

USE OF RAPD TECHNOLOGY TO IDENTIFY MAST POTENTIALS IN HARDWOOD SPECIES ¹

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Abstract. It is generally recognized that within a hardwood species population some individuals consistently produce heavy mast (acorn, nut, seed) crops while other individuals will not. Identification of an individual's mast characteristics could potentially be accomplished using molecular markers. Use of the random amplified polymorphic DNA (RAPD) marker system, based on polymerase chain reaction (PCR), is suited to this system. RAPDs are useful for distinguishing different individuals in a population.

Leaf samples were collected from 128 individuals of twelve hardwood tree species. Field observations at the time of collection showed these trees to vary widely in mast production. Species included five oaks and two hickories, as well as sugarberry, dogwood, persimmon, blackgum and swamp tupelo. Eight years of recorded mast production data are available for samples from three oak species collected on one site.

DNA was successfully extracted from the leaf material of each species and amplified using random primers. Both heavy and light mast producing tree DNAs were initially evaluated individually. We then used bulk segregant analysis experimental methods to evaluate the potential of this approach to identify mast production traits. DNAs of the heavy mast producers were combined in one aliquot and the DNAs of the light mast producers in a separate aliquot. These two groups were compared using various primers to detect distinguishing RAPD markers. Markers specific for only one bulk, either heavy or light, could correlate with the masting trait. These methods have potential for the early identification of good mast producers for selection, breeding and seed orchard use for these hardwood tree species.

Introduction

Sustaining a mixture of heavy-seeded hardwood tree species in eastern deciduous forests is a desirable but sometimes difficult task to accomplish. Poor natural regeneration often results from inherently low mast (acorn, nut, seed) productivity of individual trees, cyclic crop production, poor germination, foraging by wildlife (Gosselink and Muir, 1990), defoliation, shade and competition (Lorimer, 1992), or losses of heavy-seeded species due to disturbances that favor light-seeded species (Hook, unpublished data). When artificial regeneration is used, seedlings improved through breeding for increased mast production could be outplanted. Unfortunately breeding orchards for most of these species do not exist. In a few isolated areas individual trees are being monitored for mast production, but this process is time consuming and expensive, and the trees are not easily accessible. If techniques could be developed that would allow quick and inexpensive identification of potentially good mast producers, existing

individuals in the field could be identified and special precautions could be taken to maintain them. In addition, seedlings at the nursery could be screened for good masting potential and planted in desired locations.

Identification of an individual's masting characteristics could potentially be accomplished using DNA markers. Random amplified polymorphic DNA (RAPD) markers, based on the polymerase chain reaction (PCR), have been used to follow traits in other species (Grattapaglia et al., 1992). The DNA polymorphisms identified by the markers show up as bands on an agarose gel and are relatively simple to screen based on their presence or absence. The RAPD system offers several advantages; RAPDs are capable of distinguishing different individuals in a population (Williams et al., 1990), they are not species specific, only a small amount of DNA is required, protocols and data collection are less time consuming than restriction fragment length polymorphism (RFLP) or isozyme analysis, RAPDs

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can be combined with RFLPs to increase efficiency of genetic marker analysis (Anderson and Fairbanks, 1990), and DNA libraries, known DNA sequences and radioisotopes are unnecessary, making RAPDs ideal for small research studies.

In woody plants isolation of DNA of an adequate purity to successfully perform RAPD reactions is often difficult. Secondary compounds such as polyphenols and polysaccharides are released during tissue grinding, copurify and interact with the DNA (Katterman and Shattuck, 1983, Rogers and Bendich, 1988, Loomis, 1974) and can inhibit PCR-type reactions (Demeke and Adams, 1992).

We isolated DNA from leaf tissue of different hardwood species using a CTAB extraction method (Doyle and Doyle, 1990). We used the DNA in RAPD reactions to screen individuals separately, then in bulks for bulk segregant analysis experimental methods.

Methods and Materials

Leaves were collected in September 1991 and May 1992 from the most recent growth flush at the canopy top of 128 mature trees. Species studied included: *Quercus alba* L. (white oak); *Q. falcata* Michx. var. *falcata* (southern red oak); *Q. falcata* var. *pagodifolia* Ell. (cherrybark oak); *Q. lyrata* Walt. (overcup oak); *Q. michauxii* Nutt. (swamp chestnut oak); *Carya aquatica* (Mich. f.) Nutt. (water hickory) and *C. tomentosa* (Poir.) Nutt. (mockernut hickory), *Celtis laevigata* Willd. (sugarberry), *Cornus florida* L. (dogwood), *Diospyros virginiana* L. (persimmon), *Nyssa sylvatica* Marsh. (blackgum) and *N. sylvatica* var. *biflora* (Walt.) Sarg. (swamp tupelo). Samples represented a range of habitats across coastal and piedmont regions of South Carolina. Trees varied widely in mast production based on visual observations at the time of collection. The fall of 1991 was a bumper crop year for mast production in the southeast (Beck, 1992) representing a peak in a mast cycle. Because both extremes of the spectrum (inherently low or high mast production) would be more apparent during a peak in the mast

cycle, we collected leaves from each extreme. We feel that they would more closely reflect the true genetic nature of a tree's masting potential because of this particular time in the mast cycle.

