GENETIC LINKAGE MAP OF PECAN (Carya illinoinensis) CULTIVARS, USING RAPD AND AFLP MARKERS.

by

SUDHEER R. BEEDANAGARI

(Under Direction the of PATRICK J. CONNER)

ABSTRACT

We report here the first genetic linkage maps of pecan, using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) markers. Maps were constructed for the cultivars Pawnee and Elliot using double-pseudotestcross mapping strategy and using 120 F₁ seedlings. A total of 477 markers, including 217(45.5%) RAPD and 258 (54.0%) AFLP markers and 2 (0.5%) morphological markers were used in linkage analysis. The ‘Pawnee’ linkage map has 218 markers, comprising 176 testcross and 42 intercross markers mapped on to 16 major linkage groups and 13 minor linkage groups. The ‘Pawnee’ map covered a total distance of 2,227 cM, with an average map distance of 12.7 cM between adjacent markers. The ‘Elliot’ linkage map has 174 markers, comprising 152 testcross and 22 intercross markers, mapped to 17 major and 9 minor linkage groups. The ‘Elliot’ map covered a total distance of 1,698 cM, with an average map distance of 11.2 cM between adjacent markers. Dichogamy type and stigma color genes were tightly linked and mapped to ‘Elliot’ linkage group 16. These linkage maps are an important first step towards the detection of genes controlling
complex traits such as nut size, nut phenology, kernel quality, and disease resistance. Markers linked to these traits could serve the breeding program by allowing marker-assisted selection of seedling trees for adult characteristics.

INDEX WORDS: Molecular markers· Pecan genome· Double-pseudotestcross· Stigma color· Heterodichogamy· AFLPs· RAPDs· SSRs· RFLPs· Isozymes·
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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

1.1. PLANT DESCRIPTION:

Pecan [Carya illinoinensis (wangenh.) K. Koch] is the most economically important and widely cultivated nut crop that is native to North America. The genus Carya is the only species under tribe Hicoreae in the walnut family Jugalandaceae (Manning 1978). Though currently grown in large areas of Georgia, the native range of pecan is along the northern and western Mississippi drainage basin and southward into Mexico, principally on moist and well drained ridges in river bottoms. The leading states in pecan nut production in the United States are Georgia, Texas, New Mexico, Alabama, Louisiana, and Oklahoma. Pecans are also grown in other countries, primarily Australia, Brazil, Israel, Mexico and South Africa. Though pecans are grown outside the United States, new cultivars are developed only in the United States (Sparks, 1992).

Pecan is a deciduous tree, with alternate and compound leaves varying in size among different cultivars, with 9-17 leaflets per leaf, each measuring 5-10 cm long. Leaf color varies widely among cultivars, from the yellow-green leaves of ‘Desirable’ to extremely dark green leaves of ‘Pawnee’ (Reed and Davidson 1954; Sparks1992).

Flowering in pecan is complex with male (catkin/staminate) flowers and female (pistillate) flowers produced separately at different locations on the same tree (monoecy). Staminate, pendulous catkin inflorescences are borne at the base of shoots and along the length of the supporting one-year-old wood; whereas pistillate flowers are borne in terminal spikes on
new shoot growth (Wetzstein and Sparks 1983). The maturation of the staminate and pistillate flowers occurs at different times (dichogamy). Pecan cultivars differ in the order of occurrence and maturation of staminate and pistillate flowers (heterodichogamy) (Sparks 1992). Dichogamy favors cross-pollination, although a short period of stigma and stamen maturity overlap in a few cultivars, which may favor self-pollination. Pollen is produced in large quantities, which enhances the chances of wind pollination, the main source of pollen dispersal. Geographic location and year-to-year variations also affect dichogamy. Therefore, cultivars must be selected with great care during the establishment of new orchards, to ensure proper cross-pollination (Sparks 1992). Pecan has a long juvenile period and requires about 8-10 years of growth before producing a harvestable crop. However, once in production, pecan trees are extremely long lived and the orchards can stand for 100 years.

The economically important product of pecan is the nut, which has a high nutritional value including a high oil content (73-75%), carbohydrates (12-15%), proteins (9-10%), vitamins A and E, ascorbic acid, thiamine, riboflavin and niacin (Briston 1974). In addition, drugs, essential oils and cosmetics can be obtained from the nut’s oil. Leaves are a good source of lauric acid, jugalone (an antihemorrhagic), and proteins (Duke 1989). Pecan is also useful in several other ways, by providing wood for furniture, flooring, and as a woody ornamental shade tree (Harlow et al. 1991). Pecan nuts are comprised of the kernel enclosed by a hard shell. The shuck developed from the floral involucre encloses the nut, which upon maturity of the nut breaks open, usually into four valves. Fruit maturation occurs in the fall of the same year, and varies among cultivars and region of origin (Peterson 1990; Sparks 1992)
1.2. PECAN BREEDING:
Pecan is a genetically heterogeneous cross-pollinated crop (Madden and Malstrom 1976). Seedling trees are therefore quite variable for key horticultural traits such as nut size, kernel color quality and nut phenology. Because seedling trees are so variable, clonal propagation is an important step in the establishment of the pecan industry. The first successful graft was developed in 1846 (Taylor 1905). Since then extensive selection has resulted in more than 100 cultivars at present. Pecan cultivars are currently grafted or budded on open-pollinated seedling rootstocks (Wetzstein et al. 1989).

Pecans are attacked by a wide range of disease and insect pests, causing substantial loss to the crop. In the southeastern United States, the most economically damaging disease is scab, caused by the fungus *Cladosporium caryigenum*. Scab infection results in reduced yields and nut size. Severe infections can cause complete crop loss and defoliate trees (Sanderlin 1994). Various levels of resistance to scab are available in pecan germplasm, but only a few cultivars offer good levels of resistance combined with high horticultural quality traits. Development of varieties with combinations of disease and insect resistance could reduce the risks of epidemics when weather conditions are favorable for disease growth and unfavorable for pesticide applications (Goff et al. 1998).

Pecan cultivars have three different origins: chance seedlings, selections from seedling orchards or seedlings planted by homeowners, and breeding programs (Sparks 1992). In the period from 1890 to 1930, a large number of cultivars was selected from seedling orchards, propagated, and released. Currently, the few remaining seedling orchards are being examined with the hopes of finding genotypes with a high degree of insect and disease resistance (Goff et al. 1998).
The USDA pecan breeding program at Somerville, Texas is the largest pecan breeding effort in the USA. A second USDA pecan breeding program was initiated at the S. E. fruit and nut tree laboratory in Byron, Georgia. The USDA pecan breeding program has released many cultivars over the years. These cultivars make up a substantial percentage of the pecan acreage in the western US pecan regions. USDA selections have played a lesser role in the South-east largely due to their scab susceptibility and a tendency to bear alternately (Sparks 1992). For the Georgia pecan industry to flourish, new cultivars need to be developed that are highly resistance to scab. The University of Georgia initiated a pecan breeding program in 1998 under the supervision of Dr. Patrick Conner, to develop cultivars for the southeastern US. This program is based in the Department of Horticulture, at the University of Georgia Coastal Plain Experiment Station, in Tifton, Ga.

