: silver #1; lane 10: 'Autumn Blaze'; lane 11: silver #2; lane 12: 'Scarlet Sentinel'; lane 13: silver #5; lane 14: silver #3; lane 15: 'Redskin'; lane 16: 'Karpick'; lane 17: 'Northwood'; lane 18: 'Tim'.

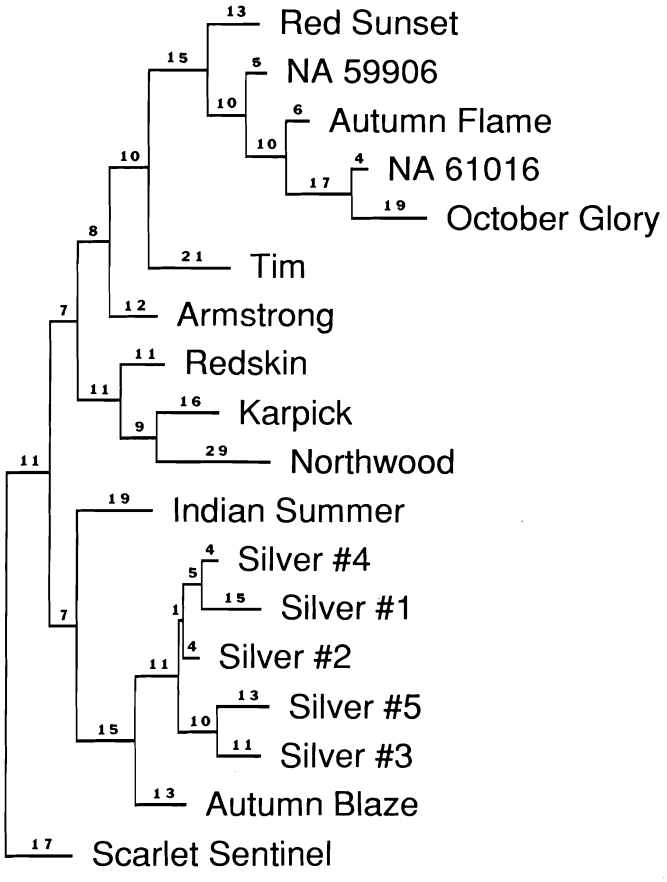

Fig. 2. Dendrogram generated using the PAUP computer program of phylogenetic anaylsis illustrating the relationships among 9 red maple cultivars, 4 interspecific (red × silver maple) cultivars, and 5 silver maple samples evaluated using single-primer amplification of genomic DNA. Relative branch lengths (numerical values) indicate relative genetic distances among taxa.

The silver maple seedlings formed a distinct and separate group from most of the red maple cultivars. However, two purported interspecific hybrid cultivars, 'Autumn Blaze' and 'Indian Summer', grouped with the silver maple cluster while the other two purported interspecific cultivars, 'Scarlet Sentinel' and 'Armstrong', did not. In fact, 'Armstrong', the interspecific hybrid morphologically most resembling silver maple, is placed in the center of the red maple cultivar cluster.

Although the silver maple seedlings did form a distinct, separate cluster from the red maple cultivar group, for the primers tested none of the bands produced were species-specific and thus could not be used to diagnose the occurrence of interspecific hybridity. Given the fact that red and silver maple interbreed readily in nature, indicating a high degree of genetic relatedness, it was not totally unexpected that their respective RAPD marker banding patterns were not entirely different. Since our study was restricted to cultivars of red maple and a very limited sample of silver maples we are unable to draw botanical conclusions about the relatedness of red and silver maple or the utility of RAPD markers in discerning interspecific hybridity of certain cultivars. Given a wider range of samples of red, silver, and suspected interspecific hybrids from the wild we believe RAPD markers would prove to be useful in phylogenetic studies.

This study indicates that RAPD markers have great utility for the identification of red maple cultivars. Whereas Tobolski and Kemery (12) were unable to separate the three cultivars 'Armstrong' (a purported interspecific hybrid), 'Red Sunset', and 'October Glory' using isozyme banding patterns, our study showed that three out of a total of nineteen primers tested produced unique banding patterns for all thirteen red maple cultivars, including 'Armstong', 'Red Sunset', and 'October Glory'. 'Red Sunset' and 'October Glory' are by far the most popular and numerous red maple cultivars propagated and sold in the U.S. and in terms of lost royalty fees the most susceptible to "pirating" by unlicensed growers. This study establishes the utility and dependability of RAPD markers for the identification of red maple cultivars regardless of growing conditions or stage of plant development. RAPD markers provide a powerful new tool for the enforcement of plant patent, trademark, and royalty protection rights.

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