GENETIC ANALYSIS OF PUTATIVELY APOMICTIC SEED FROM AMERICAN SYCAMORE

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Abstract.--While conductin g controlled pollinations of sycamore *(Platanus occidentalis L.),* it was observed that all six seed trees produced viable seed from the unpollinated flowers used as pollination controls. If the seeds proved to be of apomictic origin, exclusion of pollen would be an efficient means of cloning mature sycamore trees. We identified heterozygous loci in the five seed trees by screening for random amplified polymorphic DNA (RAPDs) markers that segregated in a 3:1 (band present:band absent) ratio in selfed progeny. Any individual seedling, or cohort, of apomictic origin should be band present for all heterozygous loci in the mother tree. We found no evidence for any of the five families of putative apomicts being of only asexual ori g in. Only five individuals out of 115 putative apomicts had the same RAPD banding patterns as the mother trees. Based on estimated gene frequencies, these five individuals are possibly of asexual origin and warrant further research. On average, these five individuals represent only 0.076% of all seeds (viable and nonviable), and only 4.3% of the viable seeds, from the unpollinated cohorts. The very low percentages of possible apomicts indicate that pollen exclusion is unlikely to be an efficient means of cloning mature sycamore trees.

Keywords: *Platanus occidentalis* L., random amplified polymorphic DNAs, RAPDs, arbitrarily primed PCR, apomixis, clone.

INTRODUCTION

The American sycamore is a common bottomland hardwood widely distributed throughout the southeastern United States, and has good potential for use in biomass production (Tuskan and De-la-Cruz 1982). Genetic improvement programs for sycamore are in progress in the southeastern United States, using methods for controlled crosses (Land 1991) and vegetative propagation (Land and Cunningham 1994). The use of vegetative propagules is of interest because they capture non-additive genetic variation in addition to

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additive genetic variation (Zobel and Talbert 1984). As Biondi and Thorpe (1982) have reviewed, techniques for vegetative propagation in forest trees (including advanced layering, rooted cuttings, grafting and budding, and tissue culture) are becoming practical reforestation methods. However, most methods of vegetative propagation are highly sensitive to the effects of maturation of the donor tree (Bonga 1982). Additionally, when compared with seed propagation, poor survival and growth of some vegetative propagules, coupled with the high cost per propagule, have offset the genetic gain advantage for many forest trees (Aimers-Halliday et al. 1991). Therefore, an efficient clonal propagation method that is able to supply large numbers of clones, is still needed.

As part of an on-going sycamore breeding program, controlled pollinations were conducted at Mississippi State University **in** the spring of 1988. It was observed that bagged flowers serving as pollination controls (i.e., no pollen was applied) averaged 2.3 percent sound seeds per bagged, globular head of pistillate flowers. These seeds could have resulted from pollen contamination, selfing, or apomixis (Bashaw 1980). If the seed proved to be of apomictic origin, the exclusion of pollen might be an efficient means for cloning mature trees.

Random amplified polymorphic DNAs (RAPDs)(Welsh and McClelland 1990, Williams et al. 1990) are a type of DNA marker based on the polymerase chain reaction (PCR). RAPDs overcome some limitations of traditional PCR (Caetano-Anolles et al. 1992) and have gained widespread acceptance and use. The RAPD technique can be used to detect genetic variation among individuals within a species (Williams et al. 1990), and has proven to be a useful genetic fingerprinting technique for parentage determination (Welsh et al. 1991), kinship relationship analysis (Hadrys et al. 1992) and pathotype identification (Goodwin and Annis, 1991). RAPDs were useful in assessing the validity of controlled crosses in hardwood species (Roy et al. 1992) and should be useful for determining clonal identity (Smith et al. 1992).

The objectives of this study were to identify RAPD markers in the seed trees used for contolled pollinations, and then to use these markers to determine the origin of the seed in the unpollinated seedlots.

MATERIALS AND METHODS

Leaf tissue was collected from the five parent trees and 15 to 30 progeny from self-pollinated and unpollinated treatments from each tree. All samples were stored at -85°C prior to use. DNA from ten grams of frozen leaf tissue from each sample was extracted by modified procedures of Murray and Thompson (1980). Two µg of DNA from each extract was further purified using the BioRad Prep-A-Gene™ DNA Purification Kit (BioRad). The RAPD reaction was performed in a modification of Williams et al. (1990) protocol consisting of the following in a 25 µl reaction volume: 2.0 ng of template DNA, 2.5 µl of 10x buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.4), 2.0 µl of $MgCl_2$ (25 mM of $MgCl_2$), 2.0 µl of dNTPs (1.25 mM of each dNTP), 1.0 µl of Operon® Primer (5 µM), 1 unit of *Taq* polymerase, and sterile distilled water. The mixture was loaded in 96 well Falcon® 3911 MicroTest III™ Flexible Assay Plate (Becton Dickinson), overlaid with 50 µl of sterile mineral oil, covered with Saran Wrap® and placed in a programmable temperature cycler.

Nelson et al. (1993). Amplified products were separated in a 1.4% agarose gel in lx TAE buffer.