The major objectives of the Georgia pecan breeding program are:

1. To develop high-quality commercial cultivars with increased disease and insect resistance.
2. To produce high-yielding cultivars with a decreased tendency to bear alternately.
3. To develop early maturing cultivars with large nut size.

Smith and Romberg (1940) first reported the techniques of making controlled crosses in pecan, and which have changed little since. Pistillate flower clusters are encased prior to receptivity in cellulose or paper pollination bags. When stigmas are receptive, pollen is blown into the bags using an aspirator. Seed from the controlled crosses are collected and stratified for 2-3 months in a cooler and planted in a greenhouse. Seedlings are usually planted in a nursery at a very close spacing for 1-2 years. Seedlings are then transplanted into a seedling orchard at close spacing, usually 10 x 15 feet. Seedlings are observed over a 10-year period and evaluated.
on the basis of tree vigor, precocity, disease and insect resistance, and nut size and quality. Superior individuals are propagated onto standard rootstocks in replicated randomized complete block orchard configurations for intensive testing for 15-20 years. Detailed records are kept of cultivar production and quality parameters. Selections performing better than test varieties are released as new cultivars.

1.3. PECAN GENETICS:

Pecan is a highly heterozygous outcrosser and most traits vary widely within and among crosses (Madden and Malstrom 1976). Heterodichogamy is the only known simply inherited trait and is controlled by a single gene, with protandry (pp) recessive to protogyny (PP or Pp) (Thompson and Romberg 1985). Substantial variation exists among cultivars in relation to tree growth, as measured by cross sectional area (Thompson et al. 1981; Thompson and Hunter 1983). Heritability estimates of tree vigor and development in pecan are difficult to estimate because the parental cultivars are grafted or budded onto seedling rootstocks while seedlings are usually grown on their own roots. Genotype-environment interactions have been demonstrated for many nut and kernel quality characteristics (Thompson et al. 1989). Few other genotype-environment studies have been conducted mainly due to the difficulty in obtaining orchards of equal age, spacing, and cultivar makeup.

Many inherent properties of pecan have made the initiation of large genetic studies difficult. Pecan is a perennial crop with a long juvenile period; this in combination with its large size makes the production of large progenies for genetic studies extremely expensive and unpractical. In addition, natural heterozygosity and inbreeding depression prevent the formation of inbred lines commonly used in genetic studies. As a result, few traits in pecan have been characterized genetically. Most important traits in pecan including yield, tree vigor, and nut size
appear to be under the control of many genes and their inheritance is poorly understood. Molecular markers are a promising tool for the study of these traits and may play an important role in the production of new cultivars.

Molecular marker analysis in pecan has been limited primarily to studies of germplasm diversity. Marquard et al. (1995, 1997) developed protocols for the analysis of five isozyme systems: malate dehydrogenase, phosphoglucose isomerase, phosphoglucomutase, leucine aminopeptidase, and diaphorase. Using these isozymes, 177 cultivars were sorted into 72 classes and the historical pedigree of some cultivars was called into question. Isozymes were also used by Grauke et al. (1995) in the evaluation of the pecan germplasm collection to designate a core subset. Xiao et al. (1998) used RAPD markers to classify 20 pecan cultivars into different groups and to suggest origins of some cultivars with unknown pedigrees. Vendrame et al. (1999, 2000) used AFLP markers to screen for genetic changes in pecan trees derived from somatic embryogenesis, and to evaluate the field performance of regenerated trees. Conner and Wood (2001) demonstrated the value of randomly amplified polymorphic markers (RAPD) markers in determining genetic relationships and fingerprinting pecan cultivars. Grauke et al. (2003) developed simple sequence repeats (SSRs) or microsatellite DNA markers and carried out an initial evaluation of SSR markers for use in genetic studies of pecan. Fjellstrom et al. (1994) developed linkage maps using Restriction Fragment Length Polymorphisms (RFLPs) in walnut (*Juglans* spp.), which is a close relative of pecan, but no genetic maps have been produced for pecan.

**1.4. MOLECULAR MARKERS:**

Classical plant breeding involves crossing parents that possess a desirable combination of alleles and then selecting superior offspring. Plant breeding deals with a wide range of traits,
both quantitative and qualitative. By definition, the difference between the qualitative and quantitative traits resides in the relative magnitudes of allele substitution effects at a genetic locus (Comstock, 1978). If the effect of substituting one allele for another is large relative to total phenotype variation, the trait is considered qualitative. The trait is considered quantitative if effect of one allele substituting another allele is small relative to total phenotype variation, because the trait is affected by several genes or because of significant portion of the phenotypic variation is environmental in origin. Many important traits such as quality and yield are quantitatively inherited whereas traits such as disease resistance and insect resistance are often qualitatively inherited. For both quantitative and qualitative traits, selection can be difficult if segregation is not clear. One promising solution to this problem is to select for another locus that shows clearer segregation and is tightly linked to the gene of interest, thus serving as a marker for the gene. Although the use of marker genes may enhance breeding efforts and extend genetic knowledge in annual crops, it is in perennial plants, such as most fruit crops, where the use of marker genes may ultimately have the most practical value, simply because breeding and genetic studies in these species are so difficult using classical breeding techniques (Janick and Moore 1975; Moore and Janick 1983). Many tree fruit and nut crops are highly heterozygous, and this along with lack of genetic knowledge of many of these species results in unpredictable progeny characters following hybridization. However the most serious impediment to fruit breeding is the long juvenile period and large plant size in many tree fruit and nut species.

1.4.1. MORPHOLOGICAL MARKERS:

Sax (1923) first proposed the concept of associating morphological markers with quantitative traits, but was unable to draw much attention of plant breeders because of several limitations associated with these markers. Unlike molecular markers, where genotypes can be
determined at plant, tissue, or cellular levels, for most morphological markers, genotypes can be ascertained only in a fully mature plant. Morphological marker traits frequently show unfavorable epistatic interactions that limit the number of segregating markers in the population, and dominant-recessive interactions often prevent screening of all genotypes associated with morphological traits. Moreover, morphological markers cause major alterations in the phenotype, which are often deleterious and undesirable, whereas most DNA markers are phenotypically neutral (Tanksley et al. 1989; Stuber 1989a, 1989b, 1989c).

With the advent of molecular markers, indirect selection became a prominent tool for plant breeders. The advantages of molecular markers over morphological markers are that they are numerous in number and phenotypically neutral. Molecular markers have no deleterious effects on plant phenotype or epistatic interaction between markers and since molecular markers are based upon protein or DNA polymorphisms, plants can be screened at the seedling stage, facilitating a pre-selection system (Conner 1996).

1.4.2. ISOZYMES:

Markert and Muller (1959) coined the term isozyme (isoenzyme) to describe the multiple molecular forms of an enzyme that are derived from the same individual and share a catalytic activity. Isozymes differ in their electrophoretic mobility due to different molecular weights and charge, thus creating polymorphisms. Isozymes are revealed by gel electrophoresis of the plant tissue extracts, followed by the immersion of gels in a solution containing enzyme-specific stains (Powell 1992). Relatively few isozyme markers are available when compared to DNA markers in a crop because the staining systems need to be worked out for each enzyme system, and many systems are not polymorphic. However isozyme markers have been used in horticulture and plant breeding for a variety of purposes such as identifying cultivars, estimating genetic
diversity, marking of monogenic and polygenic traits, and development of linkage maps (Weeden 1989).