Field samples were stored on ice during collection and transit (24-48 hours), immediately frozen in liquid nitrogen when returned to the lab and stored at -30°C until being processed further. To extract the DNA 500 mg of frozen tissue³ was placed in a prechilled (-20°C) crucible. It was ground to a fine powder under liquid nitrogen with insoluble polyvinylpyrrolidone (PVP). The powder was transferred into a Kontes tissue grinding tube (#21) to which five milliliters (mls) of preheated (65°C) CTAB extraction buffer was added. The mixture was homogenized for 1-2 min⁴ then incubated in a 65°C water bath for 30 min. It was then transferred to a centrifuge tube to which an equal volume of 24:1 chloroform:isoamyl alcohol (CIA) was added. The solution was mixed thoroughly but gently to achieve a good emulsion. After centrifugation for 10 min at $19,000 \times g$, the upper aqueous layer⁵ was transferred to a new tube and CIA was again added and mixed thoroughly. Following another centrifugation the upper aqueous layer was transferred to a new tube to which 1/10 vol 3M sodium acetate (NaAc, pH 6.0) was added. After mixing, an equal vol of -20°C isopropanol was added and mixed, precipitating the DNA. The DNA pellet was then transferred⁶ to a new tube, washed twice with 70% ethanol and air dried. It was then dissolved⁷ in 1 ml TE buffer.

Additional extractions of the DNA solution with phenol:chloroform:isoamyl alcohol (PCIA, pH 8.0) were necessary to further remove contaminants and secondary compounds which otherwise inhibited amplification in RAPD reactions. Two-hundred and fifty microliters (μl) of the DNA solution were mixed with an equal vol of Tris/NaCl buffer. An equal total vol of PCIA was added and mixed. Following centrifugation for 5 min at $16,000 \times g$ the upper aqueous layer was transferred to a new tube.

An equal vol of PCIA was again added and mixed. Following another centrifugation, the upper

³ Do not allow the tissue to thaw as oxidation of polyphenols will occur which can interact with the DNA (Couch and Fritz, 1990).

⁴ Homogenization time depends on species. Oaks and hickories took longer to grind and homogenize than the other species.

⁵ Amount of upper phase varies with species.

⁶ DNA may be transferred either by pelleting in a centrifuge 10 min at $16,000 \times g$ and carefully pouring off the supernatant, or grasped with glass pipette hook (bend tip of a glass pipette over ethanol flame to form a hook) and transferred to a new tube.

⁷ Alternatively incubate one hour at 37°C to facilitate dissolving.

aqueous layer was transferred to a new tube to which 1/10th vol 100 mM NaAc (pH 6.0) was added. After mixing, an equal vol -20°C isopropanol was added and mixed to precipitate the DNA. After a final centrifugation the supernatant was carefully poured off, and the DNA pellet was washed twice with 70% ethanol and air dried. The DNA was then dissolved in 250 μ l TE and samples were stored at 4°C. The DNA concentrations were measured (before and after PCIA) using a fluorometer.

Following extraction procedures, individual DNAs were amplified using up to 100 primers (University of British Columbia, primers 201-300). To prepare the DNAs for RAPD reactions, aliquots from each PCIA-cleaned DNA sample were diluted to a concentration of 12.5 μ g/ml in 100 μ l of water. Samples were boiled for 10 min and immediately placed on ice. Two μ l of each DNA sample were transferred to separate PCR tubes. The RAPD-reaction solution^a was mixed and 23 μ l were added to each PCR tube containing DNA. Each solution was overlaid with mineral oil and tubes were placed in a thermal cycler. Amplification took place over 45 cycles: denaturation 1 min at 94°C, annealing 1 min at 35°C, ramp at a rate of 1°C per 2 sec to 72°C, extension 2 min at 72°C (final cycle with a 5 min incubation at 72°C) and an indefinite 4°C after 45 cycles were completed. Loading dye buffer (Sambrook et al., 1989) was added to each sample, vortexed and centrifuged for 5 min at 16,000 $\times$ g. Samples were separated electrophoretically on a 1.5% agarose gel at 150 volts. The gels were stained with ethidium bromide and photographed.