Seed trees, and 15 to 30 selfed progeny from each family, were screened with primers to identify heterozygous loci in seed trees. At least ten heterozygous loci which were band present in a seed tree and segregated (both present and absent) in its selfed progeny, were identified in each family. Chi-square analysis was conducted to determine if a locus was inherited in a 3:1 (band present:band absent) ratio as expected for a dominant marker in the selfed progeny. Next, the unpollinated progeny were screened using the primers that identified heterozygous loci in the seed tree. If the putative apomicts were of somatic origin, the apomicts would be band present at all loci that are heterozygous in the seed trees. If one, or more than one, of the bands was absent in an individual, this individual was scored as not being of apomictic origin.

If an individual was band present for all identified loci, the probability that this putative apomict was of asexual origin was estimated with the following formula: for a particular locus i, the frequency of the band present allele from the female heterozygote of locus i was assumed to be 0.5. If p_i is used to represent the frequency of the band present allele of the locus in the pollen pool, then for the particular locus i, the probability that an individual resulting from pollen contamination has a particular band present is expected to be:

$$P_i = 0.5 + p_i/2.$$

Then, p_i can be estimated as:

$$p_i = 2P_i - 1.$$

Assuming that loci in each family were independent, then the probability of an individual resulting from pollen contamination (P_a) being band present for all of the loci can be estimated to be:

$$P_C = \prod_{i=1}^{n} (0.5 + P_i/2)$$

RESULTS

Ten or eleven heterozygous loci in each of the five maternal trees were identified and are listed in Table 1. A chi-square (x^2) test was performed to verify that the polymorphisms were segregating as a single genetic locus in the selfed progeny (3:1 Mendelian ratio was expected), and 15 of the 53 loci were rejected (a=0.05). In addition, inheritance of some loci in each family could not be distinguished from a 1:1 ratio. After ten or eleven heterozygous RAPD loci were identified in each seed tree, these loci were scored in the unpollinated individuals. Only five individuals, out of 115 unpollinated progeny, were band present for all identified heterozygous loci (Table 2).

Table 1. Identified heterozygous loci for each family.

Marker	Operon Primer/Fragment Size(kb.)				
	B209-08	O110-09	K110-19	H205-55	S210-19
1	A17/2.4	C8/0.72	A17/2.3	A7/0.8	A7/1.15
2	C4/0.9	X1/1.75	A17/2.0	D8/1.55	A7/0.8
3	C8/0.72	X1/1.5	A17/1.0	W2/0.9	A17/1.0
4	E2/0.95	X1/0.75	A20/1.4	W4/0.35	A17/0.75
5	W11/1.2	X18/1.15	C8/1.2	W6/0.5	X2/1.5
6	W11/0.85	Y1/0.7	C8/0.72	W11/1.1	X2/0.8
7	X4/1.25	Y1/0.62	C8/0.62	X4/0.7	X4/1.45
8	Y3/0.9	Y3/1.55	X1/1.75	X4/0.5	X9/1.5
9	Y5/1.8	Y3/1.0	Y9/0.65	Y1/0.8	Y20/1.5
10	Y13/0.6	Y3/0.9	Y13/1.4	Y1/0.7	Y20/1.4
11		Y13/0.6	Y13/0.6	Y13/1.4	

Table 2. Summary of heterozygous loci in the mother trees that are found in the putatively apomictic progeny.

Parent	Number of Heterozygous Loci in the Seed Trees	Putative Apomictic Progeny		
		Number of Progeny	Number of Loci Scored Band Present[1]	# of Individuals Band Present at All Loci[2]
B209-08	10	22	4~10	2
O110-09	11	15	7~11	2
K110-19	11	22	3~9	0
H205-55	11	27	4~10	0
S210-19	10	29	5~10	1

[1]Range in the number of loci present per individual in each cohort.
[2]Individuals that are band present for all loci segregating in the mother trees.

In order to estimate the likelihood of an asexual origin of the five individuals with all loci fixed, the frequencies of band present alleles (p,) in the pollen pool at all the 53 loci were estimated. The result indicated a 3% probability that individuals 12-110 and 12-112 from family B209-08 are from pollen contamination; individuals 22-201 and 22-305 from family 0110-09 have a 5% probability of being from pollen contamination. Additionally, individual 62-220 from S210-19 has about a 1% chance being from pollen contamination.

DISCUSSION

There are three plausible explanations for the existence of full seed obtained from unpollinated (control) flowers in this study. One possible explanation for seed production without the addition of external pollen would be the existence of hermaphroditic flower structures in sycamore. We found no published literature on the existence of hermaphrodism in sycamore, and no evidence of hermaphrodism has been noted for any of the trees used in this study. A second possible origin of the seed is pollen contamination from surrounding trees. Since the pollen exclusion bags were not placed over the buds until they began to open, and vegetative buds could be differentiated from flower buds, there was the possibility of pollen contamination. A third possible origin of the seed, and one which could simplify the clonal propagation of superior genotypes, would be the existence of apomixis.