1.4.3. RFLPs:

Restriction fragment length polymorphisms (RFLPs) are one of the common types of DNA markers in use. The detection of RFLPs depends on the natural variation in the sequences of DNA between individual genotypes. Restriction enzymes are DNA endonucleases isolated from various microorganisms that recognize and cleave specific DNA base sequences. To detect polymorphisms among individuals, genomic DNA of these individuals is digested with a restriction enzyme and the resulting fragments of DNA are separated by size by electrophoresis on an agarose gel. The DNA is denatured, transferred to a supporting membrane (nitrocellulose or nylon) by southern blotting (Southern 1975), hybridized with a radioactively labeled DNA clone (probe) in a solution, followed by visualizing and the fragments visualized using autoradiography. Polymorphisms result from differences in distribution of restriction sites in the two genotypes, creating different size fragments that are bound to the probe (Powell 1992). The differences in restriction sites may be due to one or more base pair differences or they may be due to large differences such as inversions, deletions, and translations. Thus, RFLP detection is affected by existing DNA differences as well as the restriction enzymes and probe used.

The major advantage of RFLPs over isozyme polymorphisms is that variations in DNA sequence are much more frequent than the variations in amino acid sequence, so that RFLPs are much more numerous than isozyme polymorphisms. RFLPs are more convenient than isozymes because they are DNA based and DNA can be stored and transported easily whereas isozymes often require live tissue samples. The relative ease of application and enormous number of markers available led to the wide spread use of RFLPs as genetic markers, which have been
subjected to several reviews (Tanksley et al. 1989; Burr et al. 1983; Beckman and Soller 1986). However RFLPs are tedious, and require technically sound and expensive labeling and detection methods, which only a few labs can afford.

1.4.4. RAPDs:

Random amplified polymorphic DNA (RAPDs) markers are DNA markers based on PCR amplification performed on genomic DNA template using short, arbitrary oligonucleotide primers. Each of these anonymous but reproducible fragments is derived from a region of the genome that contains two primer-binding sites located on opposite DNA strands within an amplifiable distance of each other. Polymorphisms between individuals result from sequence differences, which inhibit primer binding or otherwise interfere with amplification. They are detected as DNA fragments that are amplified from one individual but not from another. These polymorphisms can be visualized by electrophoresis on an agarose gel and staining the gel with ethidium bromide.

RAPD fingerprinting has been used extensively to detect DNA sequence polymorphisms in many plant species (Williams et al. 1990; Kresovich et al. 1992) and offers several advantages over RFLPs (Williams et al. 1990). Unlike RFLPs, RAPDs require only a single PCR reaction and gel electrophoresis to detect polymorphisms. RFLPs require probes, restriction digestion of genomic DNA, southern blotting to transfer genomic DNA to a solid support, and a means of visualization, which can be radioactive or nonradioactive. Kesseli (1992) found RAPDs require only 14 hrs of time whereas seven days are required for RFLP analysis. RAPDs allow easy and rapid regeneration of polymorphisms by using very small quantities of DNA. Moreover, unlike other PCRbased fingerprinting methods, RAPDs do not require any prior knowledge of the target template DNA sequence.
However RAPDs are scored as either band present or absent, making them dominant markers, where you cannot differentiate between homozygotes and heterozygotes. Dominant markers are disadvantageous because markers linked in repulsion provide little information for estimation of recombination frequency in F₂ analysis. Therefore, when mapping dominant markers, it is necessary to use formats in which markers are only linked in coupling as can be found in backcross or recombinant inbred populations or map markers for both the parents separately (Tingey 1992).

The reproducibility of RAPDs is an important concern with these markers. A few researchers found them too unreliable (Devos and Gale 1992; Riedy et al. 1992), whereas other researchers found the error rate within acceptable limits (Weeden 1992). RAPDs are sensitive to template DNA quality, reaction conditions, and PCR temperature profiles. The quality and quantity of template DNA have emerged to be the main factors affecting reproducibility (Williams et al. 1993; Micheli et al. 1994). Panner et al. (1993) studied the reproducibility of RAPDs across seven laboratories and found that the different laboratories amplified different size ranges of fragments using the same primers, thus resulting in poor reproducibility. The thermocycler was the major determinant in the size range of fragments produced. If temperatures are kept constant within the sample tubes across different laboratories, then reproducibility can be brought to acceptable levels across the different labs.

Some disadvantages of RAPDs can be circumvented by modifying the procedure. The utility of a desired RAPD marker can be increased by sequencing its termini and designing longer primers (>20 bp) for a specific amplification of the marker (Paran and Michelmore 1993). Such sequence-characterized amplified regions (SCARs) are similar to sequence-tagged sites (STS) (Olson et al. 1989) in construction and application. DNA sequence differences are
determined by the presence or absence of a single unique band. SCARs are more reproducible than RAPDs due to the longer primers used. Although SCARs are usually dominant markers, some SCARs can theoretically be converted to co-dominant markers by digestion with 4-bp restriction endonucleases and identification of polymorphisms by either denaturing gradient gel electrophoresis (DGEE) or single-strand conformational polymorphism (SSCP) techniques (Rafalski and Tingey 1993).

1.4.5. AFLPs:

Amplified fragment length polymorphisms (AFLPs) are DNA markers that use PCR amplification to detect genomic restriction fragments (Vos et al. 1995). AFLP analysis is particularly useful when very little information is known about the genome sequence of the plant under study. AFLP DNA analysis is a multi step procedure involving genomic DNA digestion with two restriction enzymes, Mse I and Eco RI. Eco RI is an average frequency (6-bp) cutter and Mse I is a high frequency (4-bp) cutter. The fragments formed after digestions are ligated to double stranded adapters of known sequence. The sequence of the adaptors and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments. Pre-selective primers are designed such that Mse I complimentary primer consists of an additional “C” at the 3’ end and the Eco RI complementary primer contains an additional “A” at its 3’ end. This allows only those fragments to amplify that have an Eco RI adaptor at one end and the Mse I adaptor at the other end. Addition of ‘A’ and ‘C’ nucleotides further assists in selecting the fragments. This is followed by a second selective amplification cycle with two more additional selective nucleotides at the 3’ ends of both Eco RI and Mse I primers. The amplified products are size separated on polyacrylamide gel by electrophoresis (Saunders et al. 2001) and detected by radioactive or non-radioactive methods.
The AFLP technique combines the power of RFLP with the flexibility of PCR-based technology by ligating primer-recognition sequences (adapters) to the restricted DNA. AFLP offers several advantages over other molecular marker techniques. Unlike RFLP, it requires only small amounts of DNA however; the quality and concentration of DNA must be superior in order to get best results. Unlike RAPDs that use multiple short arbitrary primers, which may lead to unreliable results, the AFLP technique uses only a few primers and generates many reproducible restriction fragments. Subsets can be amplified by changing the nucleotide extensions on the adaptor sequences, generating hundreds of reliable markers. High resolution and better reproducibility can be obtained using AFLP because of stringent PCR conditions and electrophoresis on polyacrylamide gels. The AFLP technique can be used on a variety of genomic DNA samples, even without prior knowledge of genomic sequence. The AFLP technique can generate an enormous number of polymorphic markers within a very short period of time and is useful for producing high density linkage maps.