Solutions

CTAB extraction buffer: 2% hexadecyltrimethylammonium bromide (CTAB), 1.4 NaCl, 20 mM ethylenediamine tetraacetic acid (EDTA), 100 mM Tris, pH 9.5, 0.2% 2-mercaptoethanol, 1% soluble polyvinylpolypyrrolidone (PVP), 0.1 mg/ml protease

TE buffer: 10 mM Tris, pH 8.0, 1 mM EDTA

TRIS/NaCl buffer: 200 mM NaCl, 100 mM Tris, pH 8.0, 10 mM EDTA

RAPD reaction solution: 10X buffer, 1.9 mM MgCl₂, 2.5 mM each of deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 4 μ M oligonucleotide primer, 1 unit Taq DNA polymerase, 2 μ l of 12.5 ng/ μ l DNA (add separate from reaction solution, see protocols for details), volume to 25 μ l with water. Chemicals used were obtained from Perkin Elmer and Promega.

Results and Discussion

The protocol of Doyle and Doyle (1990) worked effectively for isolation of DNA from twelve species of mature hardwoods trees. We obtained DNA from 99% of the samples with less than 1% being recalcitrant. DNA concentrations varied, shown by the following average concentrations (μ g/ml) of all samples within each species: white oak 144, southern red oak 78, cherrybark oak 158, overcup oak 69, swamp chestnut oak 88, water hickory 81, mockernut hickory 77, sugarberry 117, dogwood 49, persimmon 36, black gum 79 and swamp tupelo 34. Variations could be attributed to species, variability in leaf texture, developmental stage of the leaf, and presence of secondary compounds (Guillemaut and Marechal-Drouard, 1992).

A PCIA wash of the DNAs was a critical step in our protocols. Without it DNA extracted from late-summer leaves did not amplify successfully. Additional organic extractions have been shown to remove contaminants from DNA (Honeycutt et al., 1992). We used PCIA to clean the DNAs further. Pink and brown discoloration commonly displayed in late-summer DNAs disappeared after two to three PCIA extractions, suggesting the removal of polyphenols and other contaminants. Repeatable and consistent results were then observed for PCIA-purified DNA when tested with several primers. DNAs extracted from spring-collected samples showed no discoloration and amplified without the additional PCIA extraction. However this treatment was used on these samples to see if any changes in amplification occurred. Results revealed more bands following PCIA treatment. Therefore, PCIA purification was adopted as a routine part of the protocols for all tissues. For unknown reasons this treatment resulted in over 50% of the DNA concentration being lost. However ample DNA was still present for use in RAPD reactions.

^a This reaction solution should be made up on ice and the DNA amplified directly thereafter.

Bulk segregant analysis (BSA) has been used to screen polymorphisms in bulked groups of individuals having the same trait (Michelmore et al., 1991). By bulking the DNAs, common polymorphisms are revealed while individual and novel polymorphisms are not. Thus when an unusual polymorphism does show up in the bulk it may be informative. This method facilitates analysis because an entire genomic region, rather than an individual locus, is being evaluated for correlated markers. This in turn expedites their placement on a genetic map. BSA ideally should be done on samples derived from segregating populations that have a known genetic history. This prerequisite does not exist for the hardwood species with which we were working. The oaks with long-term mast data were the best available samples to study.

Following the completion of amplification of individual samples, we evaluated BSA experimental methods. We bulked the two groups of DNAs from each of the three species of oak trees having long-term mast data. The trees were located on one site in the coastal region of South Carolina, avoiding any differences in allelic expression due to geographic variation. For each species, DNAs from three trees being heavy mast producers were combined in one aliquot, and DNAs from three trees being light mast producers were combined in another aliquot, all in equal volumes. These two aliquots were compared, using a number of primers in RAPD reactions, to detect polymorphisms that may be present in one bulk and not in the other. When a distinguishing polymorphism was found the primer was then run with the individuals in that bulk to see if the same polymorphic band was present in each of the individual samples. With only three individuals per bulk the BSA method was not successful. We were unable to identify any informative polymorphic bands. Individual polymorphisms rather than unusual bulked polymorphisms were revealed. The BSA method is more efficient with a larger number of samples in each bulk (Michelmore et al., 1991). The dogwood species had the highest number of samples with eight per bulk. We tested these bulks using the BSA method with 12 primers. The bulks gave identical patterns except for one distinguishing marker. This primer was tested on the individual DNA samples in that bulk, but only six of the eight individuals showed the polymorphism. In evaluating our other species'

bulks, none had a sufficient number of samples to continue with the BSA method. In theory the BSA method should work with greater numbers of samples.

The sensitivity of this method was tested using two swamp chestnut DNAs, one of which contained a marker that was absent from the other sample. We ran reciprocal dilutions of the DNA samples which scored for the presence and absence of a particular marker (at 1, 2, 4, 5, 10, 20, 33 and 50%). It was found that markers present in less than 25% of the bulk samples were not detected, but above that they were. This suggests that only markers that are less than 25% recombination from a gene of interest would be detectable in this species using this method.

In conclusion, our data indicates that RAPD markers can be used for these species. Additional PCIA extractions of leaf DNA are critical for successful amplification using random primers. For the bulk segregant analysis approach however, greater numbers of individuals, preferably within a genetically defined cross or population would probably be required. The techniques presented here could provide a better understanding of hardwood tree species biology and the masting phenomenon.

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