Parthenogenic origin of the seeds in the unpollinated progeny was impossible for two reasons. First, when comparing mother trees with their unpollinated progeny, we found extra bands in the unpollinated progeny, which would be inconsistent with parthenogenic origin. Second, parthenogenic seeds should show a low germination rate and poor growth (Richards 1986). The trees resulting from the unpollinated control seeds are growing as well as open pollinated sycamore trees.

Based on the models of dominant inheritance of RAPD markers, progeny from selfed pollinations will produce banding patterns that represent a subset of the parental patterns (with heterozygous loci in the parent segregating in a 3:1 ratio in the progeny). However, we found that the selfed progeny, in comparison to the parent tree, had some non-parental bands. In fact, these additional bands were commonly found in each family. Limitations of RAPD markers, such as the possibility of co-migration, non-specific amplification or even artifact caused by mismatch at primer sites (Williams et al. 1990) may account for some of the variants we observed. Therefore, certain markers may be amplified unreliably, and may not represent useful genetic variation (Riedy et al. 1992). However, it has been confirmed that most of the RAPD markers are inherited in a Mendelian fashion and therefore will make them reliable and valuable tools (Williams et al. 1990). The reproducibility of most RAPD markers in this study excludes non-specific priming and other artifact as causes for the non-parental bands (Hadrys et **al.** 1992). To minimize artifacts of RAPD markers and exclude most of the unreliable RAPD polymorphisms, we selected only specific, reproducible heterozygous loci in mother trees to estimate levels of pollen contamination and apomictic production in sycamore.

A dominant marker (e.g. a RAPD marker) will segregate in a 3:1 ratio in the selfed progeny of a heterozygous individual. However, there are several reasons for the segregation of alleles to depart from a 3:1 ratio. One possibility, which would affect the segregation ratio, is that of self-incompatibility (Richards 1986). Furthermore, gametophyte selection or linked deleterious mutations could bias transmission or survival of those chromosomal regions bearing the RAPD markers (Echt et al. 1992). Additionally, amplification of non-nuclear DNA, such as mitochondrial DNA or chloroplast DNA, would follow the inheritance pattern of the organelles (Hadrys et al. 1992). The possibility of co-migration of fragments of the same size from non-homologous loci still exists, and cannot be ruled out without extra

analysis by Southern blotting (Hadrys et al. 1992). Due to the small sample size of selfed progeny, segregation of alleles at a locus can deviate from a 3:1 Mendelian ratio just by chance. Therefore, the loci in which bands were present in a mother tree and segregated in selfed progeny were selected to identify apomicts in unpollinated progeny if they segregated between 1:1 and a 1:0 ratio (band present:band absent). However, in family S210-19 seven of the ten loci were inherited in a 1:1 or a 1:0 ratio, and not a 3:1 ratio. This unusual departure from a 3:1 ratio was most likely the result of high levels of pollen contamination. These results also were consistent with the much higher percentage of sound seeds obtained from unpollinated flowers of S210-19; therefore, the identification of apomicts in this family is probably unreliable.

Another limitation of identifying heterozygous loci with this approach was that expected ratios in the progeny were based on the frequency of band present alleles in the pollen pool. If allele frequencies in the pollen pool approached the extreme, (i.e. a>>A or A>>a (A=band present allele, a=band absent allele)}, then the expected segregation ratio would approach 1:1 (band present: band absent) or 1:0 (all band present) in open pollinated progeny. If allele frequencies were equal in a given pollen pool, the expected ratio would be 3:1 (band present:band absent), similar to selfed progeny. Seed that resulted from open pollinated flowers would have band frequencies between 1:1 (band present:band absent) and all band present, depending upon the frequency of band present alleles in the pollen pool. Since heterozygotes could also occur from fertilization events such as open pollination described above, the presence of a maternal genotype at only one locus does not imply that the embryo is of apomictic origin. Therefore, when discussing the probability of apomixis, we could only establish a maximum value. The actual frequency of apomixis might be lower (Aly et al. 1992). By increasing the numbers of identified DNA markers, it would be possible to increase our confidence of identifying seed of apomictic origin.

Our conclusions are limited by small sample sizes; results from five families cannot represent all sycamore trees in all years. Also, the number of selfed progeny used for identifying heterozygous loci ranged from 18 to 30, and because of this small sample size, it is difficult to differentiate a 3:1 segregation ratios from 1:1 or 1:0. Furthermore, only five individuals out of 115 unpollinated progeny (approximately 4%) were identified as being possible apomicts. The small number of unpollinated seeds makes it difficult to accurately estimate the rate of apomixis. Additionally, all pro ^{g}eny from each seed tree came from one year's controlled pollinations, which were done in the same place and at the same time. Therefore, replication of this work would strengthen our conclusions.

CONCLUSIONS

Most of the putative apomicts resulted from pollen contamination. The greatly different frequencies of the band present alleles shown among different loci within a family and among the same loci from different families might be due to different pollen parents for each seed tree. The probability of an individual being produced by the apomictic process was estimated under the assumed condition of independence among the loci within a family. Only

0.076% of all seeds (both viable and nonviable seeds) collected from the unpollinated cohort might be apomicts and are unlikely to supply sufficient number of seeds for efficient cloning purposes.

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