1.4.6. Microsatellites:

Microsatellites are also referred to as simple sequence repeats (SSRs), short tandem repeats (STRs) or simple sequence length polymorphisms (SSLPs), consist of simple mono to pentanucleotide repeats, varying from a few tens of bases up to several hundred (Benet 2000). These markers are hypervariable, which in addition to their co-dominance and reproducibility makes them an excellent source of polymorphisms in eukaryotic genomes, and are well suited for genotyping and map construction studies (Staub et al. 1996; Dayanandan et al. 1998). Most SSRs are dinucleotide-repeat-based microsatellite markers [(AC)n, (AG)n, and (AT)n] (Rafalaski and Tingey 1993). The polymorphisms among individuals arise due to differences in the number of tandem repeats in a given repeat motif. In plants, (AT)n dinucleotide repeats are
relatively more abundant than any other type of repeat motifs (Akkaya et al. 1992). To detect the polymorphisms, primers are designed to amplify the microsatellite region using PCR and either fluorescently tagged or radioactively labeled. The amplified fragments are then resolved on polyacrylamide gels and are scored for the polymorphisms detected. Microsatellite markers have been used effectively in studies of diversity (Rosette et al. 1999), gene-flow and mating systems (Chase et al. 1996), paternity analysis (Streiff et al. 1999), and genetic mapping (La Rosa et al. 2003). Since microsatellites are co-dominant markers, heterozygotes can be easily identified, which is not possible with the dominant markers such as RAPDs and AFLPs. Since microsatellites are DNA-based markers amplified by PCR, they use small amounts of DNA and are applicable to high through-put systems (Robinson and Harris 1999).

1.5. LINKAGE MAPS AND MAPPING:

Before the development of molecular markers, the generation of linkage maps in plant species was primarily in the hands of breeders. The early geneticists were keen observers of morphological and developmental mutants, both natural and induced. These mutants were tested for linkage, and several of these mutants could be assembled into an impressive linkage map reflecting the arrangement of the genes on chromosomes (King 1974). The idea of using Mendelian markers to tag commercially important genes is probably as old as the concept of linkage. However, the main purpose of linkage mapping was viewed more as a book keeping device by which the various genes could be ordered on the chromosomes and predictions regarding recombination frequencies could be made (Weeden 1994). Mapping can also be defined as ordering markers, indicating the relative genetic distances between them and assigning them to their linkage groups on the basis of the recombination values from all their pairwise combinations (Jones et al. 1997). Until recently, very few genetic studies of fruit and
tree nut crops have been attempted because, like other perennial crops, breeding has been hampered by long juvenile periods, huge tree size and their highly heterozygous nature.

The development of molecular markers, particularly DNA based molecular markers, has greatly facilitated the construction of linkage maps, not only for economically important crops, but also for crops of lesser economic importance crops. Since most of the perennial fruit and nut crops are highly outbred, it is highly unlikely to obtain homozygous parents. Weeden (1994) proposed the use of double-pseudotestcross strategy for such heterozygous parents, analyzing the heterozygous loci in one parent separately from those in the other, to avoid identifying false linkages. The pseudo-testcross strategy generates two separate linkage maps for each of the parents individually. Once the maps of both parents are established, they can be compared using co-dominant markers or intercross markers.

There have been at least 212 molecular marker-based maps established for over 78 economically important plant species from 66 different genera including monocots, dicots and gymnosperms (Riera-Lizarazu et al. 2001). Several types of molecular markers have been used in generating genetic linkage maps including: isozymes RAPDs, RFLPs, AFLPs, SSRs, Single Stranded Conformational polymorphisms (SSCPs), Inter-Simple Sequence Repeats (ISSRs) and Single Nucleotide Polymorphisms (SNPs). Either a single type of molecular marker or a combination of different types of molecular markers has been used in an attempt to generate primary genetic linkage maps or to saturate the already established primary genetic linkage maps in different studies on different crop species.

A few examples of genetic linkage maps of different crops using molecular markers are; in lettuce using only AFLP markers (Jeuken et al. 2001), velvetbean using only AFLP markers (Capo-chichi et al. 2004), genus *Populus* using RAPD, AFLP and ISSR markers (Yin et al.
2002), grapes using AFLP and SSR markers (Grando et al. 2003), olive using RAPD, AFLP, RFLP and SSR markers (La Rosa et al. 2003), pears using AFLP and SSR markers (Yamamoto et al. 2002), tobacco using RFLP and RAPD markers (Lin et al. 2001), and apricot using AFLP, RAPD and RFLP markers (Hurtado et al. 2002).

1.6. OBJECTIVES OF STUDY:

Primary Objective: Develop molecular linkage maps for the cultivars ‘Pawnee’ and ‘Elliot’, two of the most important parental cultivars in the pecan breeding program, using AFLP and RAPD molecular markers.

Secondary Objective: Identify the molecular markers linked to the dichogamy type, stigma color and scab resistance traits.

Genetic maps will allow the pecan breeding program to better understand the pecan genome. Markers linked to traits of interest, especially quantitative traits, will allow segregation patterns to be observed, and provide basic information on the number of genes controlling the trait and magnitude of the effects of these genes. Markers tightly linked to advantageous genes will also be used for marker-assisted selection (MAS) of important traits to increase the efficiency of the breeding program.

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CHAPTER 2

A FIRST LINKAGE MAP OF PECAN (*Carya illinoinensis*) CULTIVARS, USING RAPD
AND AFLP MARKERS

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Abstract

We report here the first genetic linkage maps of pecan, using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) markers. Independent maps were constructed for the cultivars Pawnee and Elliot using the double-pseudotestcross mapping strategy and 120 F1 seedlings from a full-sib family. A total of 477 markers, including 217 RAPD, 258 AFLP, and 2 morphological markers were used in linkage analysis. The ‘Pawnee’ linkage map has 218 markers, comprising 176 testcross and 42 intercross markers mapped to 16 major and 13 minor (doublets and triplets) linkage groups. The ‘Pawnee’ linkage map covered a total map distance of 2227 cM, with an average map distance of 12.7 cM between adjacent markers. The ‘Elliot’ linkage map has 174 markers mapped, comprising 150 testcross and 22 intercross markers mapped to 17 major and 9 minor linkage groups. The ‘Elliot’ linkage map covered a total map distance of 1698 cM, with an average map distance of 11.2 cM between adjacent markers. Segregation ratios for dichogamy type and stigma color were not significantly different from 1:1, suggesting both traits are controlled by a single gene with protogyny and green stigmas dominant to protandry and red stigmas. Dichogamy type and stigma color loci were tightly linked (1.9cM) and mapped to ‘Elliot’ linkage group 16. These linkage maps are an important first step towards the detection of genes controlling complex traits such as nut size, nut phenology, kernel quality, and disease resistance. Markers linked to these traits could serve the breeding program by allowing marker-assisted selection of seedling trees for adult characteristics.

Keywords Molecular markers· Pecan genome· Tree· Heterodichogamy· Map Manager
Introduction

Pecan [Carya illinoinsensis (Wangenh.) K. Koch] is one of the most commercially important nut crops grown in United States of America, and the most economically important member of the genus Carya Nutt. (hickory) (Wood 1994). Pecan is one of the most important nut crops of United States of America. Most pecan cultivars are diploid, with a chromosome number of 2n=2x=32. Pecan trees are very large and long-lived trees with a juvenile period of 5-10 years. These traits in combination with a high degree of heterozygosity that is maintained through dichogamy have limited genetic studies in this crop. Consequently, the genetic control of most important traits, including disease resistance and nut quality, are not well understood.

Pecan is monoecious with male and female flowers produced on the same individual. The staminate flowers are organized into an ament or catkin, and the female flowers are borne on a spike (Wetzstein and Sparks 1986). Self-pollination in pecan is usually limited by dichogamy, whereby the male and female flowers mature at different times. Pecan cultivars differ in the order of occurrence and maturity of staminate and pistillate flowers, leading to both protandrous and protogynous patterns of flowering, termed heterodichogamy. In protogynous types, stigmas become receptive prior to pollen shed, and in protandrous types pollen is shed before the stigmas become receptive. The only trait in pecan whose genetics has been established is dichogamy type, which is controlled by a single gene where protandry (pp) is recessive to protogyne (PP or Pp) (Thompson and Romberg, 1985). ‘Pawnee’ is protandrous and has red stigma and ‘Elliot’ is protogynous and has a green stigma color.

The potential of molecular markers to increase our understanding of the pecan genome has been demonstrated in several studies. Isozyme systems have been used to study the genetic diversity of pecan populations (Marquard 1987,1989,1991a; Marquard et al. 1995). Conner et
al. (2001) used RAPDs to identify pecan cultivars and estimate their genetic relatedness. Vendrame et al. (2000) used AFLPs for molecular evaluation of pecan trees regenerated from somatic embryogenic cultures, and Grauke et al. (2003) evaluated simple sequence repeat (SSR) markers for genetic studies. Aside from these studies, little information exists on the pecan genome.

With the advent of several PCR-based methodologies, molecular markers became common tools for genetic studies of many crops. Molecular markers are particularly valuable in perennial crops for linkage map construction and mapping of qualitative and quantitative traits (Crespel et al. 2002). Molecular linkage maps are also being used successfully in many crop species for directed germplasm improvement (Pearl et al. 2004). Though most agronomically important crops have saturated or nearly saturated genetic linkage maps, mapping studies in many perennial fruit and nut crops have lagged behind. Recently molecular linkage maps of several tree fruit and nut crops have been produced, including pears (Yamamoto et al. 2002), apricot (Vilanova et al. 2003, Lambert et al. 2004), citrus (Sankar and Moore 2001), macadamia (Peace et al. 2003), apple (Conner et al. 1997), and walnut (Fjellstrom and Parfitt 1994). Generation of the maps facilitates the identification and localization of genes controlling important traits, leading to marker-assisted selection and positional cloning of genes (Staub et al. 1996, La Rosa et al. 2003).

Like many tree fruit and nut crops, pecan is an outbreeding heterozygous crop and inbreeding depression limits the ability to produce F$_2$ or backcross populations for mapping. Instead, F$_1$ progenies obtained from crossing two highly heterozygous parents are often used for mapping studies. The double-pseudotestcross strategy can be used with this type of population to generate linkage maps. This method produces two independent linkage maps, one for each of
the parents (Weeden 1994; Yin et al. 2003; Aienza et al. 2002; La Rosa et al. 2002). Molecular markers are also used for combining the genetic linkage maps (Strommer et al. 2002; Wang et al. 2002) and for comparing genetic linkage maps (Lambert et al. 2004) built from separate progenies.

In this report we describe the construction of genetic linkage maps for the pecan cultivars ‘Pawnee’ and ‘Elliot’ using a combination of RAPD and AFLP markers. Markers were identified and linked to loci controlling dichogamy type and stigma color. To our knowledge, these are the first reported genetic linkage maps of pecan. These maps are a first step towards the identification of loci controlling horticulturally important quality traits such as nut size, nut phenology and disease and insect resistance (Conner 1999).

**Materials and methods**

**Plant material**

The mapping population consisted of 120 progeny obtained from two crosses between the cultivars Pawnee and Elliot. The first progeny set consisted of 55 trees located in Byron, Ga. produced from crosses made in 1986 by Dr. Bruce Wood. The second progeny set consisted of 65 seedlings located in Tifton, Ga. produced from the same crosses made in 1999 by Dr. Patrick Conner. In both locations the trees were grown at a spacing of approximately 3.1 m by 4.6 m.

The dichogamy type of the Byron progeny was determined in the spring of 2003. Pistil receptivity was determined by dusting pollen on the stigmas and gently blowing off the excess pollen (Thompson and Romberg 1985). If the stigma was receptive, the sticky surface would retain a large number of pollen grains. Anther dehiscence was assessed biweekly, by shaking catkins and looking for the yellow pollen. Stigma color was determined visually at peak
receptivity; any sign of red or purple coloration was scored as “red” and the lack of such coloration was scored as “green”. ‘Pawnee’ produces protandrous flowers with red stigmas and ‘Elliot’ produces protogynous flowers with green stigmas.

Marker analysis

Total DNA was extracted from fresh mature leaves of parental lines and seedlings according to the protocol established for olive (Claros et al. 1999), a modified method of DNA extraction by Doyle and Doyle (1987). The DNA concentration was measured by comparison to known concentrations of Lambda-DNA by electrophoresis on agarose gels.

Primers used for RAPD analysis were synthesized by University of British Columbia, B.C., Canada. Amplifications were carried out in 25µl of solution containing 11.4 µl H2O, 2.5µl 10X buffer (20mM Tris-HCl pH 8.0, 100mM KCl, 0.1mM EDTA, 1mM DTT, 50% glycerol, 0.5% Tween 20, and 0.5% Nonidet-p40), 2.25µl of 25 mM MgCl2, 2 µl of a 2.5 mM solution of each DNTP (Promega, Inc., Madison, Wis.) 1.5µl of 10 µM primer, 5µl of a 5ng/µl DNA solution, and 0.4µl of Taq DNA polymerase (Promega, Inc. Madison, Wis.). Amplification was performed in an Eppendorf scientific thermal cycler (Eppendorf Sci., Westbury, N.Y.) programmed for 40 amplification cycles (94°C 1 min, 35°C 2 min, 72°C 2 min) followed by an 8-min extension cycle at 72°C, with maximum ramping speed between temperatures. Amplification products were resolved by electrophoresis on a gel made of 0.7% agarose and 0.3% synergel (Diversified Biotech, Boston, Ma.), run at 110V for 4 hrs in 0.5X TBE buffer (Sambrook et al. 1989). Band sizes were estimated by comparison to a 100-base-pair (bp) DNA ladder (Promega, Inc. Madison, Wis.). Gels were stained with ethidium bromide and photographed on a transilluminator, and the segregation patterns were scored manually as band
present or absent from a computer printout. The RAPD markers were named by their primer code followed by the size of the band scored (B199-1400).

The AFLP analyses were carried out as described in Vos et al. (1995) using fluorescently tagged primers. The AFLP core reagent and starter primer kit were purchased from PE-Applied Biosystems (Applied Biosystems, 2000). 200 ng of genomic DNA was digested with the restriction enzymes EcoRI and MseI, and DNA fragments were then ligated to adaptors. The pre-selective amplification cycle was carried out with one selective nucleotide (E-A, M-C), followed by a selective amplification cycle using two additional selective nucleotides. The amplification products thus obtained by using 3 selective nucleotides for both Eco RI and Mse I were run on a 6% polyacrylamide gel along with size standard Gene Scan-500 (ROX) (Applied Biosystems, Foster city, Cal.) and deionized formamide for 4-5 hrs on an ABI 377 DNA sequencer using 1X TBE buffer. Digital gel images were obtained using GENESCAN software (Applied Biosystems, Foster city, Cal.) and were manually scored for the presence or absence of bands. The AFLP bands were named with the 3 selective nucleotides of EcoRI and the 3 selective nucleotides of Msel followed by size of band scored (AGC/CTG 275).

Mapping and linkage analysis
Linkage maps were generated for both parents independently using the double-pseudotestcross mapping method (Weeden 1994). This mapping method utilizes the segregation data for polymorphic markers heterozygous in one or both parents. Testcross markers are heterozygous (band present) in one parent, homozygous recessive (band absent) in the other, and segregate in a 1:1 present:absent ratio in the progeny. Intercross markers are heterozygous in both parents and segregate in a 3:1 present:absent ratio in the progeny. All testcross markers were tested for a
Mendelian segregation ratio of 1:1 using chi-square analysis ($P<0.05$). Linkage maps were constructed using the mapping program *MAP MANAGER QTXb17* (Meer and Manley 2002). Initial linkage groups were constructed from testcross markers using the command “make linkage groups”. The criteria for linkage was a LOD threshold of 3.0 and a recombination fraction $\leq 0.35$. Then, linkages in repulsion phase of all loci were tested using the commands “flip phase” and “links”. The order of loci within linkage groups was refined as necessary by using “ripple” command and by manual rearrangement of the loci. Linkage distances were computed using the Kosambi mapping function and the “allow for segregation distortion” option.

Once the framework maps composed of testcross markers were established, intercross markers showing 3:1 ratio were mapped using the cross type designation “arbitrary” and the “links” command (Tsarouhas et al. 2002). Intercross markers were paired with the testcross marker with which they had the most significant linkage (LOD threshold of 3.0). Intercross markers were only considered accessory markers to the framework map and were not used in the estimation of linkage distances.

**Results**

**Molecular markers**

A total of 600 RAPD primers were screened using DNA from three progeny and both parents. From these 600 primers, 90 (15.0%) primers were chosen based upon the number of strong polymorphic markers they produced (Table 2.1). Using these 90 primers, RAPD analysis was performed on the entire 120 progeny, generating a total of 217 markers. The number of polymorphic RAPD markers produced per primer ranged from 1 to 6, with an average of 2.4 markers per primer. Of the 217 RAPD markers, 174 (80.2%) were testcross markers showing a
1:1 present:absent segregation ratio and the remaining 43 (19.8%) were intercross markers with a 3:1 present:absent segregation ratio. Out of 174 testcross RAPD markers generated, 82 (47.1%) and 92 (52.9%) markers were heterozygous in ‘Pawnee’ and ‘Elliot’, respectively. Distorted segregation was seen in 25 (14.4%) markers, with 11 of these heterozygous in ‘Pawnee’ and 14 heterozygous in ‘Elliot’.

AFLP selective primer screening was done using all the possible 64 combinations of primers provided in the starter kit, which includes 8 EcoRI+ 3 and 8 MseI+ 3 primers (Applied Biosystems 2000). Based on the number of polymorphic markers produced on a subset of 10 progeny and both parents, 20 highly polymorphic primer pairs were chosen for mapping (Table 2.1). Using these 20 primer-pair combinations AFLP analysis was done on the entire progeny, generating a total of 258 markers, of which 212 (82.18%) were testcross markers and 46 (18.82%) were intercross markers. Out of the 212 testcross AFLP markers generated, 116 (54.7%) and 96 (45.3%) markers were heterozygous in ‘Pawnee’ and ‘Elliot’, respectively. The number of polymorphic AFLP markers generated per primer pair ranged from 2 (E-AGC/M-CTG) to 24 (E-ACA/M-CAC) with an average of 12.9 markers (Table 2.2). In ‘Pawnee’ the number of polymorphic markers per primer pair varied from 0 (E-ACG/M-CTA) to 12 (E-ACA/M-CAC & E-AGC/M-CAG) with an average of 5.8 (Table 2.2). In ‘Elliot’ the number of polymorphic markers per primer pair varied from 2 (E-AGC/M-CTG) to 11 (E-ACA/M-CAC) with an average of 4.8. Of the 212 testcross AFLP markers, 35 (16.5%) showed distorted segregation with 22 and 13 of these heterozygous in ‘Pawnee’ and ‘Elliot’ parents, respectively.
Map construction

Independent linkage maps were constructed for each parent using the double-pseudotestcross mapping strategy (Weeden 1994). A total of 477 (Table 2.1) markers, including 217 RAPD, 258 AFLP and 2 morphological markers were available for map construction. Framework maps were created using only markers in the testcross configuration. For the ‘Pawnee’ map, 176 testcross markers were assigned to 29 linkage groups, leaving 22 markers unlinked. The 29 linkage groups include 16 major linkage groups and 13 minor linkage groups, comprising 2 triplets and 11 duplets (Fig. 2.1). Once the framework maps had been completed, 42 intercross markers were added to the maps as accessory markers, and 47 intercross markers remained unlinked. The final ‘Pawnee’ linkage map consists of 218 markers, including 176 testcross markers (80.7%) and 42 (19.3%) intercross markers. The ‘Pawnee’ map covered a total distance of 2227.7 cM, with an average distance of 12.6 cM between adjacent markers (Fig. 2.1). The size of linkage groups ranged from 302.9 cM to 14.9 cM, and the total number of markers per linkage group ranged from 2 to 23.

The ‘Elliot’ framework map was created using 152 testcross markers which were assigned to 26 linkage groups, leaving 36 markers unlinked. The 26 linkage groups include 15 major linkage groups, and 11 minor linkage groups, comprising 5 triplets and 6 duplets (Fig. 2.2). Intercross markers were used as accessory markers and 25 intercross markers were added to 17 linkage groups leaving 64 intercross markers unlinked. The 26 linkage groups covered a total map distance of 1698.4 cM, with an average distance of 11.2 cM between adjacent markers. The size of linkage groups ranged from 257 cM to 1.3 cM , and the total number of markers per linkage group ranged from 17 to 2.
A two-step process was followed in creating the linkage maps. In the first step, the framework maps were completed using only testcross markers. In the second step, the intercross markers were placed on the map next to the testcross markers to which they are most tightly linked. Intercross markers were not used in the calculation of map distances or map orders because they provide relatively little genetic information in comparison to testcross markers. However, intercross markers are useful in finding homologous linkage groups common to both the maps. The ‘Pawnee’ and ‘Elliot’ maps shared 14 of the 89 (15.7%) of the intercross markers (Table 2.3).

Phenotypic Traits
Two phenotypic traits, dichogamy type and stigma color, were evaluated in the 55 trees that make up the 1986 progeny. The 1999 progeny could not be evaluated for these traits because the trees are reproductively juvenile. In the spring of 2003 nearly all of the 1986 progeny flowered and were evaluated for these two traits. One tree did not produce any flowers and another tree was scored for stigma color but dichogamy type was not clear, thus 54 trees were scored for stigma color and 53 trees were scored for dichogamy type. Of the 53 trees scored for dichogamy type, the ratio of protandrous to protogynous was 22:31 which fit a 1:1 ratio ($\chi^2_{1 \, df} = 1.52, P \approx 0.27$), confirming the monogenic control established by Thompson and Romberg (1985). Of the 54 trees evaluated for stigma color, 21 produced green stigmas and 33 produced red stigmas which also fit a 1:1 ratio ($\chi^2_{1 \, df} = 2.67, P \approx 0.09$) suggesting monogenic inheritance.

Assuming that ‘Pawnee’ is $pp$, ‘Elliot’ must be heterozygous $Pp$ since the progeny segregated for dichogamy type. Indeed, nearly all protogynous cultivars are heterozygous for this trait since protogynous types nearly always cross with protandrous types, maintaining a 1:1
phenotypic ratio (Thompson and Romberg 1985). Because only ‘Elliot’ is heterozygous for this trait, this gene could only be mapped to the ‘Elliot’ map. Dichogamy type mapped to ‘Elliot’ linkage group 16 (Fig. 2.2) with linkage to the markers B160-800 (LOD = 10.9, 13.2 cM), B29-340 (LOD = 9.8, 11.3 cM), ACA/CAT250 (LOD = 5.6, 15.4 cM), and B160-770 (LOD = 4.6, 21.0 cM).

Stigma color appears to be tightly linked (LOD = 13.8, 1.9 cM) with dichogamy type since all protandrous seedlings produced red stigmas and nearly all protogynous seedlings produced green stigmas. Only a single recombinant seedling was detected out of the 53 progeny that could be scored for both traits (Table 2.4). Assuming green stigma color (Sc) is dominant to red stigma color (sc), the data suggest that ‘Elliot’ is heterozygous for this trait (Scsc) and ‘Pawnee’ is homozygous recessive (scsc).

**Discussion**

RAPDs and AFLPs are both PCR-based dominant type markers. They share many of the same advantages including that they require relatively little template DNA, can be resolved without the use of radioactivity, and show good levels of polymorphism in many species. Perhaps the most important advantage of these markers for use in a minor crop such as pecan is that they can be used without any prior knowledge of the target template DNA sequence. Other marker types such as RFLPs and SSRs require extensive sequencing efforts before markers can be developed. AFLP markers require greater inputs in terms of equipment and initial expense compared to RAPDs, but have the capability of returning a larger number of markers per primer pair used. We found that the AFLP markers more efficient in terms of the time spent per marker produced, but the need to use a sequencer to resolve the bands meant that the AFLP work had to be done in
a separate facility. In addition, AFLP markers are more difficult to clone and sequence than are RAPD markers and will require more effort to convert them to sequence specific markers such as Sequence Characterized Amplified Regions (Paran and Michelmore 1993).

For an outcrossing species such as pecan, the double-pseudotestcross mapping strategy is well suited for use with dominant markers. Pecan cultivars are within a couple generations of trees selected from the wild and most are highly heterozygous. Since both RAPDs and AFLPs are dominant markers, two types of segregation ratios were observed: either 1:1 present:absent segregation ratio, if the markers are heterozygous in only one parent, or 3:1 present:absent segregation ratio, if the markers were heterozygous in both the parents. The markers segregating in 1:1 ratio are called testcross markers and the markers segregating in 3:1 ratio are called intercross markers (Scalfi et al. 2004). In this study, the percentages of intercross markers for RAPDs (19.8%) and AFLPs (17.8%) were similar to those found for other intraspecific crosses of forest trees (e.g. 19% in Quercus robur, Barreneche et al. 1998, 15-18% in Fagus sylvatica, Scalfi et al. 2004).

Intercross markers are less informative than testcross markers because the dominant phenotype comprises three indistinguishable genotypes (++, +−, −+) (Crespel et al. 2002). Thus only in the homozygous recessive progeny is it unambiguous what allele came from each parent. Intercross markers are of interest, however, because they can be used as “locus bridges” to align homologous linkage groups between the two maps (Echt et al. 1994). If enough markers are in common between maps, the maps can be combined into a single integrated linkage map (Conner et al. 1997, Peace et al. 2003).

In the present study, the linkage groups were not aligned together but were instead reported and numbered individually for both parents. Though several intercross markers were
mapped to linkage groups in both parental maps, there were not enough markers in common for the maps to be merged. The difficulty in mapping intercross markers is evident from low percentage of this type of marker that could be mapped in comparison to testcross markers. In the ‘Pawnee’ map 89% of the testcross markers were mapped compared to only 47% of the intercross markers. In the ‘Elliot’ map, 80% of the testcross markers were mapped in comparison to 40% of the intercross markers. In order to align and compare the homology of both the maps effectively, either more intercross markers need to be generated and mapped on a larger progeny set or, preferably, more informative co-dominant marker types such as simple sequence repeats (SSRs) need to be placed on the maps (Powell et al. 1996).

In the present study, 16 and 15 major linkage groups were obtained in ‘Pawnee’ and ‘Elliot’, respectively, which are close to the haploid chromosomal number of pecan (n=16). However a large number of minor linkage groups remain in both maps, indicating that the maps are not saturated. Additional markers need to be mapped to resolve the data into 16 linkage groups. Clustering of AFLP and RAPD markers was sporadically observed in the linkage groups of both the parental maps, similar to those reported in other mapping studies (Crespel et al. 2002; Bednarek et al, 2003). This can be explained by a reduced recombination rate around centromeres ( Tanksley et al. 1992) and also by the tendency of some marker types such as AFLPs to map in clusters (Nilsson et al. 1997; AlonsoBlanco et al. 1998).

Two phenotypic markers, dichogamy type and stigma color, were able to be placed on the ‘Elliot’ map. The genetics of dichogamy type had already been established by Thompson and Romberg (1985), but the genetic control of stigma color had not previously been described. Dichogamy type is of great importance to pecan breeding because it is the major factor in determining pollination compatibility between cultivars. The development of cultivars of both
dichogamy types is necessary to insure that orchards can incorporate new cultivars which will effectively pollinate each other. Stigma color is characteristic for each cultivar and can be used to help identify a cultivar (Sparks 1992).

To our knowledge, the present study is the first to report genetic linkage maps of pecan. In comparison to many agronomic crops, relatively little genetic work has been done on pecan. These maps will greatly facilitate additional genetic studies in pecan. Most horticulturally important traits in pecan have a complex mode of inheritance, and genetic maps will enable us to tease apart the individual loci in control of these traits and describe their effects. Both ‘Pawnee’ and ‘Elliot’ are important cultivars to the breeding program. ‘Pawnee’ is one of the most widely planted pecan cultivars and combines desirable characteristics of large nut size, high quality, and early nut maturity (Thompson and Grauke 2000). ‘Elliot’ has a high degree of resistance to pecan scab (Goff et al. 2003) caused by the fungus *Cladosporium caryigenum*. In the short term, molecular markers linked to useful traits will allow marker-assisted selection of these traits. Marker-assisted selection will greatly improve the efficiency of the breeding program because it will allow inferior seedlings to be removed early in the breeding cycle, eliminating years of costly field maintenance. In the long term, linkage between molecular markers and valuable genes is a necessary first step towards the positional cloning of these genes.

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**Table 2.1** Markers scored in the progeny using RAPD and AFLP analysis

<table>
<thead>
<tr>
<th></th>
<th>RAPD markers</th>
<th>AFLP markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of primers/primer pairs screened</td>
<td>600</td>
<td>64</td>
</tr>
<tr>
<td>Number of primers / primer pairs used for mapping</td>
<td>90</td>
<td>20</td>
</tr>
<tr>
<td>Number of testcross markers (1:1) present in ‘Pawnee’</td>
<td>82</td>
<td>116</td>
</tr>
<tr>
<td>Number of testcross markers (1:1) present in ‘Elliot’</td>
<td>92</td>
<td>96</td>
</tr>
<tr>
<td>Number of intercross markers (3:1)</td>
<td>43</td>
<td>46</td>
</tr>
<tr>
<td>Total number of markers produced</td>
<td>217</td>
<td>258</td>
</tr>
<tr>
<td>Average number of markers obtained per primer / primer pair&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.41</td>
<td>12.9</td>
</tr>
</tbody>
</table>

<sup>a</sup>Includes both intercross and testcross markers obtained for each primer / primer pair
Table 2.2 Primer combinations used for AFLP marker analysis in pecan mapping and bands scored for linkage analysis

<table>
<thead>
<tr>
<th>Primer pair</th>
<th>Pawnee</th>
<th>Elliot</th>
<th>Total no. of bands segregating 1:1</th>
<th>Total no. of bands segregating 3:1</th>
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<td>E-AAC/M-CAC</td>
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<td>6</td>
<td>12</td>
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<td>11</td>
<td>3</td>
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<tr>
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<td>5</td>
</tr>
<tr>
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<td>9</td>
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<td>116</td>
<td>96</td>
<td>212</td>
<td>46</td>
</tr>
</tbody>
</table>

*Band present (heterozygous) in ‘Pawnee’, null in ‘Elliot’ and segregating in 1:1 in F1 progeny

*Band present (heterozygous) in ‘Elliot’, null in ‘Pawnee’ and segregating in 1:1 in F1 progeny
<table>
<thead>
<tr>
<th>Pawnee linkage group</th>
<th>Elliot linkage group</th>
<th>Marker shared</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>B283-200</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>B43-1250</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>B345-580</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>AAC/CAG157</td>
</tr>
<tr>
<td>7</td>
<td>24</td>
<td>AGC/CTA85</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>AGG/CTA210</td>
</tr>
<tr>
<td>10</td>
<td>21</td>
<td>AAC/CTG198</td>
</tr>
<tr>
<td>12</td>
<td>18</td>
<td>B223-810</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>AGG/CAA100</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>B610-700</td>
</tr>
<tr>
<td>13</td>
<td>10</td>
<td>B387-350</td>
</tr>
<tr>
<td>14</td>
<td>11</td>
<td>AGG/CAA480</td>
</tr>
<tr>
<td>14</td>
<td>8</td>
<td>B519-1350</td>
</tr>
<tr>
<td>25</td>
<td>23</td>
<td>B345-320</td>
</tr>
</tbody>
</table>
Table 2.4  Classification of the 1986 progeny that were scored for stigma color and dichogamy type

<table>
<thead>
<tr>
<th></th>
<th>Green stigma</th>
<th>Red stigma</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protogynous</td>
<td>21</td>
<td>1</td>
<td>22</td>
</tr>
<tr>
<td>Protandrous</td>
<td>0</td>
<td>31</td>
<td>31</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>32</td>
<td>32</td>
</tr>
</tbody>
</table>

* Only progeny that were scored for both traits are included in the table.
Fig. 2.1 Genetic linkage map of ‘Pawnee’ pecan consisting of RAPD and AFLP markers. Map distances in cM are indicated to the left and loci on the right of each linkage group. Testcross markers are located to the immediate right of the linkage group in normal typeface. Intercross markers are located to the right of the testcross marker with which they are most closely linked and are italicized and underlined.
Fig. 2.2 Genetic linkage map of ‘Elliot’ pecan consisting of RAPD and AFLP markers. Map distances in cM are indicated to the left and loci on the right of each linkage group. Testcross markers are located to the immediate right of the linkage group in normal typeface. Intercross markers are located to the right of the testcross marker with which they are most closely linked and are italicized and underlined.
CHAPTER 3

CONCLUSIONS

In this study we report here the first genetic linkage maps of pecan, using random amplified polymorphic DNA (RAPD) and amplified fragment length polymorphisms (AFLP) markers. Independent maps were constructed for the cultivars Pawnee and Elliot using the double-pseudotestcross mapping strategy and 120 $F_1$ seedlings from a full-sib family. A total of 477 markers, including 217 RAPD, 258 AFLP, and 2 morphological markers were used in linkage analysis. The ‘Pawnee’ linkage map has 218 markers, comprising 176 testcross and 42 intercross markers mapped to 16 major and 13 minor (doublets and triplets) linkage groups. The ‘Pawnee’ linkage map covered a total map distance of 2227 cM, with an average map distance of 12.7 cM between adjacent markers. The ‘Elliot’ linkage map has 174 markers mapped, comprising 150 testcross and 22 intercross markers mapped to 17 major and 9 minor linkage groups. The ‘Elliot’ linkage map covered a total map distance of 1698 cM, with an average map distance of 11.2 cM between adjacent markers. Segregation ratios for dichogamy type and stigma color were not significantly different from 1:1, suggesting both traits are controlled by a single gene with protogyny and green stigmas dominant to protandry and red stigmas. Dichogamy type and stigma color loci were tightly linked (1.9cM) and mapped to ‘Elliot’ linkage group 16.
Our objectives were to create linkage maps for the cultivars ‘Pawnee’ and ‘Elliot’, and use these maps to identify molecular markers linked to loci controlling dichogamy type and scab resistance. We were able to complete all of our objectives except the identification of markers linked to scab resistance genes. We have been unable to find markers for scab resistance, because it has been difficult to get reliable field data on the resistance levels of the progeny seedlings. The scab pathogen has a complex relationship with pecan host, with environmental conditions, host resistance genes, and pathogen race variability all playing a role in infection outcomes. The pecan breeding program will continue to test this progeny for resistance, and once reliable data is obtained, it is expected that the marker data from this study will enable linkages between markers and resistance genes to be identified.